An Investigation Into The Effect Of An Australian-Type Rodent Diet, With And Without Nutritional Supplementation On The Behavioural Deficits, Neuropathology And Telomere Length In An Amyloid Mouse Model Of Alzheimer's Disease.

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Summary

Alzheimer's disease (AD) is an incurable, terminal, neurodegenerative disease that occurs primarily in people over 65. Life-style interventions including diet are potential candidates for prevention and management of AD. This thesis aims to investigate the role of diet in an AD-type mouse model (APP$_{SWE}$/PSENdE9) that over expresses amyloid, called Amy mice. It is hypothesised herein that:

1. An Australian-type rodent diet accelerates the behavioural deficits and β-amyloid neuropathology that are observed in Amy mice.

2. Nutrient supplements can reduce the severity of genotype or diet-type induced behavioural deficits and β-amyloid neuropathology in Amy mice.

First, an Australian-type (Oz-AIN) diet was designed to reflect the current nutrient intake of Australians. Second, nutrient supplements that have the potential to slow progression of behavioural or neurological deficits in AD were added to the Oz-AIN diet to create the Oz-AIN Supp diet. The effects of both of these diets are compared with an optimal rodent diet, the AIN93-M diet. The rationale for these diets and their effect on weight gain, food consumption, and organ size are described. Amy mice that were fed the Oz-AIN diet were susceptible to diet-induced weight gain and obesity, which was not observed in Amy mice that were fed the Oz-AIN Supp diet, indicating that nutrient supplements prevent weight gain in Amy mice.

Spatial learning and spatial memory were assessed in the Morris Water Maze. The Oz-AIN diet impaired spatial learning in 15 month old Amy mice. This was prevented with nutrient supplementation, as Amy mice that were fed the Oz-AN Supp diet performed similarly to control mice. At 18 months, the Amy mice fed the Oz-AIN diet demonstrated intact spatial memory. This suggested that the Oz-AIN diet protects spatial memory in aging mice. Olfactory ability (sense of smell) was assessed in the Buried Chocolate Test. Olfactory ability of Amy mice that were fed the Oz-AIN diet was impaired relative to control groups at 12 months of age.

Diet did not affect amyloid load or the number of amyloid deposits. However, the Oz-AIN and Oz-AIN Supp diets were both associated with larger deposits, compared to the AIN93-M diet, suggesting that total fat content and not micro-nutrient intake, facilitates aggregation of amyloid into larger deposits.

Confocal microscopy revealed that three different β-amyloid pathologies occur in brains of Amy mice: (1) intracellular amyloid that was associated with necrotic cells; (2) extracellular diffuse deposits of amyloid; and (3) diffuse deposits that were associated with blood vessels. Whilst the Oz-AIN diet did not have an effect on pathology type, the Oz-AIN Supp diet was associated with increased diffuse deposits associated with blood vessels. It was not determined whether this was invasion or clearance of β-amyloid from the brain, and it is suggested that it is a combination of both.

Genotype and diet-type effects were observed on telomere length (aTL) and oxidative DNA damage in the brains of aging Amy mice. 18 month old Amy mice that were fed the Oz-AIN diet had significantly longer telomeres and significantly more oxidative DNA damage throughout their brains than Amy mice that were fed the an optimal rodent diet.

In conclusion, whilst nutrient supplementation prevented diet-type- and genotype induced spatial learning deficits, high total fat content conserved spatial memory in aged Amy mice. Furthermore, whilst a sub-optimal diet did not have an effect on β-amyloid pathology, nutrient supplements may alter β-amyloid clearance from the brain.

Declaration

I certify that this thesis does not incorporate without acknowledgement any material previously submitted for a degree of diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Sarah M. Brooker, February, 2014.

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Conference presentations

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Chapter 1: Literature Review.

1.1. Background.

Alzheimer's disease (AD) is a terminal neurodegenerative disorder that affects people primarily above 65 years of age. AD accounts for 50% – 75% of all cases of dementia, which is ranked the third highest cause of death in Australia [1]. Over the past decade the number of deaths in Australia due to dementia has increased substantially from 4,364 deaths in 2002 to 9,864 deaths in 2011 [1]. It is anticipated that by 2050, 1.1 million Australians will have dementia, and health expenditure will be $82.7 billion by 2062 – 2063 [2].

Clinical features of AD include severe cognitive decline, memory loss, behavioural changes and loss of the ability to carry out day-to-day self-care activities. Unfortunately, by the time that clinical diagnosis can be made AD patients are already in the moderate stages of the disease and many of the neuropathological processes involved in AD are well underway. The neuropathological hallmarks of AD are termed 'plaques' and 'tangles'. Plaques are extracellular deposits of aggregated β-amyloid. Tangles are comprised of intracellular accumulation of hyperphosphorylated *tau*. The leading hypotheses of AD propose that plaques and tangles are at the top of a cascade of events that includes high levels of oxidative stress and inflammation, neuronal and synaptic loss and brain shrinkage [3]. This leads to irreversible damage throughout regions of the brain involved in cognition and memory such as the temporal lobes, hippocampus and the neocortical association areas [4].

Despite developments that have been made over the past 30 years there is still no cure for AD and current drug and alternate treatments may be described as 'transient'

at best [5, 6]. There is an urgent need to find some means for early detection and prevention for AD. The current project aimed to investigate the effect of a modifiable risk factor, diet, on the development and progression of AD neuropathology and behavioural deficits using a mouse model of AD.

The following chapter provides a review of the current literature covering:

(i) History of Alzheimer's disease.

(ii) Hypotheses about the cause of AD.

(iii) The β-Amyloid Peptide.

(iv) Memory and Learning deficits.

(v) Anosmia.

(vi) Animal models of Alzheimer's disease.

(vii) Risk factors for Alzheimer's disease.

(viii) Diet as a means of prevention.

(ix) Telomere length and Alzheimer's disease.

1.2. History of Alzheimer's disease.

On 25th November 1901 Auguste D. was admitted to Frankfurt Hospital under the care of Dr Alois Alzheimer. Auguste's symptoms included problems with comprehension, poor memory and paranoia. Over the course of her stay in hospital, Auguste's cognitive abilities rapidly declined and she demonstrated profound behavioural changes, including jealousy, anxiousness, aggression and aphasia. The most remarkable change in her behaviour was her loss of comprehension and declarative memory. Dr Alzheimer's notes from interviews with Auguste demonstrate that while she was able to name objects she was presented with, she lost the memories of personal details about family members, her home address and what she was doing (Figure 1). Auguste's cognitive abilities continued to decline over the next five years, until she died in hospital on 8th April 1906 [7].

Figure 1. Extracts from 29thNovember, 1901.

(As viewed in Maurer et al, 1997).

> … What year is it? *Eighteen hundred*. Are you ill? *Second month*. What are the names of the patients? She answers quickly and correctly. What month is it now? *The 11th*. What is the name of the 11th month? *The last one, if not the last one*. Which one? *I don't know*. What colour is snow? *White*. Soot? *Black*. The sky? *Blue*. Meadows? *Green*. How many fingers do you have? *5*. Eyes? *2*. Legs? *2.*
>
> Writing, she does it as already described. When she has to write Mrs Auguste D, she writes Mrs and we must repeat the other words because she forgets them. The patient is not able to progress in writing and repeats, *I have lost myself*.
>
> During physical examination she cooperates and is not anxious. She suddenly says *Just now a child called, is he there*? She hears him calling…, she knows Mrs Twin. When she was brought from the isolation room to the bed she became agitated, screamed, was non-cooperative; showed great fear and repeated *I will not be cut. I do not cut myself*.

In his post-mortem analysis of Auguste's brain, Dr Alzheimer described the plaques and tangles that are now considered to be the neuropathological hallmarks of AD [8,

9]. By 1910, Dr Alzheimer and colleagues had studied three other cases that were similar to Auguste D, and published that "*a common main finding can be ascertained, namely a peculiar change in the ganglial cell fibrils and the formation of peculiar plaques, both of which appear in about the same measure and form in all four cases.*" (Perusini, 1909 as cited [9]).

Over one century has passed since AD was first described, and AD patients still live in the traumatic world experienced by Auguste D. Life expectancy after diagnosis is only 5-8 years and there is still no cure [10].

Over the past 30 years, research has made some progress toward understanding the events that occur in AD-type brains [11, 12]. It is well established that the plaques that Alois Alzheimer described contain aggregated β-amyloid [13, 14]. Specific species of β-amyloid are neurotoxic, suggesting that β-amyloid may be a causative factor in AD neuropathology. This is the basis of the leading school of thought regarding AD pathogenesis, the 'Amyloid Cascade Hypothesis' [15-17]. However, over time this hypothesis has evolved greatly. Recent developments have revealed that the aggregated β-amyloid deposits that were described by Alois Alzheimer are less toxic than the accumulation of soluble intracellular β-amyloid [18, 19]. Furthermore, aggregated β-amyloid deposits show very little association with the clinical features of AD, while intracellular soluble β-amyloid correlates strongly with behavioural deficits [20-22]. While these observations do not directly contradict the 'Amyloid Cascade Hypothesis', they do question the role of β-amyloid deposits in AD pathogenesis.

It is also clear that the neurofibrillary tangles that were observed by Alois Alzheimer are comprised of hyperphosphorylated tau [23, 24]. Tau is a microtubule associated protein and has physiological roles in normal cell functioning [23]. However, hyper-

phosphorylation of tau causes disruption to axonal trafficking and mitochondrial function, contributing to impaired cellular metabolism and subsequent cell death, all of which are cytotoxic events that occur in AD [25, 26]. Tau pathologies can develop independently of β-amyloid deposits and correlate well with cognitive decline [24, 25, 27].

1.3. Hypotheses about the cause of Alzheimer's disease.

Despite the increasing body of knowledge in regards to the neuropathological processes involved in AD progression, there is still little consensus about the origins of the disease [28]. In general the theories that describe the onset of AD can be separated into two broad camps: those who support the 'Amyloid Cascade Hypothesis', and those who support the 'Tau Hypothesis' [12].

The 'Amyloid Cascade Hypothesis' was first put forward by Hardy and Allsop in 1991 [29]. In their initial hypothesis, Hardy and Alsop proposed that insoluble β-amyloid was secreted into the extracellular space, where it aggregated into plaques. These insoluble plaques then led to enhanced oxidative stress, resulting in tau phosphorylation and tangle formation, neuro-inflammation and eventually causing neuronal death [29].

Mutations in genes that code for machinery involved in β-amyloid production cause familial AD [30, 31], adding support to the 'Amyloid Cascade Hypothesis'. Mutations to genes that code for amyloid precursor protein and presenilins 1 and 2 result in an increase in β-amyloid [30, 32]. Patients that carry these mutations develop familial AD by the time they are 65 [30, 32]. However, familial AD makes up less than 5% of AD cases. Over 95% of AD cases are sporadic and are not associated with mutations in APP, presenilin 1 or 2 [32].

The most well established genetic risk factor for sporadic AD is expression of different alleles of ApoE, which plays a role in cholesterol transport [33]. ApoE isoforms can bind and transport β-amyloid across the blood brain barrier, and may therefore play a role in β-amyloid clearance [33, 34]. Different alleles of ApoE are associated with different levels of AD risk. Expression of the ApoE2 allele is associated with a reduced risk for AD, whilst expression of the ApoE4 allele increases risk of AD [35]. Furthermore, ApoE4 is the least efficient β-amyloid transporter [34] and is associated with increased β-amyloid accumulation [36]. This indicates that the increased risk of developing AD that is associated with ApoE4 is due to impaired β-amyloid clearance. However, not all cases of sporadic AD express the Apoe4 allele, suggesting that whilst it may accelerate pathogenesis, the β-amyloid accumulation that arises from impaired clearance and trafficking is not a causative factor for AD.

The 'Tau Hypothesis' proposes that tau hyperphosphorylation is an early event in AD pathogenesis, and causes neuronal death independently of β-amyloid [26, 37]. Tau is a microtubule associated protein that is required to maintain internal architecture of the cytoplasm [25, 26, 38]. Hyperphosphorylation of tau decreases its affinity for microtubules, resulting in microtubule instability and formation of neurofibrillary tangles. The impaired microtubule transport disrupts normal neuronal function and metabolism [25, 26]. This contributes to neuronal death [25, 26]. Tau hyperphosphorylation is also associated with neurodegeneration in Parkinson's disease and West syndrome [39, 40]. Collectively these observations suggest that tau hyperphosphorylation, and not β-amyloid deposition, is responsible for the neuronal cell death and cognitive decline in AD [38, 41].

The uncertainty between whether tau hyperphosphorylation or β-amyloid accumulation are at the top of the cascade of events leading to AD [37, 42], or whether they are separate independent pathologies that contribute to AD progression [43], continues to fuel debate within the current literature. Nonetheless, the 'Amyloid Cascade Hypothesis' remains the leading school of thought to explain the onset and progression of AD [44].

1.4. The Amyloid peptide.

Amyloid precursor protein may be processed down one of two pathways: the amyloidogenic or non-amyloidogenic pathway [45-47] (Figure 2). In the non-amyloidogenic pathway, amyloid precursor protein is cleaved by α-secretase to produce α-carboxyl terminal fragment and soluble APP-α. Soluble APP-α is released into the extracellular space where it plays a role in neural proliferation, repair and growth [47, 48]. The α-carboxyl terminal fragment is cleaved by γ-secretase to produce P3 and amyloid intracellular domain. The neuropathological function of P3 is unknown [45]. The amyloid intracellular domain has an important role in nuclear signalling, transcription regulation and negative modulation neurogenesis [46, 49, 50] (Figure 2). These beneficial roles that the non-amyloidogenic pathway products play indicate that the non-amyloidogenic pathway is important for neuroprotection, synaptic plasticity, nuclear signalling and maintaining neuronal viability [51].

In the amyloidogenic pathway, amyloid precursor protein is cleaved by β-secretase to produce soluble APP-β and β-carboxyl terminal fragment [52]. Like soluble APP-α, soluble APP-β can influence neuronal proliferation. Soluble APP-β also mediates microglial proliferation, suggesting that it plays a role in neuro-inflammation [47]. The β-carboxyl terminal fragment is cleaved by γ-secretase to produce amyloid intracellular domain and β-amyloid peptides. Amongst the different species of β-

amyloid peptides, β-amyloid 40 is the most commonly found in the brain. However, β-amyloid 42 is more common in AD brains and is considerably more toxic [53, 54]. Beta-amyloid 42 can impair mitochondrial functions and neurotoxicity in PC12 cells [53]. Furthermore, β-amyloid 42 aggregates into large plaques much easier than β-amyloid 40, a property that correlates well with its neurotoxic capabilities [53, 54]. After cleavage from amyloid precursor protein, β-amyloid 42 can be secreted into the extracellular space, where it rapidly aggregates into extracellular plaques which can lead to synaptic dysfunction and neuronal death [55, 56] (Figure 2).

However, the majority of amyloid precursor protein processing down the amyloidogenic pathway occurs after amyloid precursor protein is internalised by endosomes [52, 57]. Amyloid precursor protein is cleaved by β-secretase and γ-secretase in the late endosomal and lysosomal compartments to produce β-amyloid 40 and β-amyloid 42 which then undergo proteolysis [19]. At high levels, β-amyloid 42 may overwhelm the clearance mechanisms in the late endosome [19] and accumulate within the cell, jeopardizing functions of the endoplasmic reticulum and mitochondria [52, 58]. This causes accumulation of nitric oxide, reduced levels of ATP and a subsequent increase in oxidative stress, impaired axonal transport and apoptosis [58].

While β-amyloid plaques are considered a hallmark of AD, the intracellular oligomeric species of β-amyloid are the most toxic species of amyloid [59]. Zhang *et al.* report that a mouse model of intraneuronal accumulation of β-amyloid oligomers without amyloid plaques, have impaired synaptic placticity, reduced neuronal numbers and memory dysfunction compared to age matched controls [60]. Domert *et al.* report that oligomeric amyloid can be transferred between cells. Furthermore, they report that impaired clearance of oligomeric β-amyloid 42 in the recipient cell,

leads to build up of oligomeric β-amyloid 42, cellular toxicity and neurodegeneration [61]. Similar reports that β-amyloid oligomers may play a role in behavioural deficits [62-65] and pathological events [59, 66] in AD have been communicated by other groups as well. Collectively, these studies indicate that intracellular amyloid oligomers, and not fibrils, may be the key players in AD pathogenesis.

Accumulation of intracellular β-amyloid 42 correlates with loss of cholinergic neurons in brain regions most affected in AD [67]. Treatments for AD have been developed that target these cholinergic systems which slows down loss of cholinergic neurons and delays the progression of behavioural deficits [3]. However, there has been limited success with medical intervention in AD [68], and focus is now also being given to the role of modifiable risk factors such as diet or exercise in AD prevention [69-71] .

Figure 2. Processing of amyloid precursor protein down the non-amyloidogenic or the amyloidogenic path.

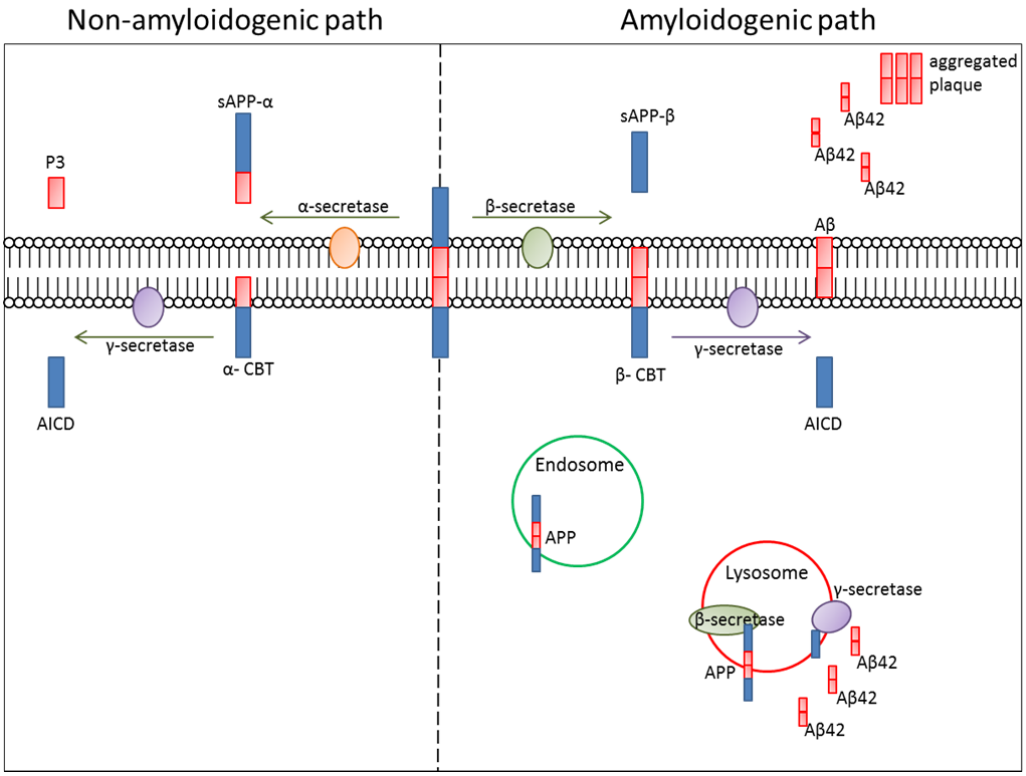


Figure 2. In the non-amyloidogenic pathway (left), Amyloid precursor protein (APP) is cleaved by α-secretase to produce α-carboxyl terminal fragment (α-CBT) and soluble APP-α (sAPP-α). α-CBT is cleaved by γ-secretase to produce P3 and amyloid intracellular domain (AICD). In the amyloidogenic pathway (right), APP is cleaved by β-secretase to produce soluble APP-β (sAPP-β) and β-carboxyl terminal fragment (β-CBT). β-CBT is then cleaved by γ-secretase to produce amyloid intracellular domain (AICD) and β-amyloid 40 (Aβ-40) or 42 (Aβ-42). Aβ-42 may then be secreted into the extracellular space where it rapidly aggregates into extracellular plaques that are a characteristic feature of the AD brain. Alternatively, APP may be taken up by the endosome, where it undergoes β-secretase cleavage to form Aβ-42 that is secreted into the intracellular space.

1.5. Memory and learning.

The key clinical features of AD include memory loss, profound behavioural changes and cognitive impairment. Memory loss is one of the earliest clinical features to present in AD. However, the subtleties of the early signs of memory loss in AD are hard to distinguish from non-critical, normal age-related change or impairments [10]. As a result, the disease goes undiagnosed and untreated until patients reach mild or moderate stage AD, when the underlying neuropathological processes are well underway. At this stage of AD progression, memory loss is more pronounced and is accompanied by severe cognitive decline, loss of language skills, and loss of self-care [4, 10, 72, 73].

Other clinical features of AD include non-cognitive changes such as pronounced motor, sensory and co-ordination deficits, aphasia (loss of speech), apathy (loss of motivation), depression and anosmia (loss of sense of smell). In the later stages of the disease, AD patients may also lose social inhibition, experience hallucinations and become aggressive [4, 10].

Memory is classified as working memory or long term memory [74, 75]. Working memory includes tasks such as attention, short term memory and information processing [76]. Long term memories may be either declarative or non-declarative, depending of the type of information that they encode. Non-declarative memory refers to information that can be unconsciously recalled, such as the skills required to successfully ride a bicycle. Declarative memory, on the other hand, refers to information that is consciously recalled and can be either semantic memory (factual information) or episodic memory (previous experiences) [75].

There is strong evidence that different types of memory are carried out in different regions of the brain [77]. In human and rodent models, auditory fear conditioning response is associated with the amygdala [78, 79]. Spatial learning is dependent on the cholinergic system of the hippocampus and cortex [80]. Episodic memory and spatial memory are also hippocampal dependent. However, once consolidated, semantic memories can be recalled independent of hippocampal function [77, 81, 82]. Depending on the type of semantic memories, semantic processing occurs in different regions of the lateral and anterior temporal cortex, and ventro-lateral prefrontal cortex [81].

Hippocampal dependent processes such as spatial learning, spatial memory and episodic memory are lost in the early stages of AD [83]. Memory loss and cognitive decline can be studied using rodents, because rodents process and remember complex relationships in a similar way to humans [84, 85]. Rodent models of AD have demonstrated clear correlations between hippocampal damage and impaired spatial learning and spatial memory in a manner that is similar to that observed in AD patients [82, 86, 87].

Rodent behavioural tests such as the Radial Arm Maze (RAM) and the Morris Water Maze (MWM) can both be used to assess spatial learning and memory [88-91]. The two tests differ in their primary outcomes, sources of motivation to complete the tests and protocols available. While both are designed to assess spatial learning and memory, they may assess different components of these cognitive processes and therefore may produce conflicting results [85, 88, 92]. This does not mean that one test is more reliable than the other for research into AD-associated behaviours. It means that researchers must be aware of the limitations and benefits of each, before deciding which test is most applicable to their specific research question [93, 94].

The RAM is used to assess rodent spatial working memory, and provides insight into short term memory and information processing [62, 88]. The maze consists of eight arms, with a food reward at the end of each arm. The task requires the animal to visit each arm once and collect all eight rewards. Working memory is assessed through accuracy at visiting each arm once to receive a reward. Time taken to complete the task is also recorded to validate motivation and how fast the mouse can make decisions about where the remaining food pellets are located [88, 92]. Long term memories may also be measured in the RAM whereby food rewards are only placed down four of the eight possible arms. The number of entries the mouse makes into arms that do not contain the reward are counted as reference memory errors, as they indicate that the mouse has not recalled where the food pellets initially were located [88, 92].

The benefit of the RAM is that the primary outcome measures are easily defined and they are committal i.e. it is clearly apparent when a mouse moves down an arm of the maze. However, this type of testing is easily confounded by non-spatial cues. For example mice may use scent to track where they have already been, which would improve their performance in the maze and mask any memory deficits [85, 88].

The MWM is a commonly used test to assess spatial learning, spatial memory and cognitive flexibility. The MWM consists of a pool of opaque water, which is surrounded by controlled visual cues and contains a submerged platform. During a three to five day acquisition phase, mice are trained to escape the pool by finding the submerged platform. The latency to escape the pool is the primary measure of improved performance and hence spatial learning in the MWM [87-89]. However, escape latency may not necessarily reflect spatial learning as it is easily distorted by

factors such as speed and motivation. Therefore, average distance travelled over each of the training days is also recorded [95].

Spatial memory is assessed the day following the training period. The platform is removed from the pool and the mouse is given a free swim lasting from 30 seconds (s) to 2 minutes (min). The number of passes the mouse makes over the platform and the time spent in the Test Quadrant are the key indicators of whether or not the mouse remembers the location of the platform [85, 88, 89, 95]. Cognitive flexibility is tested in reversal trials. These trials are conducted the day after the acquisition phase and mice are required to learn a new platform location. A probe test is used as a control, where latency and distance to a visible platform are recorded. This control confirms motivation levels and validates that mice can use visual cues to locate the platform [85, 88, 89, 95].

While the MWM is not limited by environmental factors such as scent, it is an aversive test as mice must escape an uncomfortable environment. This may cause anxiety and stress, and impair memory in some rodent strains [96]. However, the benefit of this type of testing is that mice are highly motivated to escape the pool. Trials often last less than 120 s, which is much faster than trials in dry mazes such as the RAM. This allows for higher through-put, and therefore is an attractive test to use when large numbers of animals are required [97].

Other rodent behavioural tests have been designed to assess different aspects of cognitive abilities. For example, in operant learning tasks mice learn to associate the performance of a task (i.e. pressing a bar) with a reward (i.e. a cookie). This can be used to measure learning and memory. However, operant learning tasks were originally designed for rats, and require behaviours that are not natural for mice (such as pressing on a bar). This has been overcome with the introduction of touch

screens that count the times a mouse investigates a hole or an image with their nose, which is more natural for a mouse as they rely heavily on their sense of smell [98, 99]. Behavioural tests such as fear conditioning tests make use of cued fear, contextual fear, or passive avoidance to assess learning and memory of an environment or an event [98, 99]. However, these are aversive tests and therefore, may not be appropriate for some mouse models.

The current study will make use of the MWM to assess changes in spatial learning and spatial memory in AD-type mice. The benefits of the MWM are that it may be used in long-term dietary studies, and that it is possible to do tests on large numbers of mice. Furthermore, mice do not have to be fasted throughout testing, as there is no food-reward stimulus to complete the test. This last point is important to consider for any dietary study.

Nutrient intake and calorie consumption can affect cognitive deficits [100-102], neuropathology [103, 104] and telomere attrition [105, 106] in normal and AD-type mice. Halagappa *et al.* demonstrated that both caloric restriction (by 40%) and intermittent feeding (24 hours without food every second day) for 17 months conserved spatial learning and memory in an AD-mouse model using APP/PSEN1 mice [101]. The caloric restriction also reduced amyloid and tau pathology, demonstrating that nutrient intake alters behaviour and neuropathology in mice [101]. By avoiding the requirement for overnight fasting, the MWM is an ideal tool to use to assess spatial learning and memory in mice in this dietary study.

1.6. Anosmia.

Anosmia, loss of sense of smell, is reported to precede memory loss and cognitive decline in some cases of AD [107]. Although anosmia is a natural phenomenon of

aging, olfactory decline is more noticeable amongst AD patients [108-112]. As a result, olfactory detection tests may be a useful diagnostic tool for AD. However, the limited understanding of the relationship between anosmia and AD has prevented the usefulness of olfactory tests in early AD detection.

Odour detection, discrimination and identification tests have been developed in a wide range of rodent models of anosmia [113-115]. These tests have enabled a clearer understanding of olfactory pathways in social behaviours, food consumption and neurodegenerative diseases [113-115]. Before their contribution to each field can be further investigated, it is important to understand the differences between testing paradigms, and how they can be influenced by different mouse models [113-117].

Simple olfactory sensitivity tests aim to detect the threshold at which a mouse can detect a particular scent [114]. The simplicity of these tasks means that they do not necessarily place demands on the mouse with respect to mobility, cognitive ability or memory. Latency to the scent is not used as a measurement so movement has minimal impact on performance in the test. Likewise, the mouse is not required to learn the location of the odorant, or to solve a puzzle in order to locate the odorant. Therefore simple olfactory tests may be ideal for assessing olfactory detection and sensitivity in aging and AD mouse models, whose cognitive abilities may be compromised. However, because of their simplicity, this type of testing rests on the assumption that mice are calm during the testing period. Therefore, each test requires long periods of habituation, making each testing period extremely laborious [114].

The Buried Food Pellet Test (BFPT) makes use of a rodent's natural tendency to scavenge and dig for food to assess olfactory detection [114, 115]. Mice are required to locate a hidden food pellet in an arena with no visual cues. Latency and distance travelled to the food pellet are used as measures of olfactory detection. Mice are

often fasted overnight or placed on a food restricted diet to ensure motivation to locate the food pellet [113, 115, 118]. The BFPT is not biased by anxiety, as it only requires tasks that are natural for mice (such as digging). This removes the need for long periods of habituation [119, 120]. Olfactory performance has been demonstrated to be unrelated to overall exploratory behaviour in an APP/PSEN1 mouse model of AD, confirming that performance in the BFPT is a true reflection of olfactory detection [120]**.** As a result of this, the BFPT is a commonly used assessment of olfactory detection in rodent models of anosmia [113, 115, 118, 120, 121].

Rey *et al.* report that in APP/PSEN1 mice, olfactory dysfunction and β-amyloid deposition in the olfactory bulb are both enhanced by loss of the noradrenergic neurons that project from the locus coeruleus to the olfactory bulb [120]. This finding is important as it suggests a mechanism and a link between AD neuropathology and olfactory decline [120].

Other behavioural tests can provide more detail than the BFPT as they can distinguish between different types of olfactory loss (i.e. detection versus discrimination of a scent). However, they are limited in their application to AD-mouse models as they require intact cognitive functioning. Behavioural tests such as odour preference tests, learned avoidance tasks and habituation / dis-habituation tests do not rely on declarative hippocampal memory, but they do assume equal cognitive abilities between groups and therefore may not be easy to interpret in models of cognitive decline [119, 121]. For example, habituation / dis-habituation tasks have returned different results depending on the AD mouse model used, and the severity of cognitive discrepancies at the time of the olfactory tasks. Impaired odour habituation and discrimination has not been consistently demonstrated in different AD-mouse models. While olfactory deficits have been demonstrated in the

commonly used Tg2576 and APP/PSEN1 mouse strains [120, 122], they have not been replicated in the APP23 mouse model [123].

Until a better understanding of the relationship between olfactory decline and AD is established, it may be more useful to utilize simple olfactory tests of odour detection and sensitivity.

The current study has made use of a modified version of the BFPT, whereby mice are required to locate a buried piece of chocolate rather than a buried cookie. The reasoning for using chocolate in these tests was due to reports from others that chocolate searching behaviours are relatively stable in mice [124]. The modified version of the BFPT will be called the Buried Chocolate Test (BCT) for the remainder of this thesis.

1.7. Animal models of Alzheimer's disease.

The underlying neuropathological processes of AD are well underway before clinical diagnosis of AD can be made [125]. Coupled with ethical and moral constraints, this provides limited opportunity to study the onset and progression of AD in humans. Therefore, animal models are used to study the neuropathological and clinical features of AD [56, 126-137].

Mouse models of AD have several advantages: (i) mice are small and easy to rear, which enables studies of large cohorts of mice; (ii) mice have a short gestation period with large litter sizes, which makes mouse numbers easily accessible; and (iii) the mouse genome has been completely mapped [138]. The single, double and triple transgenic mouse models that have been developed target genes that are associated with familial AD [134]. The limitation with the use of these mouse models is that no single mouse model exists that encapsulates all the features of AD [138, 139].

Nonetheless, mouse models are useful tools to enhance the understanding of the processes involved in AD.

1.7.1. Amyloid mutations.

The first transgenic mice that successfully presented with an AD-type phenotype were developed by Games *et al.* in 1995 at Athena Neurosciences, Inc., California [140]. The PDAPP mice that were developed by Games *et al.* were bred to overexpress APP. They generate ten times the amount of endogenous β-amyloid, and form β-amyloid deposits and neuritic plaques. The synaptic loss, astrogliosis, microgliosis and shrinkage of the hippocampus that occurs in PDAPP mice reflects that seen in the brains of AD patients [140]. Similarly, DPAPP mice also develop age related cognitive decline. Whilst the β-amyloid neuropathology and behavioural deficits are similar to AD, the PDAPP mice do not develop tangles nor have significant neuronal loss. Therefore, while PDAPP mice are a good model to investigate β-amyloid neuropathology, they do not reflect AD *per se* [134, 138, 141]. Nonetheless, PDAPP mice are still a commonly used mouse model [84, 134, 138, 141].

Shortly after the development of the PDAPP mouse model, another mouse model of AD, the Tg2576 mouse was developed at University of Minnesota, Minneapolis [142]. The Tg2576 mice over-expresses amyloid precursor protein and develop β-amyloid neuropathology that is similar to that observed in AD patients. By the time Tg2576 mice are five months old, there are plaques present throughout the frontal, temporal, and entorhinal cortices [84, 134, 141]. By the time they are ten months old they demonstrate memory loss in the MWM [143, 144]. Similar to the PDAPP mice, Tg2576 do not develop neuronal loss, tangles, nor demonstrate a clear correlation between β-amyloid deposition with aging and cognitive decline [84, 138, 141].

1.7.2. Presenilin 1 and 2 mutations.

Presenilins 1 and 2 form the two active subunits of γ-secretase, and therefore play an important role in amyloid precursor protein processing. Presenilin mutations accelerate amyloid precursor protein processing and as a result, presenilin mutants have higher levels of intracellular β-amyloid. However they do not develop β-amyloid plaques [84, 134, 138]. Furthermore, while they do demonstrate modest cognitive decline, this is not significant [134]. The benefit to using presenilin mutants is that unlike the amyloid precursor protein mouse models, presenilin mutants present with neuronal loss [138].

1.7.3. Tau mutations.

Transgenic mouse models that either express single mutant variations or overexpress human tau isoforms, have been developed to investigate the role of tau in AD [126, 145-147]. The tau transgenic mice develop neurofibrillary tangles throughout the brain and neuronal loss throughout the hippocampus [126, 134, 145-147]. Transgenic tau mice are traditionally used to model neurodegenerative diseases such as Parkinson's Disease and develop severe motor deficits [147]. Recent models have been developed that have the cognitive deficits typical of AD by ten to twelve months without motor impairments [134, 145]. There are significant correlations between tau hyperphosphorylation and cognitive decline in transgenic tau mutants [126, 127, 134]. The main limitation with the transgenic tau models is that they do not develop β-amyloid deposits. However, intracerebral injections of β-amyloid 40 and β-amyloid 42 into the brains of these mice provide the opportunity to clearly demonstrate interactions between β-amyloid and tau, such as the ability of amyloid to accelerate tau production and hyperphosphorylation [148, 149].

1.7.4. Double transgenic mice.

There are a wide variety of double transgenic mouse models that overexpress amyloid precursor protein and presenilin 1 [84, 128, 141], or amyloid precursor protein and *tau* [150]. To cover all of them and compare their differences is beyond the purposes of this chapter. However, it is worthwhile to appreciate the differences in the mouse models that are available.

Mice that overexpress amyloid precursor protein and presenilin 1 are more aggressive models of β-amyloid deposition and develop β-amyloid plaques faster than single transgenic mice [138, 141]. The behavioural deficits and plaque deposition in these mice are similar to that observed in AD [128]. The double transgenic mice that overexpress amyloid precursor protein and tau develop β-amyloid deposits, hyperphosphorylated tau and neurofibrillary tangles. Depending on the mutations used, amyloid deposits, neurofibrillary tangles and neuronal loss can occur in varying degrees, enabling different aspects of the disease process to be studied [84]. Irrespective of the mutations, the double transgenic mouse models tend to develop cognitive deficits at a faster rate than single transgenic mice [84].

1.7.5. Triple transgenic mice.

Triple transgenic mice that express APP, tau, and presenilin 1 mutations, have been developed to replicate all three pathologies in AD (plaques, tangles, and neurodegeneration). While the onset of neuropathology is rapid, it is preceded by behavioural deficits. Furthermore, the behavioural deficits observed are not typical of AD mouse models, in that they reflect impaired retention and recall, rather than impaired learning which does not occur until mice are 16 months old [138]. In this respect, these models may not be useful for investigating behavioural deficits in AD.

The onset of neuropathology is rapid, but it does enable the investigation of plaques, tangles and neuronal loss [84]. However, mice do not present with behavioural deficits characteristic of AD [141].

1.7.6. Background strain selection.

The background strain is also a feature that can alter AD-type neuropathologies and behavioural deficits, as different background strains can have different effects on behaviour and neuropathological processes in subsequent mouse models [99, 151]. For example, transgenic mice that are built off a C57bl/6 strain are often used for behavioural tests [152, 153]. This is because although chronological age has a moderate effect on performance in behavioural tasks of C57bl/6 mice, they respond better to behavioural testing than other wild type mice [154, 155].

Fröhlich *et al.* demonstrated that mice overexpressing amyloid precursor protein, from either a C57bl/6 or FVB/N background developed β-amyloid pathologies at different rates. While mice from a C57bl/6 background developed more plaques, and had an exponential increase in plaque number, the FVB/N mice had an initial rapid increase in plaque numbers with a plateau at around 5 months [151]. This demonstrates that not only does changing the mutation or expression of a gene alter AD pathology, but careful consideration must also be given to selecting the appropriate background strain to answer a specific research question.

1.7.7. Mouse model used in the current thesis

The current project makes use of the double transgenic mouse model APPswe/PSEN1dE9 [156] and have been bred from a C57bl/6 background, making them more suitable for behavioral testing. These mice were bred at Flinders University, from stock that were originally obtained from The Jackson laboratory

(Maine, USA), and have been used by the Department of Human Physiology and Centre for Neuroscience, Flinders University and CSIRO Food Science Australia [106, 157].

APPswe/PSEN1dE9 mice are genetically predisposed to overexpress a chimeric mouse/human amyloid precursor protein and a mutant human presenilin 1 which has a deletion at exon 9. The over expression of amyloid precursor protein allows the mouse to secrete high levels of β-amyloid. This is further enhanced by the PSEN1dE9 mutation, which increases activity of γ-secretase, and drives processing of amyloid precursor protein down the amyloidogenic pathway [156]. Mice develop β-amyloid deposits by 6 months of age [141] and have spatial memory deficits in the MWM by 8 months [156, 158, 159]. Similar to other models of amyloid precursor protein over expression, APPswe/PSEN1dE9 (Amy) mice do not develop neurofibrillary tangles [141]. However, there do appear to be discrepancies in the literature about whether or not these mice undergo neurodegeneration [141, 160] . For example, in a review of AD-type mouse models, Lee and Han report that Amy mice do not undergo neurodegeneration [134]. However, Broersen et al. clearly demonstrate that aging wild type and Amy mice both undergo some neuronal loss, but that is more pronounced in the brains of Amy mice [160].

It must be stressed that, similar to many AD-type mouse models, the APPswe/PSEN1dE9 mice do not develop AD *per se*. They are designed to over express Amyloid Precursor Protein and consequently have increased β-amyloid levels. Therefore, this model, at best, reflects aspects of familial AD, and is better to be considered an amyloid-over expression model. To maintain clarity that this is an amyloid mouse model, and that it does not encompass all features of AD, these mice will be referred to as Amy mice (amyloid mice) for the remainder of this thesis.

1.8. Risk factors for Alzheimer's disease.

Risk factors for AD include age, susceptibility genes, gender, cardiovascular health, social stress, exercise, diabetes, diet and weight gain [161-171]. The strongest risk factor for AD is age. Of all AD cases, less than 1% are 65 years, and over 23% are 100 years old [172]. Gender is also a large determinant of AD risk [173]. Women make up 64% of AD cases in Australia [172]. In human and animal studies, aged female brains are more susceptible to β-amyloid- induced up-regulation of reactive oxygen species and oxidative stress [174]. It has been suggested that this is due to the decline in oestrogen levels with aging. However, medical intervention such as hormone replacement therapy has not been successful in AD prevention [175].

Stroke and atherosclerosis are also associated with increased AD risk [165, 176]. In a post-mortem analysis of 200 patients with dementia (aged 87.6±7.1 years), intracranial atherosclerosis but not coronary or aortic atherosclerosis correlated well with dementia, suggesting that the cerebrovasculature may play a key role in AD development [176]. This is not surprising, given that in AD development and progression, the endothelial cells lining the blood brain barrier are subject to high levels of oxidative damage, leading to unwanted movement of toxins across the blood brain barrier into the brain [177].

The most established genetic risk factors are mutations to genes encoding APP, presenilin 1 and 2. However, these are associated with familial AD, which accounts for less than 5% of all AD cases [178]. Genetic risk factors for sporadic AD have been identified, however they do not occur in all cases. For example, carriers of the ApoE4 allele are at higher risk of developing sporadic AD, but the ApoE4 allele itself is not a determinant of AD [171]. Recent research has revealed a number of other genetic mutations that may increase risk of developing AD. These include

genes for other lipid chaperones or genes that are involved in β-amyloid formation or that are associated with other dementias [35, 178, 179].

An understanding of age, gender and genetics as risk factors for AD enables clarification of populations most at risk, which may enable early intervention strategies. However, age, gender or genetics cannot be modified *themselves* in any meaningful way to reduce the risk for AD. On the other hand, lifestyle factors, such as diet and exercise can be modified in a manner to potentially reduce AD risk [166, 167].

1.9. The role of diet as a modifiable risk factor.

Epidemiological studies and animal studies have provided strong evidence for the role of diet in AD risk and prevention [167, 180-182]. The Mediterranean style diet is characterised by high intake of vegetables, cereals, fish and olive oil (as the primary source of monounsaturated fats) and limited red meat and poultry (and therefore low in saturated fats) and with moderate intake of red wine. This diet is associated with a decreased risk for AD [180-184]. On the other hand, a typical Western-style diet, which is a high-energy diet that is high in saturated fats, red meat, and sugary desserts [185, 186], is associated with an increased risk for AD [183, 186, 187]. This suggests that macronutrients such as saturated fats and cholesterol increase AD risk, whilst polyunsaturated fats (such as the ω-3 fatty acids found in fish oil) and anti-oxidants reduce AD risk. The question then becomes *how do these different dietary elements influence AD risk?*

Dietary Fats, Endothelial Function and Risk for Alzheimer's disease.

Dietary cholesterol and saturated fats form deposits along vascular walls, leading to atherosclerosis and endothelial dysfunction [188]. Similar processes may occur in the

cerebral vasculature, impairing the microvascular walls that line the blood brain barrier. Consistent with this, high fat diets impair blood brain barrier integrity in rats [189] and microvascular pathology in mice [190], and enable infiltration of inflammatory markers from the periphery across the blood brain barrier in rat brains [189]. This suggests that in AD, high-fat diets may lead to cerebral vasculature dysfunction, impairing blood brain barrier integrity and enabling β-amyloid transport from the periphery and into the central nervous system [191, 192]. This may result in increased β-amyloid deposition and the increased inflammation and oxidative damage that is characteristic of AD brains.

Dietary Modifications to Cellular Membranes and Their Role in Alzheimer's disease.

Cellular membranes are organised into domains. These domains reflect lipid-lipid or lipid-protein affinity and aversion. Lipid rafts are domains that are enriched in saturated fatty acids and contain lipid linked signalling proteins. Lipid rafts are 'glued' together by cholesterol [193, 194]. Movement of cholesterol within the membrane can alter the formation of lipid rafts, and subsequently alter membrane assembly and accessibility of membrane bound proteins that are essential for normal cellular function [194]. For example, the movement of cholesterol from raft- to non-raft domains is accompanied by a shift of membrane bound proteins, such as γ-secretase and β-secretase. The movement of γ-secretase and β-secretase limits that amount of γ-secretase and β-secretase that are available to APP, and subsequently limits amyloid precursor protein processing down the amyloidogenic pathway [194, 195].

Cholesterol in the CNS is synthesised *de novo* in astrocytes rather than being obtained from the diet [196]. However, a high-cholesterol diet alters cholesterol

levels and amyloid precursor protein processing in AD-type mouse brains [197, 198]. Refolo *et al.* demonstrated that diet-induced hypercholesterolemia caused significant increases in cholesterol levels, increased β-amyloid levels, and reduced soluble APP-α in AD-type mice [197]. This suggests that dietary cholesterol can alter amyloid precursor protein processing and promote β-amyloid formation, accelerating onset of AD.

At the same time, cholesterol plays a role in β-amyloid clearance. Beta-amyloid is extremely lipophilic and is attracted to cholesterol in the membrane. Meleleo *et al*. suggest that in this context, cholesterol may actually be neuroprotective as it also promotes uptake of β-amyloid into the membrane, facilitating clearance of intracellular β-amyloid [193]. However, accumulation of β-amyloid in the cellular membrane disrupts their organised structure and increases production of reactive oxygen species, resulting in cell death [56, 199, 200].

This understanding of the interaction between cholesterol and β-amyloid in neuronal membranes has led to the development of diets that are designed to maintain neuronal membrane integrity, reduce β-amyloid build up, enhance synaptic function and stability, and prevent neuronal death [160, 201-204]. An example of such a diet is the multinutrient intervention Fortasyn™ Connect [201]. Fortasyn™ Connect contains precursors and cofactors that are required to ensure membrane stability. This includes uridine-mono-phosphate, docosahexaenoic acid and choline, which are required for phospholipid synthesis; eicosapentaenoic acid as an additional source of docosahexaenoic acid in the brain; B vitamins and other antioxidants which enable or support biological processes involved in phospholipid synthesis [160, 205].

Fortasyn™ Connect has been demonstrated to be successful in rodent models of AD and in patients with mild AD [201, 205-207]. In human trials Fortasyn™ Connect

was supplied in a medical food, Souvenaid® (Nutricia N.V., Zoetermeer, The Netherlands) [201, 206, 207], which is designed to meet the nutritional requirements of people with a specific condition i.e. aged AD patients [180]. The Fortasyn™ Connect Souvenaid® diet improved neuronal membrane stability and cognitive abilities of patients with mild AD [160, 205]. In rodent models of AD, Fortasyn™ Connect was added to the AIN93-M diet, which is designed to meet all the nutrient requirements of normal laboratory mice [160, 205]. This nutrient supplemented diet improved membrane stability and cognitive abilities in mice and reduced β-amyloid neuropathology and neuronal loss [160, 205].

Parachikova *et al.* developed a different nutrient supplement cocktail for rodents. They report that supplementing an optimal rodent diet with curcumin, piperine, epigallocatechin gallate, α-lipoic acid, N-acetylcysteine, B vitamins, vitamin C, and folate improves cognitive abilities and reduces β-amyloid neuropathology in AD-type mice [204].

These studies have demonstrated that modifying a diet can be beneficial in reducing neuropathology and behavioural deficits in AD. However, these diets were also designed to meet the nutritional requirements of the participants and animals in their respective studies [160, 201, 204-207]. Unfortunately, in a household setting, normal healthy individuals may not eat diets that meet their nutritional requirements. For example, a typical Australian-type diet is high in total fats, with an imbalanced polyunsaturated: monounsaturated; saturated fat ratio that favours saturated fats (1.0: 2.4: 2.7) [185]. A typical Australian-type diet is also low in essential nutrients such as folate, calcium and magnesium [185], and diets that are deficient in folate are associated with an increased risk for AD [171, 208]. The introduction of voluntary fortification of food in 1995 has improved folate status in some populations in

Australia [209]. However, folate deficiency has continued to increase in lower socioeconomic and remote communities [210]. Many AD patients are often malnourished [208], which puts their dietary habits at the opposite end of the spectrum to those used by Parachikova *et al*. or in the Fortasyn™ Connect studies [160, 201, 204-207].

It may not be conventional to create an entire diet that promotes neuroprotection against AD, as this would also require changing behavioural eating patterns. Compliance to changes in eating behaviours can be low even when patients are aware of the risks or benefits associated with certain dietary patterns [211, 212]. Dietary supplementations, on the other hand, may enable specific populations to reduce their risk of AD with minimal impact on normal dietary patterns.

Polyphenols are small, plant derived compounds that activate intracellular processes to protect neurons from inflammatory and oxidative damage [213]. Owing to the fact that oxidative stress plays a large role in AD pathogenesis, polyphenolic compounds are good candidates as nutrient supplements in prevention of AD [214].

Curcumin, the orange colouring in turmeric, is a polyphenolic compound that has several sites of action in preventing AD [213, 215]. Curcumin crosses the blood brain barrier with ease, where it has potent antioxidant and anti-inflammatory potential, reduces genomic instability events, decreases β-amyloid production and enhances β-amyloid clearance [106, 213-217]. Similar to other polyphenolic compounds, curcumin is a scavenger of reactive oxygen species and inhibits lipid peroxidation [215]. Curcumin mediates microglial induced phagocytosis of β-amyloid and prevents production of inflammatory cytokines [215]. By reducing inflammation and oxidative stress, curcumin also limits inflammatory mediated β-secretase activation and subsequent β-amyloid production [215]. It would seem then, that curcumin is an

ideal nutrient supplement in AD prevention. However, curcumin is extremely hydrophobic and its unstable nature means that very little curcumin is absorbed from diet. Therefore, on its own, curcumin may not be of benefit as a dietary supplement in AD prevention [213].

Fish oil may also offer some means of protection against AD. Fish oil is an excellent source of the polyunsaturated fatty acids docosahexaenoic acid and eicosapentaenoic acid. Docosahexaenoic acid is the most abundant ω-3 polyunsaturated fatty acid in the brain [218]. Docosahexaenoic acid supplements increase activity of protein kinase B, an anti-apoptotic '*pro-survival*' kinase, in AD-type mice [219] and reduce pro-inflammatory cytokines such as interleukin-6 and tumor necrosis factor-α in aging mice [220]. The anti-inflammatory capability of docosahexaenoic acid has been demonstrated to have beneficial effects in reducing AD neuropathology and behavioural deficits in rodent models of AD [219, 221].

Docosahexaenoic acid also plays important roles in membrane stabilisation, neuronal signalling, and neuron proliferation. Docosahexaenoic acid is taken up into lipid rafts and displaces cholesterol [222]. High levels of docosahexaenoic acid lead to fewer cholesterol-rich lipid rafts, less β-secretase available to amyloid precursor protein and consequently less β-amyloid produced in the brain. The balance between lipid rafts (and the proteins they carry) and docosahexaenoic acid rafts (which are non-structured and low in cholesterol) is important for maintenance of membrane integrity and cellular function [223]. Wassall & Stillwell suggest that in the context of AD the primary benefit of docosahexaenoic acid is that it displaces cholesterol from the membrane, shifting this balance in favour of membrane stability [223].

Broersen *et al.* investigated the effects of optimal rodent diets that were supplemented with Fortasyn^{TM} Connect, docosahexaenoic acid, uridine-mono-

phosphate or a combination of docosahexaenoic acid and uridine-mono-phosphate [160]. All of these supplements have the capabilities of improving membrane stability. Broersen *et al.* report that all four diets increased cerebral docosahexaenoic acid and altered lipid profiles within the mouse brain. However, docosahexaenoic acid supplements did not produce significant changes in β-amyloid neuropathology [160]. This suggests that although docosahexaenoic acid is easily taken up into the neuronal membrane, it does not necessarily mean it can alter β-amyloid pathology. These findings also suggest that the benefits of docosahexaenoic acid in AD mouse models may depend on the type of the diet that docosahexaenoic acid supplements are added to.

There is evidence to suggest that in the context of a high-fat diet, docosahexaenoic acid enhances inflammation, endothelial dysfunction and allows invasion of proteins across the blood brain barrier [191]. This is because once taken up into the brain, docosahexaenoic acid undergoes rapid oxidation [224]. Therefore, supplementing a high-fat diet with docosahexaenoic acid in the absence of antioxidants may be futile.

Supplementing high-fat diets with docosahexaenoic acid in combination with antioxidants has proven to be highly successful in preventing β-amyloid neuropathology and behavioural deficits in AD mouse models [215]. Combinations of fish oil and polyphenolic compounds such as curcumin may be particularly useful. While the potent antioxidant abilities of curcumin may reduce oxidation of docosahexaenoic acid, the use of fish oil may also increase bioavailability of curcumin [217, 225].

Ma *et al.* fed triple transgenic AD-type mice high-fat diets that were supplemented with either curcumin, fish oil or a combination of curcumin and fish oil. The fat content of the diet used by Ma *et al.* was similar to Western-style rodent diets (21%

fat). They report that mice that received supplements had better spatial learning abilities than mice that were fed high-saturated fat diets. These effects were most pronounced in mice that were fed the combination of curcumin and fish oil. This demonstrates that the combination of a powerful antioxidant (curcumin) with a polyunsaturated fatty acid (docosahexaenoic acid) has a more potent effect than either supplement alone, and is especially beneficial in the context of a high-fat diet [217].

While there is substantial evidence that dietary supplements may play a role in AD prevention, there is little consensus regarding which dietary elements will be the most beneficial. This is partially because many potential supplements have been trialled in conjunction with diets that already meet nutritional requirements. If nutritional supplements are to be investigated in a meaningful way, they need to be assessed alongside a diet that reflects nutrient intake of the population at risk of developing AD.

This project will investigate the potential of nutrient supplements in prevention of AD against a diet that is typically consumed by adult Australian women. Women make up 64% of cases of AD cases in Australia (at 65 years of age) [172] and female brains are more susceptible to β-amyloid induced up-regulation of reactive oxygen species and oxidative stress [174]. While hormone replacement therapy has not proven successful in prevention of AD [175], adjusting diet may offer some means of prevention, especially considering that the diets that are typically consumed by Australian women share several features with diets that increase risk for AD, such as being low in folate [171, 185]. Therefore, a better understanding of the role that these diets may play in AD development, at least in Australian women, may enable a means to AD prevention.

1.10. Telomere length and Alzheimer's disease.

Telomeres are specialized DNA sequences at the end of chromosomes that are associated with risk factors for AD, such as aging and oxidative damage [226-235]. There is an increasing body of knowledge regarding telomere attrition with aging in replicative tissues such as lymphocytes and hepatic cells [236-242]. However, very little is understood about telomere length in the aged brain or what implications telomere attrition in the brain may have on AD. This project presented the opportunity for a small exploratory study that investigated telomere length in the mouse brain at two stages of late adulthood in normal and AD-type mice.

Telomeres are specialised sequences of DNA (TTA GGG) that cap the ends of chromosomes and distinguish between the natural end of a chromosome or a DNA strand break which may trigger senescence. One of the primary roles of telomeres is to prevent the loss of coding DNA during replication. DNA polymerases are unable to synthesise to the 3' end of the template DNA strand, therefore, rather than losing coding DNA sequence, a fragment of telomere sequence is lost with every replication [228, 231, 237]. Humans have telomeres that are anywhere from 15 to 30 kbp long, and lose around 50 bp per year [238, 243]. Mice have much longer telomeres and undergo a faster telomere attrition rate than humans, of around 7000 bp per year [244]. Telomere length and the rate of telomere attrition is important because once telomeres shorten to a 'critical length' they lose their protective function against DNA damage. Cells then enter a stage of cellular senescence and can no longer replicate. In this capacity telomeres may act as a 'biological clock', whereby telomere length provides some indication of cellular age [226-232]. However, there are important species to species and tissue to tissue variations in telomere length and the picture is by no means clear.

Telomeres are susceptible to oxidative DNA damage as they contain a high number of repeats of the highly oxidisable nucleic base, guanine [245, 246]. Pérez-Rivero *et al.* report that shorter telomeres are associated with loss of catalase activity and with an increase in reactive oxygen species and oxidative stress [247]. This suggests that while telomeres themselves are vulnerable to oxidative damage, loss of telomere integrity can promote oxidative stress further due to reduced activity of antioxidant defence mechanisms [247]. In line with this, diseases that are associated with high levels of oxidative damage, such as AD and Parkinson's disease are also associated with accelerated telomere shortening in proliferative tissues [242, 245].

There is lack of consensus on telomere length in the brains of AD patients. Some researchers argue that neuronal telomere length does not change with age in adulthood, as neurons in the adult brain are in a post-mitotic state and do not replicate [228]. Others argue that not only does telomere length decrease with aging in the brain, but that telomeres in hippocampal neurons of AD patients are shorter than those of normal healthy controls [248]. Thomas *et al*. confirm that telomeres in replicative tissues from AD patients are shorter than those from controls. However, they also report that telomeres from hippocampal tissue from AD patients are *longer* than those from controls [241]. This suggests that telomere dynamics in the AD brain is different to the rest of the body.

Mouse models have been used to elucidate telomere dynamics in the aging brain. Telomere shortening does not occur in the brain of aging mice [240]. In fact telomere length has been demonstrated to increase with age in rodent brains [234, 235]. Rolyan *et al.* used a transgenic mouse model to demonstrate that AD-type mice that were also genetically predisposed to develop short telomeres had less amyloid neuropathology and reactive microgliosis than control AD-type mice [233]. This

suggests that there may be associative evidence that short telomeres are protective against β-amyloid neuropathology.

Diet can also have an effect on telomere shortening. High ω-6 fatty acid consumption and increased caloric intake are both associated with shortened telomeres in leukocytes [249, 250]. The use of dietary supplements to increase the plasma ω-3: ω-6 ratio increases telomere length in adult human leukocytes [251]. Similar reports have been made about telomere length in mice. Increased antioxidant intake and caloric restriction have both been associated with increased telomere length in lens epithelium, white blood cells and buccal cells in rodents [106, 241, 252].

Dietary antioxidants such as vitamin C, folate, and vitamin B12 are associated with increased telomere length in peripheral blood lymphocytes [253, 254]. As AD progresses, telomeres in replicative tissues are at increased risk of accelerated shortening as a consequence of increased oxidative stress [242, 245]. Panossian *et al.* report that telomere length in T cells correlates with AD status [242]. The question then arises whether or not dietary supplementation with antioxidants that have the potential to prevent neuropathology and behavioural deficits in AD may also prevent telomere shortening in AD.

Without a better understanding of the telomere dynamics that occur in the aging brain, there is limited potential to effectively investigate the role of diet on telomere attrition in AD. Therefore, one of the aims of this thesis is conduct a small exploratory study of telomere length in normal and Amy mice at two stages of adulthood long after β-amyloid neuropathology has been established [132]. Owing to the evidence that antioxidants may play a role in telomere length protection, the potential effects of nutritional supplementation on telomere length in the brains of adult mice was also investigated.

1.11. Hypotheses, Aims and Outline of thesis.

The current thesis investigates the role of diet on the behavioural deficits and β-amyloid neuropathology in an AD mouse model. The thesis focuses on the effects of an Australian-type rodent diet, either alone or with nutritional supplementation, on the behavioural deficits and amyloid neuropathology in a double transgenic AD mouse model of AD (APPswe/PSEN1dE9). These mice are called Amy mice through-out this thesis.

It is hypothesised herein:

1. That an Australian-type rodent diet accelerates the behavioural deficits and β-amyloid neuropathology that are observed in Amy mice.

2. That nutrient supplements can reduce the severity of genotype or diet-type induced behavioural deficits and β-amyloid neuropathology in Amy mice.

Design of Diets.

Two rodent diets have been developed to address these hypotheses. The first diet is called the Oz-AIN diet and was designed to reflect the deficiencies and excesses of macronutrients and micronutrients in the diets that are typically consumed by Australian women. The second diet is called the Oz-AIN Supp diet, which has been constructed from the Oz-AIN diet with additional nutrient supplements that have potentially beneficial effects in AD prevention. The effects of these diets on food intake, body weight and the weight of major fat deposits and organs are described in normal and Amy mice.

Animal Model.

The Amy mouse model (APPswe/PSEN1dE9) was chosen to address the hypotheses of this project. These mice develop amyloid deposits at 6 months [141] and

demonstrate cognitive behavioural deficits by 8 months [156, 158, 159]. The non-aggressive nature of AD progression in this mouse strain makes them well suited to dietary intervention studies. These mice have been bred from a C57bl/6 background, which are better suited to behavioural tests than other background strains [154, 155].

Behavioural Deficits.

Three behavioural deficits that are associated with AD were assessed in this study, spatial learning, spatial memory and anosmia. Spatial learning and spatial memory were assessed using the Morris Water Maze, which is a well established test of rodent cognitive abilities. Anosmia was assessed using a simple Buried Food Pellet Test, which is called the Buried Chocolate Test. This test is appropriate to use to assess olfactory abilities in AD-type mice, as it does not require intact learning abilities but relies on behaviours that are natural for mice (i.e. digging).

Beta-Amyloid Neuropathology.

Amyloid neuropathology in Amy mouse brains have been investigated in this project using immunohistochemical and immunofluorescent techniques. First, amyloid deposits throughout the brains of Amy mice are characterised. Then the effect of diet on these deposits is investigated.

Telomere Length.

Owing to the limited amount of literature on telomere length in the brains of AD-type mice a small exploratory study was also conducted to investigate telomere length in the brains of 15 and 18 month old Amy mice.

Chapter 2: The design of the Oz-AIN rodent diet and characterisation of its effects on food intake, weight gain and obesity in normal and Amy mice.

2. Background.

Diet is a major modifiable risk factor for AD [255]. Undernutrition and malnutrition are associated with increased AD risk in the elderly and accelerate cognitive decline and neuropathology in AD rodent models [101, 256-259]. Dietary supplementation delays the onset of AD neuropathology and slows cognitive decline in rodent models of AD [157, 220].

The aims of this research address the hypotheses that:

1. An Australian-type rodent diet accelerates the behavioural deficits and β-amyloid neuropathology that are observed in Amy mice.

2. Nutrient supplements can reduce the severity of genotype or diet-type induced behavioural deficits and β-amyloid neuropathology in this mouse model.

The Australian-type rodent diet designed to test these hypotheses was based on diets that are typically consumed by Australian adults, in particular, Australian women. Sixty-three percent of AD patients above 65 years of age are women [172]. Nutritional intakes and requirements of Australian women differ substantially from those of Australian men [185, 260, 261]. Diets that are typically eaten by Australian women are high in total fat (33% kcal) with an imbalanced polyunsaturated: monounsaturated: saturated fats ratio (P:M:S) (1.0: 2.4: 2.7) and are also low in

essential vitamins such as folate and calcium [262]. These are also characteristics of diets that have been associated with increased AD risk [183, 208, 255, 263-265]. Therefore, the Australian-type diet that was developed for this research was based on the typical diet consumed by Australian women.

Humans and rodents have different metabolic rates and nutritional requirements [266-270]. It is not appropriate to develop rodent diets that contain the same levels of macronutrients and micronutrients as those in diets consumed by people. For example, unlike humans, mice do not require dietary vitamin C as a component of their ordinary diet [266] and altering dietary vitamin C in a rodent diet many not have the same effect that it does in humans. Therefore, two novel rodent diets have been designed in preparation of this thesis, to investigate the effect of nutrition on the development and progression of AD in a mouse model.

The first of these diets is the 'Oz-AIN diet'. The Oz-AIN diet reflects the degree that macronutrients and micronutrients that are typically consumed by Australian women vary from recommended levels (Figure 2). For example, Australian women only obtain three quarters of the recommended amount of calcium from their diet [185]. Therefore, the Oz-AIN diet only contains three quarters of the amount of calcium required to maintain optimal health in rodents. This diet has been fed to mice to investigate the effect of an Australian-type diet on behavioural deficits and β-amyloid neuropathology in AD, and is used to test the first hypothesis of this thesis. The design and formulation of the Oz-AIN diet and the characterisation of its effects on food consumption, weight gain and adiposity in normal and Amy mice are addressed in the current chapter.

The second diet is called the 'Oz-AIN Supp diet', and has been constructed from the Oz-AIN diet with additional nutrient supplements. The Oz-AIN Supp diet was

designed to explore the potential benefits of nutrient supplements on β-amyloid neuropathology and behavioural deficits in a mouse model of AD, testing the second hypothesis of this research. The design and formulation of the Oz-AIN Supp diet and the characterisation of its effects on normal and Amy mice is addressed in Chapter 3.

The third diet used in this study is the AIN93-M diet for rodents. The AIN93-M diet is well established as a standard rodent chow and is often used in rodent studies as a control diet [106, 266, 271]. It is a fabricated rodent diet that has been designed to maintain optimal laboratory rodent health [266].

The first half of this chapter describes the design and construction of the Oz-AIN diet. The second half of this chapter aims to characterise the effect Oz-AIN diet when it is fed to normal mice and Amy mice from weaning. Investigations are made into the effect of genotype (normal versus Amy mice) and diet-type (AIN93-M diet versus Oz-AIN diet) on food consumption, weight gain, and the weight of fat deposit and major organs at death.

One of the primary findings of the study described in chapter is that while the Oz-AIN diet induced weight gain in normal and Amy mice, this was substantially pronounced in Amy mice. Amy mice that were fed the Oz-AIN diet reached weights that were comparable with those in models of diet-induced obesity [272-274]. This weight gain was not as rapid as observed in diet-induced obesity models, probably due to the fact that the Oz-AIN diet contained 33.0% (kcal) fat, whilst diet-induced obesity models can be above 45% (kcal) fat [272-274]. However, the Oz-AIN diet was not intended to be a model of diet-induced obesity. Potential dietary factors driving the rapid weight gain of normal and Amy mice fed the Oz-AIN diet include total fat content, P:M:S ratio, and increased levels of micronutrients such as zinc, which have been demonstrated to enhance weight gain [275].

It is concluded that the Oz-AIN diet is a good model of dietary patterns of Australian women. Furthermore, the finding that Amy mice that were fed the Oz-AIN diet were susceptible to diet-induced obesity and adiposity suggests that the Oz-AIN diet was able to modify risk factors for AD, and may also affect other processes in AD pathogenesis.

2.1. Methods.

2.1.1. Animals.

All experiments were approved by the Commonwealth Scientific and Industrial Research Organisation (CSIRO) Animal Welfare Committee, Australia in accordance with National Health and Medical Research Council guidelines.

Female Amy (APPswe/PSEN1dE9) mice and their female normal (C57bl/6) littermates were bred at Flinders University Animal Facility, Bedford Park, South Australia. Genotype was confirmed by PCR and agarose gel electrophoresis, as described in Appendix I. Amy and normal mice were each fed either the Oz-AIN diet or the AIN93-M diet from weaning until 18 months of age. Aging mice developed bald spots on the back of the neck and abdomen, as a result of over-grooming by dominant cage mates. Less-dominant mice developed open sores in the balding areas, which became infected. Attempts to treat infection (such as isolation and application of Neotopic-H lotion (Delvit, NSW)) were unsuccessful, and lead to increased inflammation and death. Therefore, group sizes at 75 weeks are smaller than at the start of the study. Group sizes every 10 weeks post-weaning are reported in Table 1.

Body weight was measured twice weekly. Mice were housed in cages that were lined with sawdust and had tunnels and tissues as environmental enrichment and had free

access to food and water (n<6 per cage) (Figure 1A). Mice had access to their respective diets from either the top of the cage and from food bowls that had been placed within the cage (Figure 1B). This approach provided two benefits:

(i) Food at the top of the cage was kept clean and free from saw-dust.

(ii) Food in bowls is easily accessible, and easier for older mice to reach.

Twice a week the amount of diet remaining in the food bowls and on the top of the cage was recorded and fresh diet was returned to the cage. This minimized oxidation of the fats in the Oz-AIN diet.

At the end of the experiment mice were anaesthetised with isoflurane and killed by exsanguination from the abdominal aorta. Mice were perfused with PBS and fat tissue, liver, kidneys, heart, and spleen were collected and weighed. Organs were frozen in liquid nitrogen and stored in the CSIRO bio-bank. Uterine fat tissue, renal fat tissue, and fat tissue lining the skin were stored in RNAlater Stabilization Reagent (76106, Qiagen).

Table 1. Group sizes every 10 weeks post weaning of normal and Amy mice that were fed either the AIN93-M diet or the Oz-AIN diet for 18 months.

	Normal mice AIN93-M diet	Normal mice Oz-AIN diet	Amy mice AIN93-M diet	Amy mice Oz-AIN diet
5 weeks	n = 12	n = 12	n = 9	n = 9
15 weeks	n = 12	n = 12	n = 8	n = 9
25 weeks	n = 12	n = 12	n = 8	n = 9
35 weeks	n = 12	n = 12	n = 8	n = 9
45 weeks	n = 12	n = 12	n = 8	n = 9
55 weeks	n = 12	n = 12	n = 8	n = 9
65 weeks	n = 9	n = 12	n = 5	n = 7
75 weeks	n = 9	n = 12	n = 5	n = 7

Figure 1. Mouse housing conditions.

Figure 1B. Access to food and water.

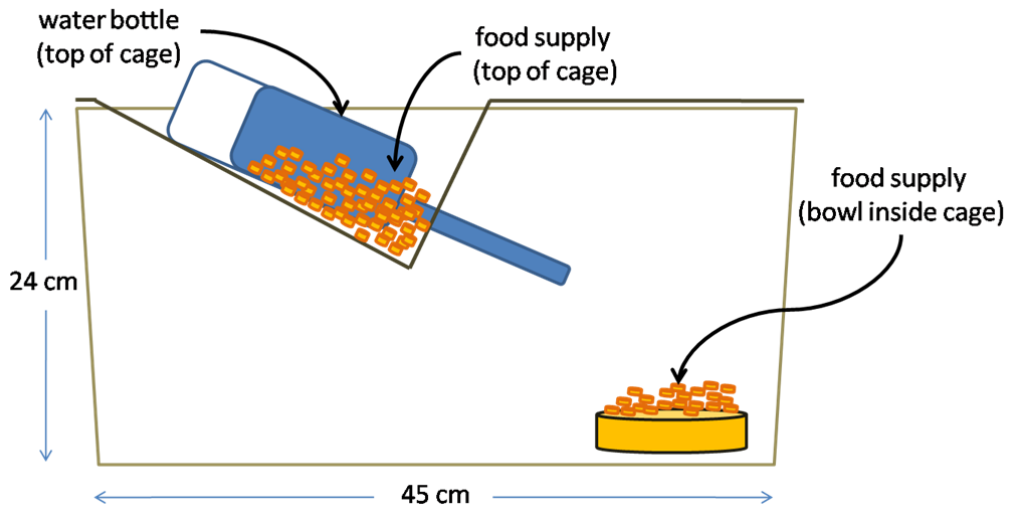


Figure 1A. Mice were housed (n<6) in sawdust lined cages with free access to food and water. Plastic tunnels and a tissue were also placed in the cage to provide environmental enrichment. The yellow card in the figure is an identification card that was attached to the top of the cage to enable identification of mice.
Figure 1B. Mice had access to their food from the top of the cage and from bowls placed inside the cage.

2.1.2. Study design.

To characterise the effects of the Oz-AIN diet on food consumption, weight gain and the weight of major organs at death, this study made use of a two-factorial design. One factorial was genotype (normal versus Amy mice) and the other was diet-type (AIN93-M diet versus Oz-AIN diet).

2.1.3. Data analysis.

Every five weeks, comparisons of mean (±SEM) mouse body weight (g) and food consumption (g/day) were made between diet-matched mice and genotype-type matched mice using Student's *t*-tests. Diet-type effects were determined by comparisons between genotype-matched mice that were fed either the AIN93-M or the Oz-AIN diet. Genotype effects were determined by comparisons between diet-matched normal and Amy mice.

The area under the curves for food intake and energy intake were used to estimate overall food consumption (g) and energy intake (kJ). Genotype and diet-type effects overall food consumption (g) and energy intake (kJ) were determined using two-way ANOVA's and Bonferroni post tests. Genotype and diet-type effects on mean (±SEM) organ weight (g) and fat deposits (g) were also determined using two-way ANOVA and Bonferroni post tests. Unless otherwise indicated statistical significance was established at $p<0.05$.

2.2. Design of the Oz-AIN diet.

The Oz-AIN diet is a rodent diet has been designed to reflect the nutrient content in diets typically consumed by Australian women [185]. It is not appropriate to feed mice the same levels of 'actual' nutrients as described in the 1995 NNS, due to the differing metabolism and nutrient requirements between mice and humans [261, 266-270]. Therefore, where possible, the amount of each micronutrient that is considered optimal to maintain rodent health was modified by the degree that the 'actual' nutrient consumption by middle aged Australian women varies from 'recommended' nutrient intake and used to create the Oz-AIN diet (Figure 2).

The macronutrient content was modelled as best as could be calculated, to reflect the carbohydrate (% kcal), fat (% kcal) and protein (% kcal) in the diets consumed by Australian women [185]. Macronutrient levels could not be adjusted to reflect the degree that consumption varies from recommended levels because there are no recommended dietary intakes for adult consumption of carbohydrates and total fats [261]. The NHMRC Nutrient Reference Values for Australians and New Zealand (NRV) refers consumers to the US:Canadian DRI review recommendations for guidelines regarding optimal fat and carbohydrate intake [261]. The US:Canadian DRI review recommendations advise that consumption of these macronutrients be ".. a*s low as possible while consuming a nutritionally adequate diet";* and that carbohydrate intake be "… *no more than 25% of total energy."* [276]. However, no further guidelines are described. Therefore, the macronutrient content of the Oz-AIN diet is a direct reflection of protein, carbohydrates and dietary fats within diets that are typically consumed by Australian women (Figure 3, Table 2) [185].

The micronutrient content reflects the degree that vitamin and mineral content in a diet typically consumed by Australian women varies from recommended levels. The

amount of each micronutrient that is considered optimal to maintain rodent health was modified by the amount that the 'actual' micronutrient consumption by middle aged Australian women varies from 'recommended' micronutrient intakes (Figure 2) [185, 261, 266].

In all calculations, levels of nutrients that are considered optimal to maintain rodent health are the same as those outlined by the American Institution of Nutrition and are described in the AIN93-M diet for rodents [266]. This diet is currently considered gold standard for maintaining optimal health in laboratory rodents and is therefore commonly used in a variety of fields of research, including nutrition. While the AIN93-M diet has been used to represent an "optimal" rodent diet throughout the current thesis, its design and formulation are not discussed in detail. However, the micro-nutrient contents of the AIN93-M diet are presented alongside the contents of the Oz-AIN diet in Tables 6 and 7 of the current chapter. The macronutrient content and energy content are described in Chapter 3, Table 6.

Figure 2. Formula used to calculate the amount of each adjusted micronutrient in the Oz-AIN diet.

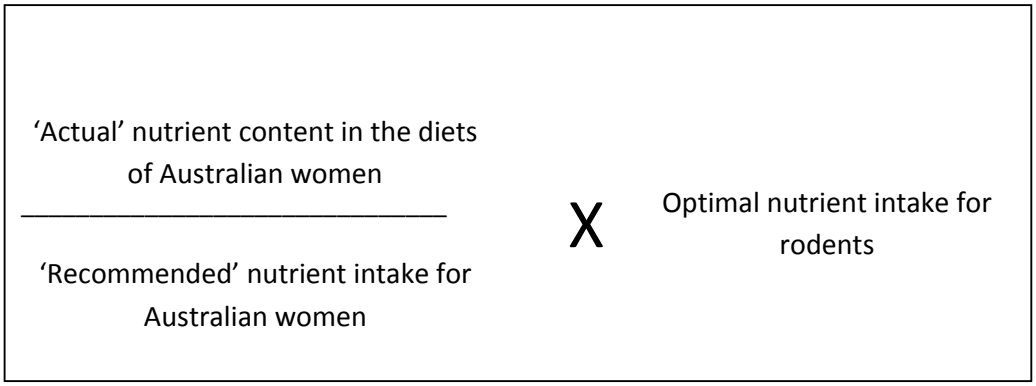


Figure 2. The amount of each adjusted micronutrient in the Oz-AIN diet was calculated by calculating a ratio of the 'actual' nutrient intakes by Australian women to 'recommended' nutrient intakes, and applying that ratio to 'optimal nutrient intakes' for rodents.

2.2.1. Determining 'recommended' nutrient intake for Australian women.

The recommended amounts of vitamin and mineral intake for Australian women aged between 30 and 51 years old as described in the 2005 Nutrient Reference Values for Australia and New Zealand [261] were used to define 'recommended' vitamin and mineral intake for Australian women (Table 3, Table 4 respectively). These values describe the "*average daily intake level that is sufficient to meet the nutrient requirements of nearly all (97 – 98 per cent) healthy individuals in a particular life stage and gender group*" [261] and are therefore an appropriate representative of nutrient requirements for healthy Australian women.

2.2.2. Determining 'actual' nutrient intake for Australian women.

Data from the 1995 National Nutritional Survey (1995 NNS) was used to define the 'actual' nutrient intake in the diets consumed by Australian women, and thus to calculate the nutrient content in the Oz-AIN diet (Table 2, Table 3, Table 4) [185]. The 1995 NNS was an Australia-wide, 24-hour food recall survey to assess macronutrient (fat, carbohydrate, and protein) and micronutrient (vitamin and mineral) intake for Australian men, women and children. The diet consumed by Australian women aged between 25 and 44 years old was used to represent the 'actual' nutrient intake by Australian women (Table 2, Table 3, Table 4) [185]. Later studies by Mishra are in agreement with the findings of the 1995 NNS, validating the use of this data to represent the 'actual' nutrient intake of Australian women [260, 277].

In the 1995 NNS, a diet that is typically consumed by Australian women is comprised of 46.7% kcal carbohydrates (20.2% from sugars and 26.5% from starch),

16.8% kcal protein, and 33.0% kcal total fats. The total fat content of the diet had a P:M:S ratio of 1.0:2.4:2.7 (Table 2, Table 5). The ω-3: ω-6 fatty acid ratio may also play a role in diet-induced diseases. However, the 'actual' ω-3: ω-6 fatty acids ratio in the diets that are typically consumed by Australian women was not reported in the 1995 NNS. Studies of polyunsaturated fat content in Australian-type diets have estimated that the ω-3: ω-6 ratio in typical Australian diets is 1.0:9.0 [278, 279]. However, these studies describe nutrient intakes of the Australian population as a whole, and have not reported the ω-3: ω-6 ratios in diets specifically eaten by Australian women. Harris *et al.* report that docosahexaenoic acid and eicosapentaenoic acid intake tends to be lower in females than males, suggesting that their ω-3: ω-6 ratio is lower than 1.0:9.0. Therefore, the ω-3: ω-6 ratio of the diets typically consumed by Australian women was estimated to be 1.0:10.0.

The vitamin and mineral levels in diets typically consumed by Australian women, as described in the 1995 NNS were also used to represent 'actual' micronutrient levels for calculating micronutrient content of the Oz-AIN diet. The vitamins described by the 1995 NNS were: vitamin A, thiamin, riboflavin, niacin, folate, and vitamin C (Table 3). The minerals described by the 1995 NNS include: calcium, phosphorus, magnesium, zinc, iron and potassium (Table 4) [185]. Whilst the 1995 NNS is a comprehensive dietary study, there was no data available to describe mean daily intake of vitamin B6, pantothenic acid, biotin, vitamin D, vitamin E, vitamin K, choline, iodine, selenium, molybdenum, chromium, fluoride and sodium. Therefore, these components were not adjusted whilst formulating the Oz-AIN diet (Table 3, Table 4). The 1995 NNS reported that Australian women consume more than twice the recommended amount of vitamin C. However vitamin C was excluded from the Oz-AIN vitamin mix as mice do not require Vitamin C as a component of their diet and it is not a component of the AIN93-M diet [185, 261, 266].

Table 2. Macronutrient content in the diets described in the 1995 National Nutrition Survey for Australian women.

Macronutrient	**1995 National Nutrition Survey**
Protein (% total energy)	16.80%
Carbohydrate (% total energy)	46.70%
Sugar (% total energy)	20.20%
Starch (% total energy)	26.50%
Total fat (% total energy)	33.00%
Saturated fat (% total energy)	28.70%
Monounsaturated fat (% total energy)	26.00%
Polyunsaturated fat (% total energy)	10.80%

Table 3. Comparing the recommended vitamin intake in the 2005 Nutrient Reference Values for Australian women[Ξ] with the vitamin intake described in the 1995 National Nutrition Survey[Θ].

Vitamin	2005 Nutrient Reference Values[Ξ]	1995 National Nutrition Survey[Θ]	% difference
Vitamin A (Retinol Equivalent) (µg/day)	700.00	1024.40	46.34 %
Vitamin B1 (Thiamin) (mg/day)	1.10	1.40	27.27 %
Vitamin B2 (Riboflavin) (mg/day)	1.10	1.80	63.64 %
Vitamin B6 (Pyridoxine) (mg/day)	1.30	N/A[†]	N/A[†]
Vitamin B12 (Cobalamin) (µg/day)	2.40	3.41	42.08 %
Folate Equivalent (µg/day)	400.00	227.00	-43.25 %
Vitamin C (Ascorbic acid) (mg/day)	45.00	108.50	141.11 %
Vitamin D (Cholecalciferol) (µg/day)	5.00	N/A[†]	N/A[†]
Vitamin E (Tocopherol) (mg/day)	7.00	N/A[†]	N/A[†]
Biotin (µg/day)	25.00	N/A[†]	N/A[†]
Vitamin K (µg/day)	60.00	N/A[†]	N/A[†]

(Ξ) Data from the 2005 Nutrient Reference values describe the recommended dietary intake values that are sufficient to meet the nutrient requirements of 19-30 year old Australian women.

(Θ) Data from the 1995 National Nutrition Survey describe the mean daily intake of nutrients of Australian women aged 25-44 years old.

(†) Mean daily intake for vitamin B6, vitamin D, vitamin E, biotin and vitamin K were not available.

Table 4. Comparing the recommended mineral intake in the 2005 Nutrient Reference Values for Australian women[Ξ] with the mineral intake described in the 1995 National Nutrition Survey[Θ].

Mineral	2005 Nutrient Reference Values[Ξ]	1995 National Nutrition Survey[Θ]	% difference
Calcium (mg/day)	1000.00	762.10	-23.79 %
Choline (mg/day)	425.00	N/A[†]	N/A[†]
Chromium (µg/day)	25.00	N/A[†]	N/A[†]
Copper (mg/day)	1.20	1.16	-3.33 %
Fluoride (mg/day)	3.00	N/A[†]	N/A[†]
Iodine (µg/day)	150.00	N/A[†]	N/A[†]
Iron (mg/day)	18.00	12.00	-33.33 %
Magnesium (mg/day)	310.00	283.60	-8.52 %
Manganese (mg/day)	5.00	3.27	-34.60 %
Molybdenum (µg/day)	45.00	N/A[†]	N/A[†]
Niacin Equivalent (mg/day)	14.00	35.30	152.14 %
Pantothenic Acid (mg/day)	4.00	N/A[†]	N/A[†]
Phosphorus (mg/day)	1000.00	1299.80	29.98 %
Potassium (mg/day)	2800.00	2816.30	0.58 %
Selenium (µg/day)	60.00	N/A[†]	N/A[†]
Sodium (mg/day)	460-920	N/A[†]	N/A[†]
Zinc (mg/day)	8.00	9.90	23.7 %

(Ξ) Data from the 2005 Nutrient Reference Values describe the recommended dietary intake values that are sufficient to meet the nutrient requirements of 19-30 year old Australian women.

(Θ) Data from the 1995 National Nutrition Survey describe the mean daily intake of nutrients of Australian women aged 25-44 years old.

(†) Mean daily intake for choline, chromium, fluoride, iodine, molybdenum, pantothenic acid, selenium and sodium were not available.

Figure 3. Macronutrient content of the Oz-AIN diet as a percentage of total energy.

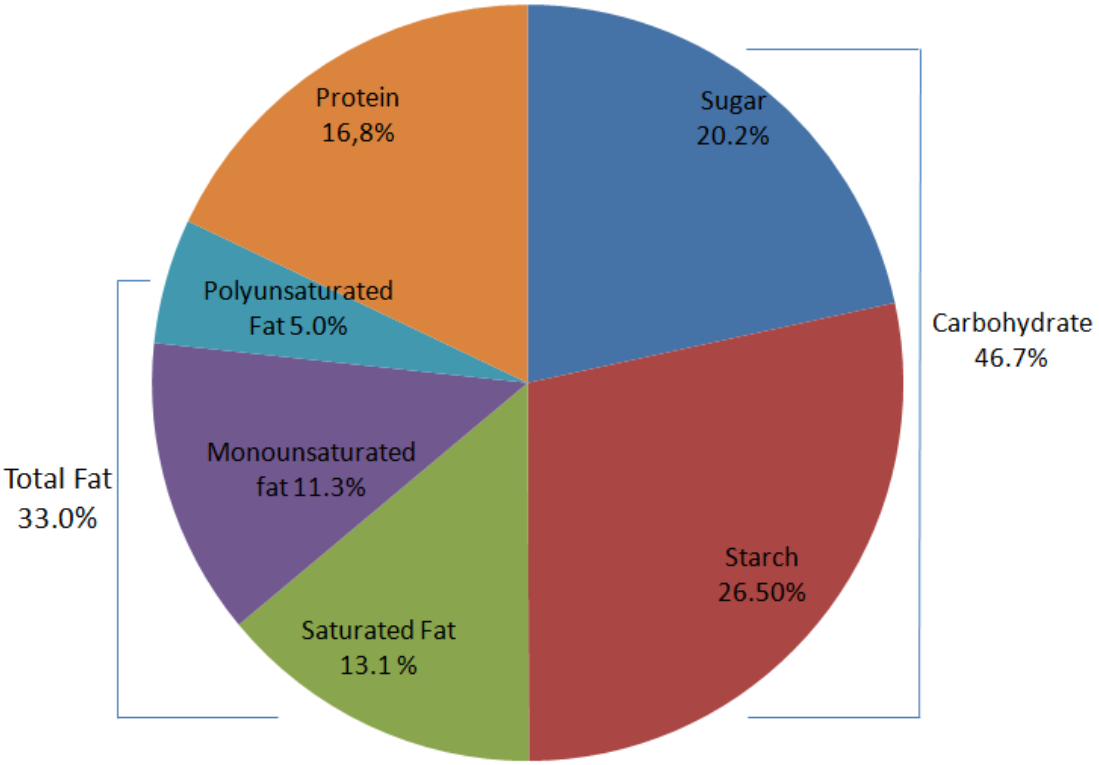


Figure 3. The macronutrient content of the Oz-AIN diet was developed to directly reflect the macronutrient content of diets that are typically consumed by Australian women, as defined by the 1995 National Nutrition Survey.

2.2.3. Designing the macronutrient content of the Oz-AIN diet.

The macronutrient content of the Oz-AIN diet was designed to directly reflect the specific macronutrient content in a diet typically consumed by Australian women (Table 2, Figure 3). Whilst optimal dietary intake of fats and carbohydrates has been identified for laboratory rodents [266], no such values exist for human consumption in Australia [261]. Therefore, it was not possible to determine the degree to which macronutrient intake varied from dietary requirements. The values for each macronutrient in the Oz-AIN diet were set to reflect the amount of total energy in the diet typically consumed by Australian women that was derived from sugars, starch, protein, and dietary fats. Careful attention was also made to replicate the P:M:S ratio and the ω-3: ω-6 content of these diets.

Similar to other westernised rodent diets, the Oz-AIN diet is higher in total fats and lower in carbohydrates than rodent diets. Standard rodent diets provide around 10% energy from fat, 75% energy from carbohydrate and 14% energy from protein [266]. Typical westernised rodent diets are generally high in fat (30% - 40% kcal) at the expense of carbohydrate (40% - 50% kcal) [274, 280, 281]. These westernised rodent diets do not necessarily characterise the type of fats in a diet typically consumed in western cultures, which has 2.7 times more saturated fat than polyunsaturated fat and an ω-3: ω-6 ratio of approximately 1:10 (Table 5)[185, 280-282].

The fat, protein and carbohydrate content of the Oz-AIN diet directly reflect the carbohydrate: fat: protein ratio (% kcal) in diets consumed by Australian women [185]. As such, the Oz-AIN diet is 33.00% (kcal) fat, 16.8% (kcal) protein and 46.7% (kcal) carbohydrate (Figure 3, Table 2). Casein was used as the source of protein, and white sucrose and starch provided total carbohydrate. Total fats were derived from a combination of melted lard (P:M:S of 1.0:4.4:4.2) and coconut oil

(P:M:S of 1.0:3.0:41.5), vegetable oil (P:M:S of 5.3:10.3:1.0), sunflower oil (P:M:S of 5.8:1.6:1.0), and olive oil (P:M:S of 1.0:6.5:1.5) (Table 7).

The different oils and fats were combined in amounts based on their P:M:S ratios to achieve a final P:M:S ratio of 1.0:2.4:2.7, which reflects the P:M:S ratio in the diets consumed by Australia women (Table 5)[185]. Saturated fats were predominantly derived from the melted lard and coconut oil. Oils such as palm oil (P:M:S of 1.0:4.0:5:3) or hydrogenated palm oil (P:M:S ratio of 1:5.4:6.3) are also used as a source of saturated fats rodent diets, owing to their high saturated fat content [283-285]. However, palm oil was excluded from construction of the Oz-AIN diet, as it is also relatively high in monounsaturated fats, which could potentially confound the efforts to manipulate the P:M:S ratio of the Oz-AIN diet. Instead, coconut oil (P:M:S ratio of 1.0:3.0:41.5) was used as the primary source of saturated fats, as it has negligible levels of monounsaturated and polyunsaturated fats. Coconut oil also has a high melting point and remains solid at room temperature. This enabled construction of a hard diet that was less prone to crumble or dissolve into the sawdust bedding in mouse cages. Sunflower oil provided most of the polyunsaturated fat content, particularly ω-6 fats, lowering the ω-3: ω-6 ratio to the desired level of 1:10 (Table 5). The final P:M:S ratio is 1.0:2.4:2.7, which represents the P:M:S ratio of fats consumed by Australian women [185] (Table 2, Table 5).

2.2.4. Designing the micronutrient content of the Oz-AIN diet.

The micronutrient content of the Oz-AIN diet was designed by calculating the 'actual' nutrient intake [185, 277] to 'recommended' nutrient intake [261] ratios for the vitamins and minerals typically found in diets consumed by Australian women (Figure 2, Table 2). These ratios were used to adjust nutrient levels that are recommended to maintain optimal health of laboratory mice [266], which determined

the vitamin and mineral levels in the vitamin mix and the mineral mix used in the Oz-AIN diet (Table 6, Table 8, Table 9).

The most striking differences between the micronutrient content of the Oz-AIN diet and the AIN93-M diet is the imbalance of B vitamins in the Oz-AIN diet. The Oz-AIN diet is deficient the essential vitamin folate (43% less), and contains excessive amounts of vitamin B12 (excess of 42.08%), niacin (excess of 152.13%), and riboflavin (excess of 63.67%).

Niacin is recognised for its roles in lipid metabolism, and is discussed further in this capacity on page 87. Dietary niacin may also play a role in AD prevention. In a prospective study of independently living adults (>65 years old), Morris *et al.* report that dietary niacin intake was associated with better cognitive function and low AD incidence [286]. This suggests that dietary niacin, within normal recommended intakes, may have a role in AD prevention.

Folate and vitamin B12 are powerful antioxidants and play important roles in DNA and RNA synthesis, and in homocysteine remethylation [287]. Folate and vitamin B12 deficiency are associated with increased AD risk [287-291]. Low folate status is associated with impaired memory in humans and mice [292-295], and folate, vitamin B6 and vitamin B12 deficiencies increase β-amyloid deposition in AD-type mice [296].

The detrimental effects of folate and vitamin B12 deficiency are likely to be due to their roles in homocysteine metabolism [295], as elevated plasma homocysteine is associated with neurological impairments such as dementia, learning difficulties, brain atrophy and AD [289, 297, 298]. However, the detrimental effects of imbalanced B vitamin intake may also occur through mechanisms independent of

homocysteine remethylation [288, 295]. Folate is required for *de novo* synthesis of purine and thymidylate synthase building blocks for DNA and RNA synthesis [287, 299]. Therefore, folate may be beneficial in preventing DNA damage with aging, and reduce AD pathology. Consistent with this The Oz-AIN diet is low in folate, which may have an effect on cognitive functioning and β-amyloid pathology.

Table 5. Ratio of the fats and oils required to give a final P:M:S ratio of 1.0:2.4:2.7 and an ω-3: ω-6 ratio of 1:10.

	grams	Polyunsaturated Fats	Monounsaturated Fats	Saturated Fats	ω-3	ω-6	ω-3: ω-6
Lard	0.80	8	35.2	33.6	0	8	
Canola oil	0.40	8.8	24.8	2.4	4	8.8	
Sunflower oil	0.30	20.4	5.7	3.6	0.3	20.4	
Coconut oil	1.10	2.2	6.6	91.3	0	2.2	
Olive oil	0.70	11.2	49.7	7	0.7	11.2	
TOTAL	3.30	50.6	122	137.9	5	50.6	1:10

Table 6. Comparison of the micronutrients in the AIN93-M and Oz-AIN diet.

Micro-nutrient	AIN93-M diet (mg/kg)	Oz-AIN diet (mg/kg)	Δ %
Vitamin A (Retinol)	2.40	3.50	45.83 %
Vitamin B1 (Thiamin)	5.00	6.36	27.20 %
Vitamin B2 (Riboflavin)	6.00	9.82	63.67 %
Vitamin B6 (Pyridoxine)	6.00	6.00	0.00 %
Vitamin B12 (Cyancobalamin)	0.025	0.035	42.08 %
Folate (Folic acid)	2.00	1.14	-43.00 %
Vitamin D (Cholecalciferol)	2.50	2.50	0.00 %
Vitamin E (α-tocopherol)	33.80	33.75	-0.15 %
Vitamin K (Menadione)	0.90	0.90	0.00 %
Biotin	0.20	0.20	0.00 %
Choline	1000.00	1000.00	0.00 %
Niacin (Nicotinic acid)	30.00	75.64	152.13 %
Pantothenic acid	15.00	15.00	0.00 %
Calcium	5000.0	3810.5	-23.79 %
Phosphorus	3000.0	3899.4	29.98 %
Sodium	1033.0	1033.0	0.00 %
Chloride	1613.0	1613.0	0.00 %
Potassium	3600.0	3621.0	0.58 %
Magnesium	511.0	452.9	-11.37 %
Sulphur	300.0	300.0	0.00 %
Zinc	35.0	43.3	23.71 %
Iron	45.0	30.0	-33.33 %
Iodine	0.2	0.2	0.00 %
Manganese	10.0	6.5	-35.00 %

(continued over page)

Table 6. Comparison of the micronutrients in the AIN93-M and Oz-AIN diet *(continued)*.

Copper	6.0	6.0	0.00 %
Molybdenum	0.15	0.15	0.00 %
Selenium	0.17	0.17	0.00 %
Chromium	1.0	1.0	0.00 %
Fluoride	1.0	1.0	0.00 %
Lithium	0.1	0.1	0.00 %
Boron	0.5	0.5	0.00 %
Nickel	0.5	0.5	0.00 %
Vanadium	0.1	0.1	0.00 %
Iodine	0.2	0.2	0.00 %
Silicon	5.0	5.0	0.00 %

Table 7. Composition of the Oz-AIN diet.

Ingredient	**g / kg**
Sugar (SUGW/25, FTA Food Solutions, Vic, Aus)	243.8
Starch (National Starch, Lane Cove, NSW, Aus)	309.6
Lard (Conroys Small Goods, Bowden, SA, Aus)	44.8
Canola Oil (Coles Supermarkets Australia)	22.4
Sunflower Oil (Crisco; Goodman Fielder, North Ryde, NSW, Aus)	16.8
Coconut Oil (Nui; African Pacific Pty. Ltd., Terry Hills, NSW, Aus)	61.6
Olive Oil (Coles Supermarkets Australia)	39.2
Protein (acid casein 1704896-6, Fonterra Ltd., Auckland, NZ)	193.9
Fibre (α-cellulose, C-8002, Sigma-Aldrich)	38.0
Vitamin mix	15.0
Mineral mix	15.0
TOTAL	**1000.0**

Table 8.Vitamin Mix for the Oz-AIN diet.

Vitamin	IU / 10 kg diet	mg / 10 kg diet
Vitamin A (Retinol) (IU) (Thompsons Auckland, NZ)	58,540.0	58,540.0
Vitamin D (Cholecalciferol) (IU) (Blackmores, NSW, Aus)	10,000.0	10,000.0
Vitamin E (α-tocopherol) (Sigma-Aldrich, Aus)		338.0
Vitamin K (Menadione) (47775, Supelco, Aus)		9.0
Choline (C7017, Sigma-Aldrich, Aus)		10,000.0
Vitamin B1 (Thiamin) (T4625, Sigma-Aldrich, Aus)		64.0
Vitamin B2 (Riboflavin) (R-4500, Sigma, Aus)		98.0
Niacin (Nicotinic acid) (N0761, Sigma, Aus)		756.0
Vitamin B6 (Pyridoxine) (P5669, Sigma, Aus)		60.0
Pantothenic acid (P5155, Sigma, Aus)		150.0
Folate (Folic acid) (F8758, Sigma-Aldrich, Aus)		11.0
Vitamin B12 (Cyancobalamin) (Blackmores, NSW, Aus)		0.4
Biotin (B4639, Sigma, Aus)		2.0
Sucrose (SUGW/25, FTA Food Solutions, Vic, Aus)		69,971.6
TOTAL		**150,000.0**

Table 9. Mineral Mix for the Oz-AIN diet.

Mineral	mg / 10 kg diet
Calcium Carbonate (10068, BDH Chemicals, Aus)	40,810.5
Potassium Phosphate (P5379, Sigma, Aus)	38,219.4
Potassium Citrate●H_2O (60153, Sigma-Aldrich, Aus)	1,903.8
Sodium Chloride (S9888, Sigma-Aldrich, Aus)	11,100.0
Potassium Sulphate (P9458, Sigma-Aldrich, Aus)	7,025.0
Magnesium Oxide (243388, Sigma-Aldrich, Aus)	3,190.5
Ferric Citrate (F-6129, Sigma-Aldrich, Aus)	606.0
Zinc Carbonate (ZL004, Chem Supply, SA, Aus)	306.3
Manganous Carbonate (29136, BDH Chemicals, Aus)	61.8
Copper (II) Carbonate (CL035, Fluka, Gilman, SA, Aus)	45.0
Potassium Iodide (60399, Fluka, Gilman, SA, Aus)	1.5
Sodium Selenate (S5261, Sigma, Aus)	1.5
Ammonium Paramolybdate●$4H_2O$ (09878, Fluka, Gilman, SA. Aus)	1.2
Sodium Metasilicate (S4392, Sigma, Aus)	217.5
Chromium Potassium Sulphate $12H_2O$ (S243361, Sigma, Aus)	41.5
Lithium Chloride (LL036, Chem Supply, SA, Aus)	2.6
Boric Acid (B6768, Sigma, Aus)	12.2
Sodium Fluoride (10246, AnalaR, British Drug Houses, Eng)	9.5
Nickel Carbonate (NL007, Chem Supply, SA, Aus)	4.8
Ammonium Vanadate (AL072, Chem Supply, SA, Aus)	1.0
Sucrose (SUGW/25, FTA Food Solutions, Vic, Aus)	46,438.4
TOTAL	**150,000.0**

2.2.5. Production of the vitamin and mineral mixes in the Oz-AIN diet.

The Oz-AIN vitamin mix and Oz-AIN mineral mix were constructed independently as per Table 8 and Table 9 respectively. Both mixes were made up to 15 g /kg with commercially available sucrose that is used for human consumption. To prevent oxidation of vitamins and minerals within each mix, both mixes were stored in airtight bags at 4°C until required.

2.2.6. Production of the Oz-AIN diet.

The Oz-AIN diet was constructed as per Table 7. Starch, casein, fibre, Oz-AIN vitamin mix, and Oz-AIN mineral mix were combined at room temperature. Lard and coconut oil were gently melted and combined with vegetable oil, sunflower oil, and olive oil in a stainless steel bowl placed in a 37°C water bath, before being added to the powdered components of the diet. Sugar was then dissolved in 80 mL water and added to the diet mix. The diet was then solidified by adding the dissolved sugar and set at 4°C.

Oz-AIN diet was made weekly in one kg batches and stored in sealed glass containers 4°C for no longer than 7 days to minimize the likelihood of oxidation.

2.3. Characterisation of the effect of Oz-AIN diet and AIN93-M diet on food consumption and energy intake for normal and Amy mice.

2.3.1. An evaluation of the amount of food eaten by normal and Amy mice throughout the study.

A two-way ANOVA revealed significant genotype ($p=0.001$) and diet-type ($p=0.02$) effects on the overall amount of food eaten by normal and Amy mice that had been fed either the AIN93-M diet or the Oz-AIN diet (Table 10). Genotype accounted for 58.02% of the overall variance of food eaten and diet-type accounted for 21.28% of overall variation. There were no significant interaction effects ($p=0.72$, Table 10).

Comparisons between treatment groups suggested that over 18 months, normal mice that were fed the AIN93-M diet ate the least and Amy mice that were fed the Oz-AIN diet ate the most food (Table 10). While this was significantly different ($p=0.004$) these two groups are not easily comparable as they do not share a common factor and there was no significant interaction. Bonferroni post tests revealed that genotype effects could be attributed to the differences in the amount of food eaten by normal and Amy mice that were fed the AIN93-M diet, as the normal mice that were fed the AIN93-M diet ate significantly less than Amy mice fed the AIN93-M diet ($p=0.04$, Table 10).

Table 10. The effect of diet-type or genotype on the amount of food eaten (g) over 18 months by normal and Amy mice that were fed the AIN93-M or the Oz-AIN diet

	Normal mice	Amy mice
AIN93-M diet	1500.67 ±28.09 g $^{\Delta\,\Phi}$	1669.33 ±44.66 g $^{\Delta}$
Oz-AIN diet	1607.67 ±29.63 g	1751.67 ±24.92 g $^{\Phi}$

The overall amount of food eaten by normal or Amy mice was calculated from the area under the curve. Values are mean ±SEM. A two-way ANOVA detected significant diet-type effects (p=0.02), genotype effects (p=0.001). Values with matching symbols are significantly different with Bonferroni post tests. (Φ) p=0.004. (Δ) p=0.04.

2.3.2. The effect of genotype on the amount of food eaten by normal and Amy mice that were fed either the AIN93-M diet or the Oz-AIN diet.

Normal and Amy mice that were fed the AIN93-M diet.

Amy mice that were fed the AIN93-M diet ate more than normal mice that were fed the AIN93-M diet every five weeks until they were 40 weeks old (Figure 4A). Comparisons using Student's *t*-tests revealed that this difference was significant at 20 weeks (p=0.05, Figure 4A), 35 weeks (p=0.009, Figure 4A), 40 weeks (p=0.04, Figure 4A), and 45 weeks (p=0.04, Figure 4A). The normal and Amy mice that were fed the AIN93-M diet ate similar amounts of food for the remainder of the study, except for at 70 weeks, when food consumption by normal mice abruptly dropped and they ate significantly less than Amy mice that were fed the AIN93-M diet (p=0.0003, Figure 4A). These results indicate that there are clear genotype effects on the amount of food eaten throughout the first half of life by normal and Amy mice that are fed an ideal rodent diet such as the AIN93-M diet (Figure 4A).

Normal and Amy mice that were fed the Oz-AIN diet.

In contrast to the genotype effects on amount of diet eaten by mice that are fed the AIN93-M diet, the normal and Amy mice that were fed the Oz-AIN diet ate similar amounts of food until very old age. Amy mice that were fed the Oz-AIN diet ate significantly more food than normal mice that were fed the Oz-AIN diet at 65 weeks ($p=0.03$, Figure 4B), 70 weeks ($p=0.03$, Figure 4B), and 75 weeks ($p=0.03$, Figure 4B). These results indicate that there are no genotype effects on the amount of food eaten by normal and Amy mice that are fed a high-fat, sub-optimal rodent diet such as the Oz-AIN diet (Figure 4B).

Figure 4A. The effect of genotype on food intake (g/day) of normal and Amy mice that were fed the AIN93-M diet.

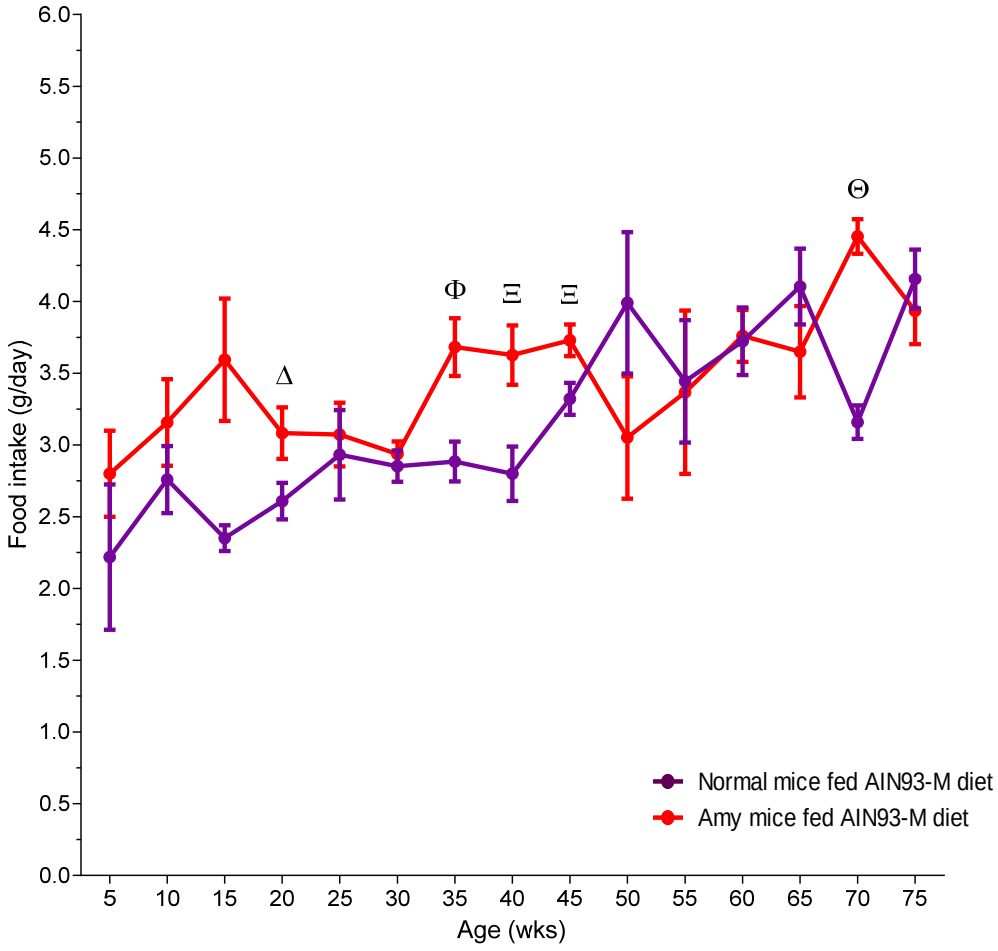


Figure 4A. Estimated AIN93-M diet intake (g/day) by normal mice (purple line, wk 5: n=12, wk 75: n=9) and Amy mice (red line, wk 5: n=9, wk 75: n=5). Error bars are mean ±SEM. Significant differences were detected using Student's *t*-tests. (Θ) p=0.0003. (Φ) p=0.009. (Ξ) p=0.04. (Δ) p=0.05.

Figure 4B. The effect of genotype on food intake (g/day) of normal and Amy mice that were fed the Oz-AIN diet.

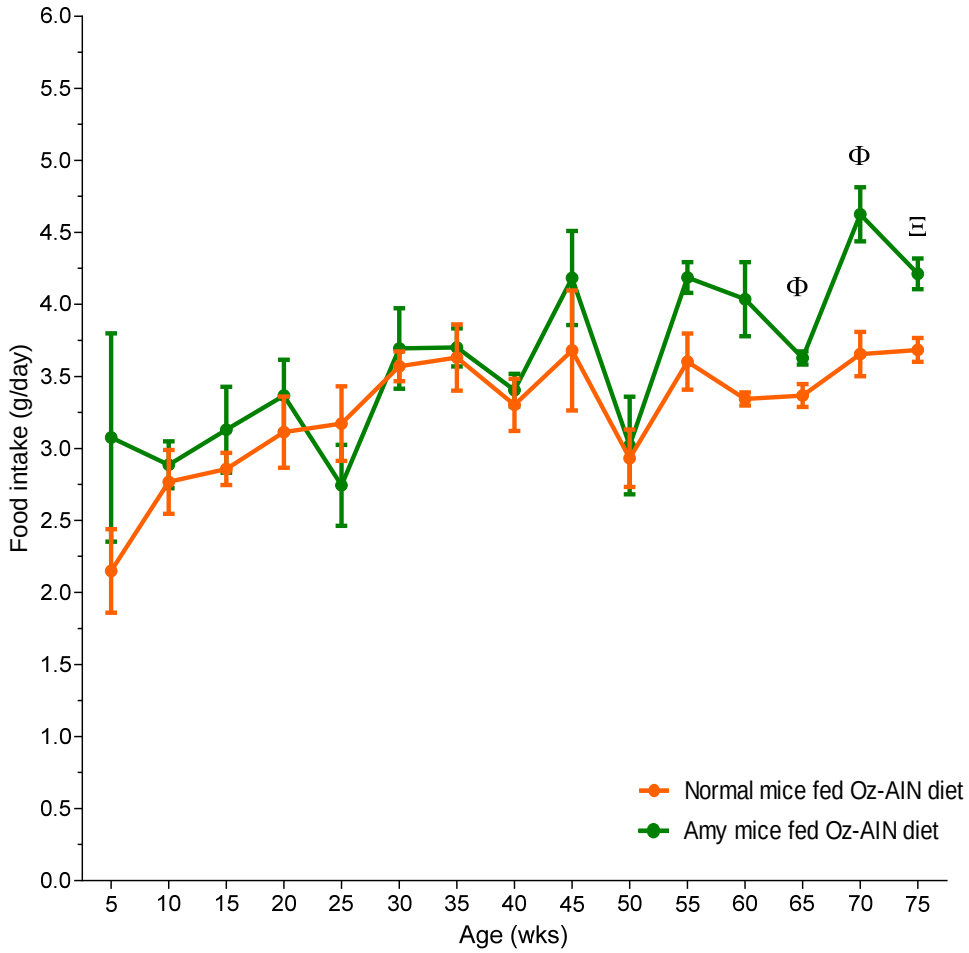


Figure 4B. Estimated Oz-AIN diet intake (g/day) by normal mice (orange line, wk 5: n=12, wk 75: n=12) and Amy mice (green line, wk 5: n=9, wk 75: n=7). Error bars are mean ±SEM. Significant differences were detected using Student's *t*-tests. (Ξ) p=0.01. (Φ) p=0.03.

2.3.3. The effect of diet-type on the amount of food eaten by normal and Amy mice that were fed either the AIN93-M diet or the Oz-AIN diet.

Normal mice that were fed the AIN93-M diet or the Oz-AIN diet.

The normal mice that were fed the Oz-AIN diet ate more food than normal mice that were fed the AIN93-M diet from 15 to 40 weeks old (Figure 5A). This was significant at 15 weeks ($p=0.006$, Figure 5A), 30 weeks ($p=0.0006$, Figure 5A) and 35 weeks ($p=0.02$, Figure 5A). After 45 weeks, significant differences were next detected at 70 weeks, when normal mice that were fed the AIN93-M diet ate significantly more than those fed the Oz-AIN diet ($p=0.05$, Figure 5A).

Amy mice that were fed the AIN93-M diet or the Oz-AIN diet.

At 30 weeks Amy mice that were fed the Oz-AIN diet ate more than Amy mice that were fed the AIN93-M diet ($p=0.04$, Figure 5B). The amount of food eaten by Amy mice that were fed the AIN93-M diet or the Oz-AIN diet did not differ on any other week. These results indicate that diet-type does not have an effect on the amount of food eaten by Amy mice.

Figure 5A. The effect of diet-type on food intake (g/day) of normal mice that were fed the AIN93-M diet or the Oz-AIN diet.

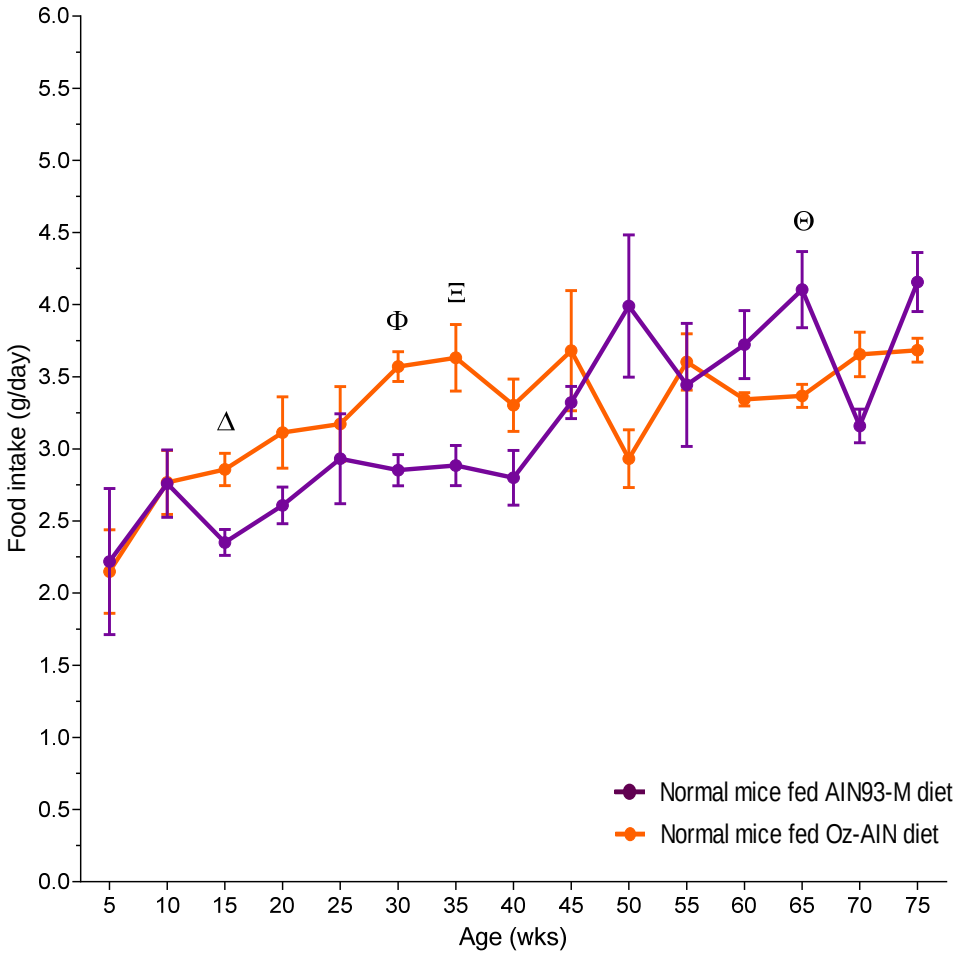


Figure 5A. Estimated food intake (g/day) every five weeks by normal mice fed the AIN93-M diet (purple line, wk 5: n=12, wk 75: n=9) or the Oz-AIN93-M diet (orange line, wk 5: n=12, wk 75: n=12). Error bars are mean ±SEM. Significant differences were detected using Student's *t*-tests. (Φ) p=0.0006. (Δ) p=0.006. (Ξ) p=0.02. (Θ) p=0.05.

Figure 5B. The effect of diet-type on food intake (g/day) of Amy mice that were fed the AIN93-M diet or the Oz-AIN diet.

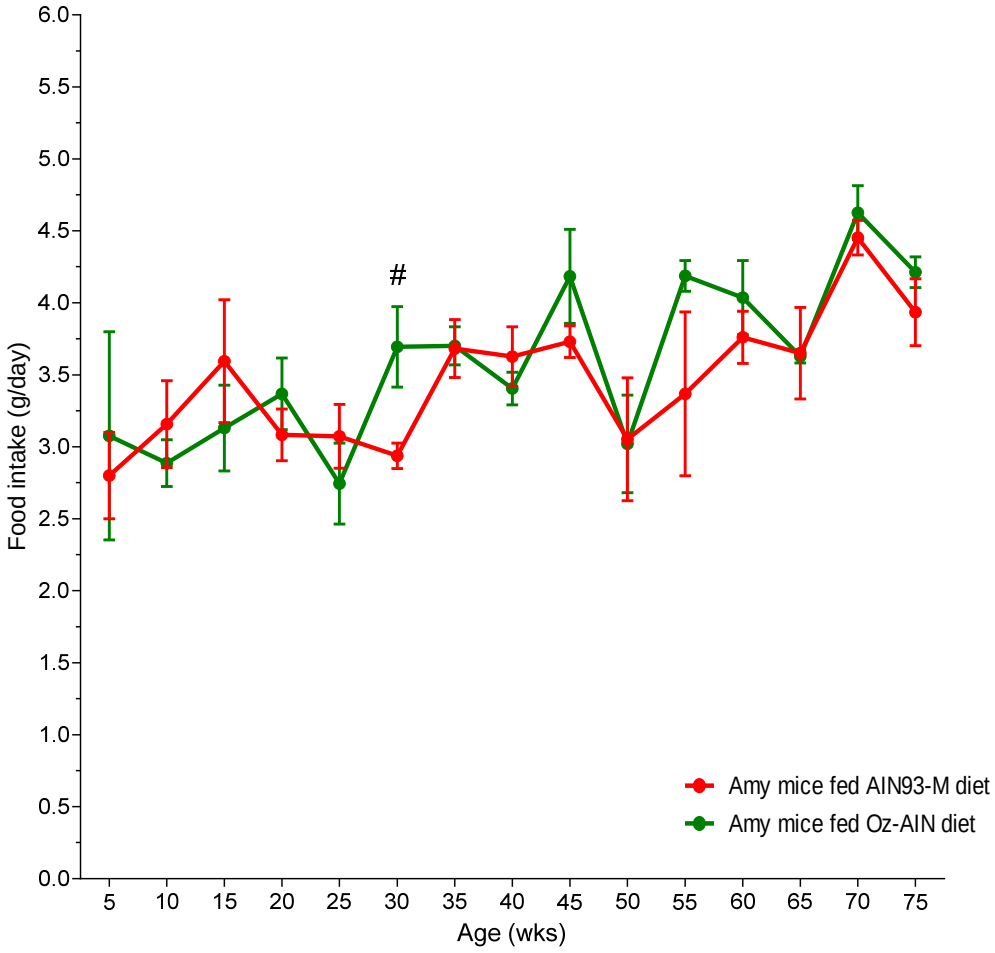


Figure 5B. Estimated food intake (g/day) by Amy mice fed the AIN93-M diet (red line, wk 5: n=9, wk 75: n=5) or the Oz-AIN diet (green line, wk 5: n=9, wk 75: n=7) for 75 weeks. Error bars are mean ±SEM. Significant differences were detected using Student's *t*-tests. (#) p=0.04.

2.3.4. An evaluation of the estimated overall energy intake of normal and Amy mice that were fed either the AIN93-M diet or the Oz-AIN diet.

The energy intake from diets was calculated by multiplying food consumed by energy content of each diet (Table 11).

Table 11. Energy content of the AIN93-M diet and the Oz-AIN diet.

Diet	Energy content (kcal)
AIN93-M diet	16.66 kcal
Oz-AIN diet	20.11 kcal

Energy content has been calculated based on the macronutrient content of each diet. Total fat content was multiplied by 37.7 kcal; Total carbohydrate content was multiplied by 16.7 kcal; Total protein content was multiplied by 16.7 kcal.

There were no significant genotype ($p=0.39$, Table 12) or interactive effects ($p=0.82$, Table 12) on the estimated overall energy intake throughout the study. A two-way ANOVA revealed that diet-type accounted for 35.66% of the overall variance of energy intake, however this failed to achieve significance at $p<0.05$ ($p=0.057$, Table 12). This suggests that there were trends for diet-type effects on overall energy intake for normal and Amy mice over 18 months, there are trends for diet-type effects.

Table 12. The effect of diet-type or genotype on the energy intake (kJ) over 18 months by normal and Amy mice that were fed the AIN93-M or the Oz-AIN diet

	Normal mice	Amy mice
AIN93-M diet	26347 ±3054 kJ	28394 ±2942 kJ
Oz-AIN diet	32330 ±2691 kJ	35783 ±3328 kJ

The overall energy intake of normal or Amy mice was calculated from the area under the curve. Values are mean ±SEM.

2.3.5. The effect of genotype on energy intake (kJ) every five weeks by normal and Amy mice that were fed either the AIN93-M diet or the Oz-AIN diet.

Normal and Amy mice that were fed the AIN93-M diet.

Similar to the genotype effect on the amount of food eaten, there were genotype effects on energy intake by normal and Amy mice that were fed the AIN93-M diet. Amy mice that were fed the AIN93-M diet had higher energy intakes than normal mice that were fed the AIN93-M diet until they were 45 weeks old (Figure 6A). Student's *t*-tests revealed that this difference was significant when mice were 20 weeks ($p=0.05$, Figure 6A), 35 weeks ($p=0.009$, Figure 6A), 40 weeks ($p=0.04$, Figure 6A), and 45 weeks ($p=0.04$, Figure 6A). There were no differences between energy intakes of normal mice that were fed the AIN93-M diet or the Oz-AIN diet after 45 weeks of age, apart from at 70 weeks, when normal mice that were fed the Oz-AIN diet had higher energy intakes than mice that were fed the AIN93-M diet ($p=0.0003$, Figure 6A). This is consistent with the genotype effect on the amount of food eaten, and suggests that there is a genotype effect on energy intake of normal and Amy mice that have been fed an optimal rodent diet (AIN93-M diet) throughout the first half of life.

Normal and Amy mice that were fed the Oz-AIN diet.

Consistent with the minimal effect of genotype on food eaten, there were no genotype effects on energy intake of normal and Amy mice that were fed the Oz-AIN diet until late life (Figure 6B). Student's *t*-tests indicated that normal mice and Amy mice that were fed the Oz-AIN diet had similar energy intakes until they were 55 weeks of age. Amy mice that were fed the Oz-AIN diet had higher energy intake than normal mice

that were fed the Oz-AIN diet from 55 weeks onwards. This was significant when mice were 65 weeks ($p=0.03$, Figure 6B), 70 week ($p=0.03$, Figure 6B), and 75 weeks ($p=0.01$, Figure 6B). This suggests that genotype does not have an effect on energy intake of normal and Amy mice that are fed a high-fat diet until very old age.

Figure 6A. The effect of genotype on estimated energy intake (kJ/day) of normal mice and Amy mice that were fed the AIN93-M diet.

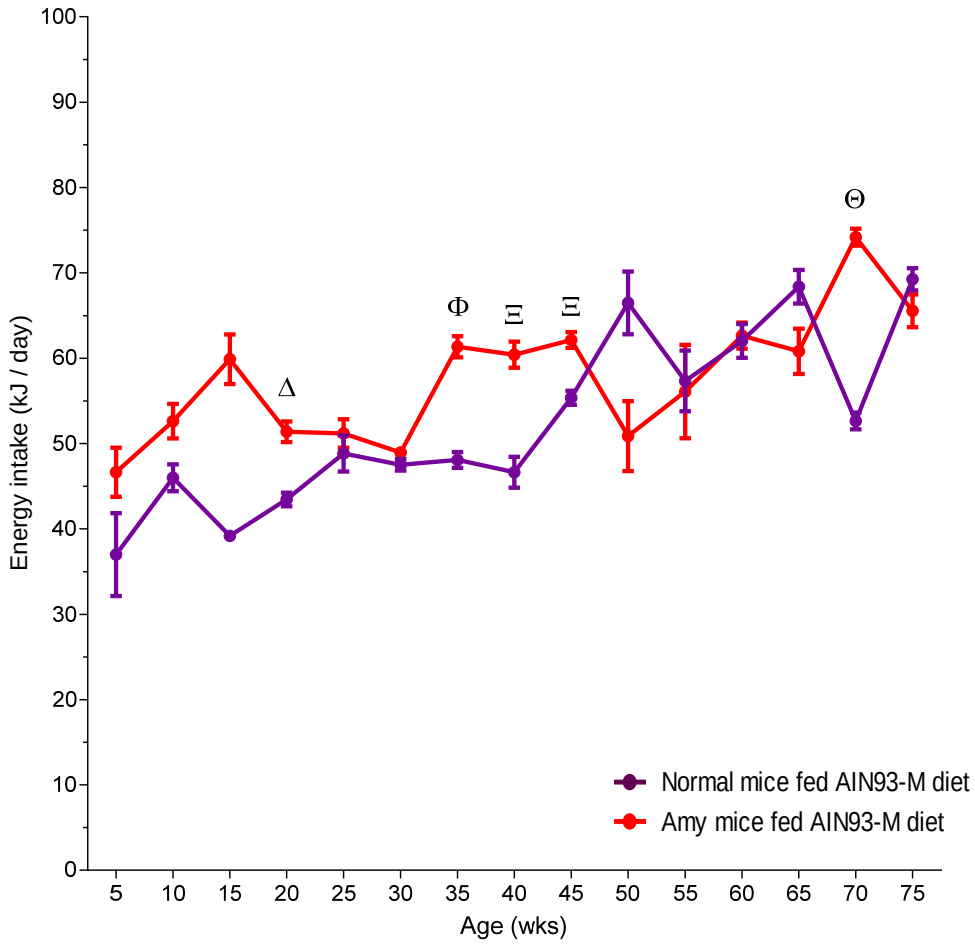


Figure 6A. Estimated energy intake (kJ/day) from the AIN93-M diet by normal mice (purple line, wk 5: n=12, wk 75: n=9) and Amy mice (red line, wk 5: n=9, wk 75: n=5). Error bars are mean ±SEM. Significant differences were detected using Student's *t*-tests. (Θ) p=0.0003. (Φ) p= 0.009. (Ξ) p=0.04. (Δ) p=0.05.

Figure 6B. The effect of genotype on estimated energy intake (kJ/day) of normal mice and Amy mice that were fed the Oz-AIN diet.

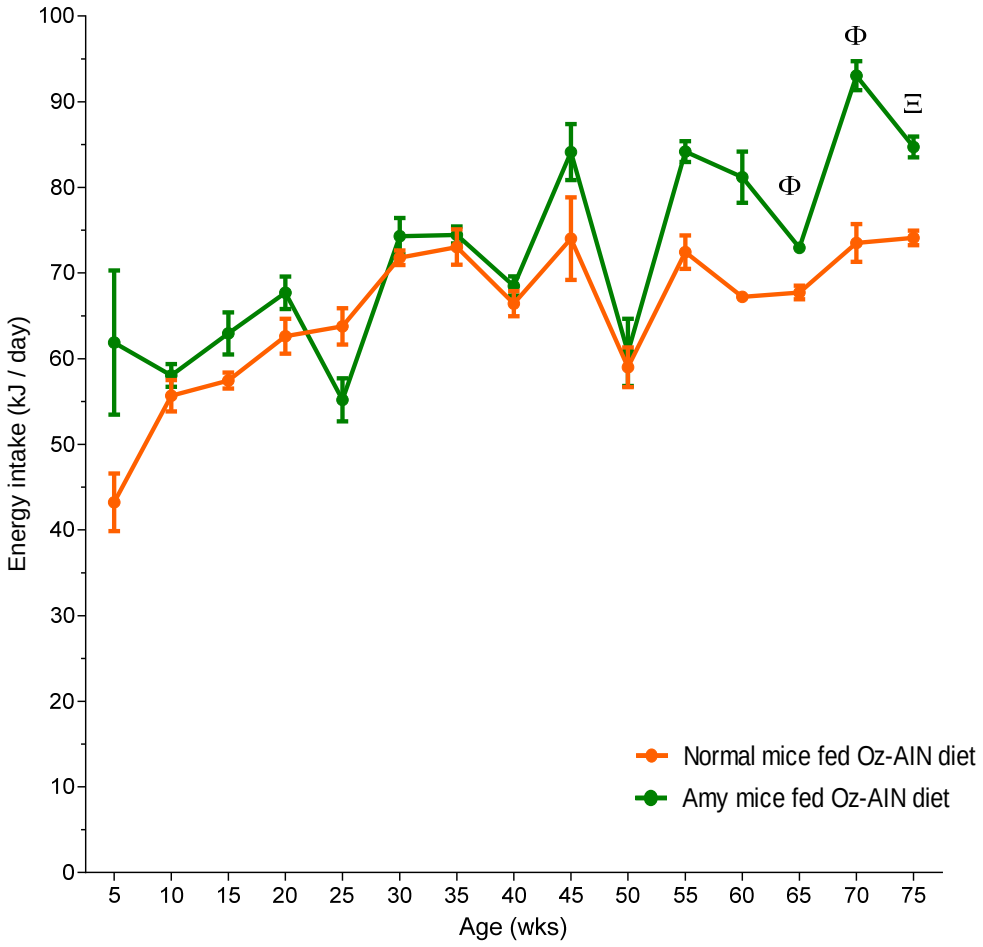


Figure 6B. Estimated energy intake (kJ/day) from the Oz-AIN diet by normal mice (orange line, wk 5: n=12, wk 75: n=12) and Amy mice (green line, wk 5: n=9, wk 75: n=7) for 75 weeks. Error bars are mean ±SEM. Significant differences were detected using Student's *t*-tests. (Ξ) p=0.01. (Φ) p=0.03.

2.3.6. The effect of diet-type on energy intake (kJ) every five weeks by normal and Amy mice that were fed either the AIN93-M diet or the Oz-AIN diet.

Normal mice that were fed the AIN93-M diet or the Oz-AIN diet.

Student's *t*-tests revealed that there were significant diet-type effects on the energy intakes of normal mice that were fed the AIN93-M diet or the Oz-AIN diet (Figure 7A). Normal mice that were fed the Oz-AIN diet had higher energy intakes than normal mice that were fed the AIN93-M diet until they were 45 weeks old. This was significant at 15 weeks ($p<0.0001$, Figure 7A), 20 weeks ($p=0.003$, Figure 7A), 30 weeks ($p<0.0001$, Figure 7A), 35 weeks ($p=0.0007$, Figure 7A), 40 weeks ($p=0.01$, Figure 7A), and 45 weeks ($p=0.03$, Figure 7A). After 45 weeks of age, normal mice that were fed the AIN93-M diet or the Oz-AIN diet had similar energy intakes, except for at 70 weeks, when the normal mice that were fed the AIN93-M diet ate less food, and subsequently had lower energy intake ($p=0.004$, Figure 7A). These results indicate that diet-type has an effect on energy intake of normal and Amy mice in the first half of life, when they are fed an ideal diet.

There are two possible reasons for this outcome. First, the normal mice that were fed the Oz-AIN diet tended to eat more food early in life compared to normal mice that were fed the AIN93-M diet, and would have had higher energy intakes. The second reason is that the Oz-AIN diet is a higher energy diet than the AIN93-M diet (Table 11). Therefore, whether or not mice had eaten more of the Oz-AIN diet is almost irrelevant, as mice received more energy per gram eaten. Nonetheless, the finding that mice that were fed the Oz-AIN diet consistently had higher energy intakes than

normal mice that were fed the AIN93-M diet suggests that energy content of food does not drive food intake in normal mice.

Amy mice that were fed the AIN93-M diet or the Oz-AIN diet.

Amy mice that were fed the Oz-AIN diet ate the same amount of food as Amy mice that were fed the AIN93-M diet. As a result of this, they had higher energy intakes throughout the study. This was significant on almost every week throughout the study (Figure 7B). As discussed above the higher energy intakes of mice that were fed the Oz-AIN diet can be attributed to the higher energy content of the Oz-AIN diet. This suggests that diet-type does have a significant effect on food intake of Amy mice and that energy content of a diet does not necessarily alter food intake.

Figure 7A. The effect of diet-type on estimated energy intake (kJ/day) of normal mice that were fed the AIN93-M diet or the Oz-AIN diet.

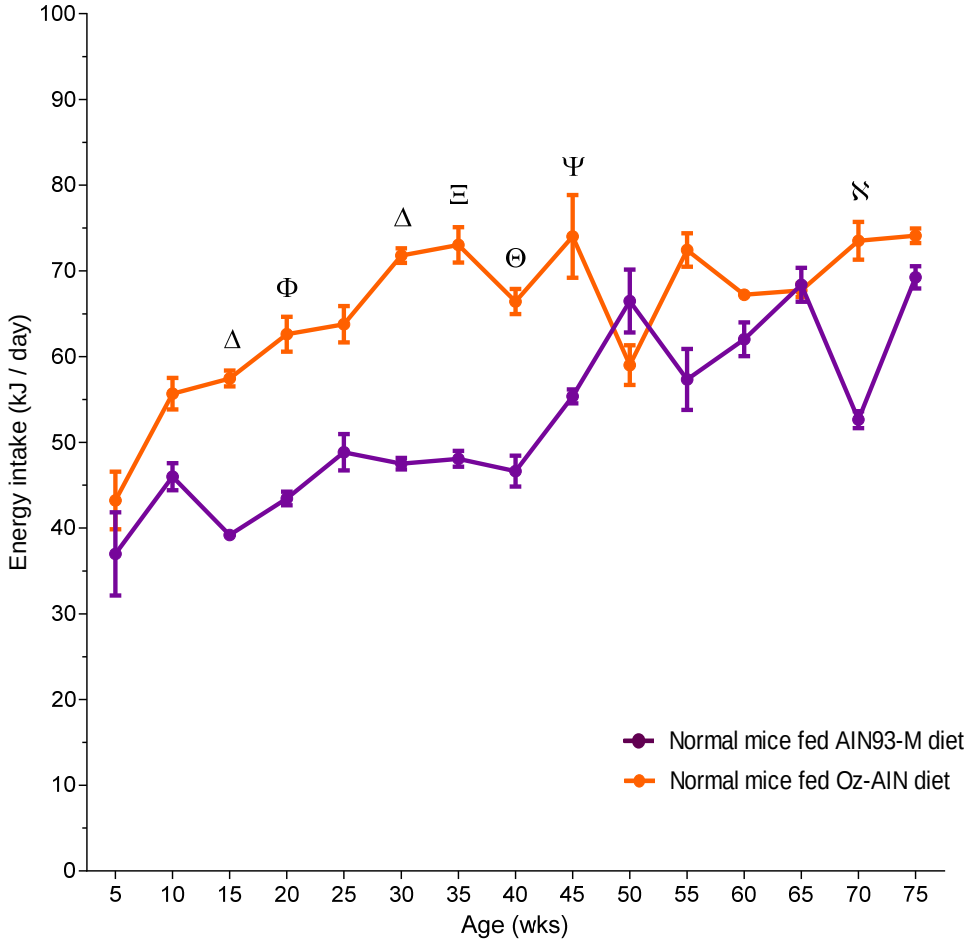


Figure 7A. Estimated energy intake (kJ/day) by normal mice fed the AIN93-M diet (purple line, wk 5: n=12, wk 75: n=9) or the Oz-AIN diet (orange line, wk 5: n=12, wk 75: n=12). Error bars are mean ±SEM. Significant differences were detected using Student's *t*-tests. (Δ) p<0.0001. (Ξ) p=0.0007. (Φ) p=0.003. (ϰ) p=0.004. (Θ) p=0.01. (Ψ) p=0.03.

Figure 7B. The effect of diet-type on estimated energy intake (kJ/day) of Amy mice that were fed the AIN93-M diet or the Oz-AIN diet.

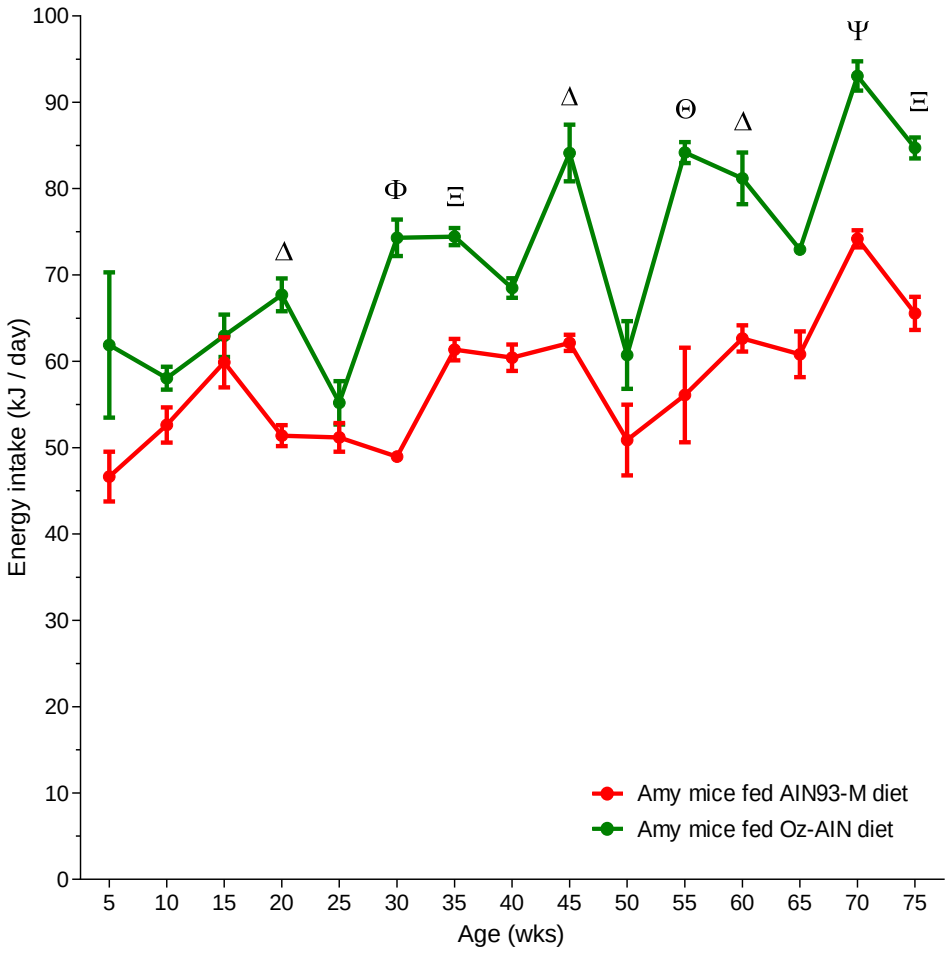


Figure 7B. Estimated energy intake (kJ/day) by Amy mice fed the AIN93-M diet (red line, wk 5: n=9, wk 75: n=5) or the Oz-AIN diet (green line, wk 5: n=9, wk 75: n=7). Error bars are mean ±SEM. Significant differences were detected using Student's *t*-tests. (Δ) p=0.02. (Φ) p=0.002. (Ξ) p=0.01. (Θ) p=0.04. (Ψ) p=0.005.

2.4. Characterisation of the Oz-AIN diet in terms of body weight, fat deposition and organ size in normal and Amy mice.

2.4.1. The effect of diet-type on body weight.

Two-way ANOVAs indicated that diet-type accounted for 14.9% of the overall variation of body weight at 20 weeks of age ($p=0.05$, Figure 8). These significant diet-type effects persisted throughout the remainder of the study (Figure 8). While Bonferroni post tests detected significant interactions from 25 weeks, significant diet-type effects were not apparent until 50 weeks, 55 weeks and 60 weeks.

Student's *t*-tests between genotype matched groups indicated that diet-type effects on body weight in mice may occur earlier than 20 weeks old. Normal mice that were fed the Oz-AIN diet were significantly heavier than genotype-matched mice that were fed the AIN93-M diet at 15 weeks old ($p=0.02$, Figure 8). Similarly, Amy mice that were fed the Oz-AIN diet were also significantly heavier than Amy mice that were fed the AIN93-M diet at 15 weeks ($p=0.02$, Figure 8).

The rapid weight gain of mice that were fed the Oz-AIN diet may largely be attributed to the high energy content (20.11 kcal, Table 11) and high total fat content of the Oz-AIN diet (33.0% kcal, Figure 3). Weight gain may also be attributed to an imbalance of micronutrients [275, 281, 300]. The Oz-AIN diet contains almost twice the recommended intake of niacin (152.13% increase), and is also high in zinc (23.71%) and deficient in calcium (-23.79%) (Table 6). The high levels of dietary zinc that were consumed by mice may have contributed to weight gain. Excessive

dietary zinc increases zinc-mediated nutrient uptake leading to enhanced growth of fat cells and weight gain [275].

While calcium and niacin are also both associated with increased obesity risk, the levels of each that are used in the Oz-AIN diet would be expected to lower weight, rather than lead to obesity [281, 300]. Niacin has powerful lipid lowering effects, and as such has potential benefits against disorders such as diabetes, hypercholesterolemia, metabolic syndrome, dyslipidemia and cardiovascular disease [301-304]. As the benefits of niacin in weight control are centred on its abilities to increase high-density lipoprotein-C and lower low-density lipoprotein and decrease adipose deposition, the effect of niacin in the Oz-AIN diet will be discussed further in the context of the effect of diet on fat mass in mice (p. 86).

Low dietary calcium was expected to reduce weight gain, rather than enhance it [281]. Bastie *et al.* report that normal mice fed a high-fat diet that was rich in calcium and vitamin D had increased body mass compared to mice fed a high-fat diet that was low calcium and vitamin D. They demonstrated that the mice that were fed the high calcium-high vitamin D diet utilized lipids more efficiently through enhanced fatty acid metabolism. However, this resulted in storage, rather than oxidation of lipids, resulting in enhanced body mass and weight gain [281]. It is possible that the adjusted calcium levels in the Oz-AIN diet were not low enough to be considered ‘deficient’, and therefore calcium levels may have had little bearing on weight gain or fat metabolism of mice that were fed the Oz-AIN diet.

2.4.2. The effect of genotype on body weight.

Two-way ANOVAs did not reveal significant genotype effects on body weight of normal and Amy mice until very old age ($p=0.006$, 70 weeks, Figure 8).

However, Amy mice were consistently heavier than normal mice, and this may have been associated with the onset of other AD pathologies. In the Amy mouse model, amyloid deposition begins around 6 months of age [141]. Student's *t*-tests revealed that at 6 months, old Amy mice that were fed the AIN93-M diet were significantly heavier than normal mice that were fed the same diet ($p=0.02$, Figure 8) and Amy mice that were fed the Oz-AIN diet were significantly heavier than normal mice that were fed the same diet ($p=0.001$, Figure 8). These results suggest that at the same age that AD neuropathologies begin to develop in the brain, Amy mice are heavier than diet-matched normal mice. This may imply that there may be an association between body weight gain and onset of AD pathology. However, this needs further research to be confirmed, as the current study has not evaluated amyloid pathology in 6 month old mice. Nonetheless, the finding that Amy mice appear to be more vulnerable to weight gain than normal mice is supported by reports from other groups that have linked AD pathology to increased obesity risk [305, 306].

Potentially, this may be associated with increased insulin resistance in AD-type mice [305]. It has been proposed that peripheral insulin resistance may also result in cerebral insulin resistance, and this may lead to impaired degradation of β-amyloid by insulin degrading enzyme [305, 307, 308]. Mody *et al.* report that double transgenic APP/PSEN1 mice gain weight and develop insulin resistance faster than single transgenic PSEN1 mice or control mice, after short-term high-fat diet feeding [305]. Mody *et al.* also report that the APP/PSEN1 mice had higher levels of brain PTP1B, which is a negative regulator of insulin and leptin receptor signalling. This suggests a potential mechanism through which AD-type mice may be susceptible to weight gain. [305].

Figure 8. Weight gain (g) of normal and Amy mice fed the AIN93-M or the Oz-AIN diet.

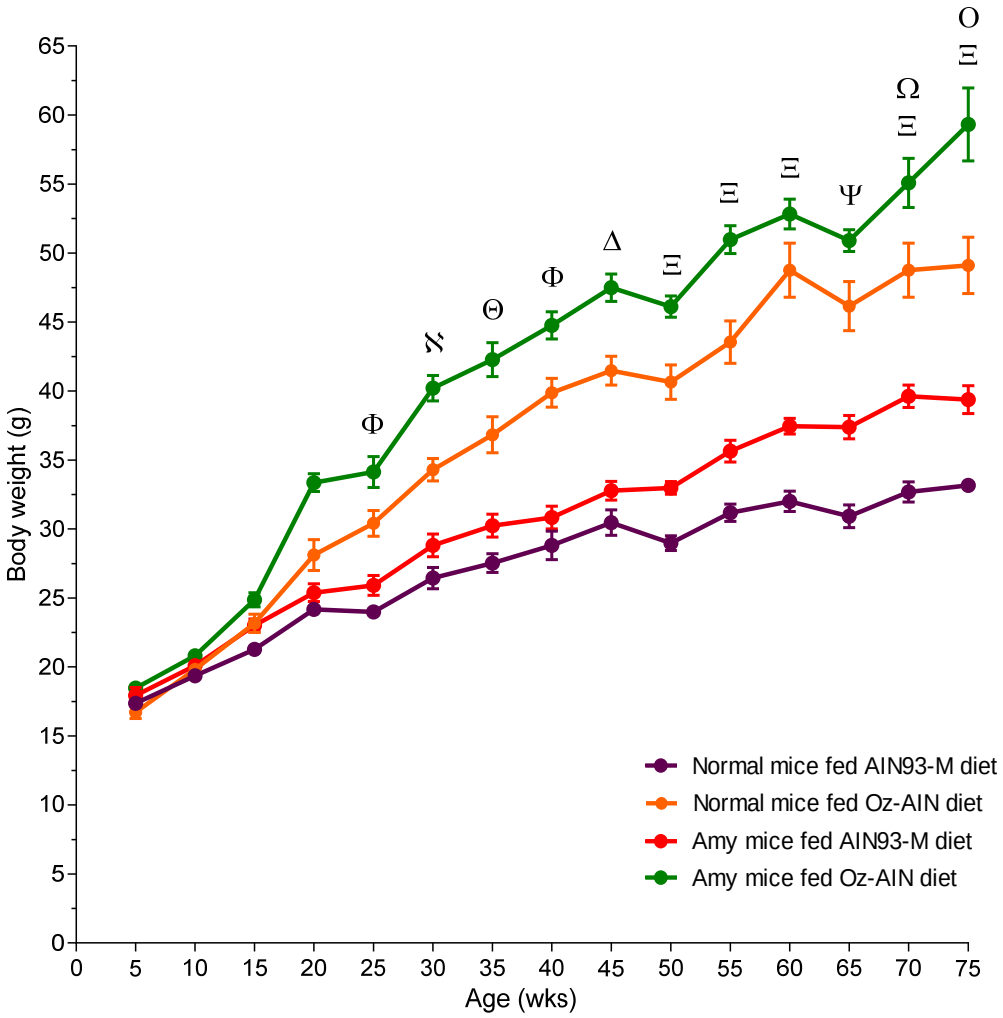


Figure 8. Weight gain (g) of normal mice fed the AIN93-M diet (purple line, wk 5: n=12, wk 75: n=9), normal mice fed the Oz-AIN diet (orange line, wk 5: n=12, wk 75: n=12), Amy mice fed the AIN93-M diet (red line, wk 5: n=9, wk 75: n=5), and Amy mice fed the Oz-AIN diet (green line, wk 5: n=9, wk 75: n=7). Error bars are mean ±SEM. Two-way ANOVA every five weeks detected significant diet-type and genotype effects.
Diet-type effects: (Θ) p=0.02. (Φ) p=0.009. (χ) p=0.004. (Δ) p=0.002. (Ψ) p=0.001. (Ξ) p<0.0001.
Genotype effects: (Ω) p=0.006. (O) p=0.0003.

Contrary to the findings of the present study, others have reported that AD-type mice are in a hypermetabolic state and are prone to weight loss [123]. Vloeberghs *et al*., for example, report that male APP23 mice fed a normal diet weigh less but drink and eat more than wild type controls [123]. However, three major differences between Vloeberghs *et al*.'s methodology and that of the present study could explain the differences in findings. First, the different mouse models may have contributed to a difference in sensitivity to weight gain from dietary sources. Vloeberghs *et al*. used a single transgenic mouse model of AD (APP23) [123], whereas the Amy mouse model that is used in the present study is a double transgenic mouse (APP/PSEN1). Consistent with this, others have reported that APP/PSEN1, but not APP or normal mice were susceptible to weight gain from a high-fat diet [305].

The second difference between the Vloeberghs *et al.* study and the present one relates to the types of fats used in each diet [123]. The source of dietary fat can have a different effect on fat metabolism, and can also alter fat deposition and weight gain in mice [309]. Dietary fat derived from animal sources can cause greater visceral fat deposition than those derived from vegetable sources [309]. The Oz-AIN diet uses a high level of animal fats as the primary source of dietary fats (44.8 g/kg lard, Table 7) but also combines vegetable oils (canola oil, 22.4 g/kg, sunflower oil 16.8 kg/g. coconut oil 61.6 g/kg, olive oil 39.2 g/kg, Table 7). The diet used in Vloeberghs *et al.* contains oilseed products and milk oils (Carfil, Oud-Turnhout, Belgium) [123]. It is possible that the different sources of dietary fats in the two studies may have had a different effect on weight gain and fat deposition in mice used.

The third difference between the Vloeberghs *et al.* study and the present one is that the present study used female mice, whilst Vloebergh *el al*. used male mice [123]. Female mice are more susceptible to glucose intolerance, insulin resistance and

weight gain [305, 309], particularly in the Amy mouse model used here [305]. This may partially explain the increased weight gain of Amy mice in response to the AIN93-M and Oz-AIN diet observed in the present study.

2.4.3. The effect of diet and genotype on the weight of fat deposits.

2.4.3.1. TOTAL FAT deposits.

A two-way ANOVA revealed that there were significant diet-type effects ($p<0.0001$) and genotype effects ($p=0.008$) on the total weight of fat deposits in 18 month old mice (Figure 9). Diet-type accounted for 67.60% of the variance of fat weight, and genotype accounted for 7.13% of variance. There were no effects of a genotype-diet-type interaction ($p=0.18$, Figure 9). Bonferroni post tests revealed that there were diet-type effects between normal mice that were fed the AIN93-M diet or the Oz-AIN diet ($p<0.0001$, Figure 9), and Amy mice that were fed either the AIN93-M diet or the Oz-AIN diet ($p=0.001$, Figure 9), and that there were genotype effects between normal and Amy mice that were fed the AIN93-M diet ($p=0.02$, Figure 9). The Oz-AIN diet did not have an effect on the weight of total fat collected from normal or Amy mice ($p>0.99$, Figure 9).

2.4.3.1.1. Diet-type effect on fat weight (g).

The finding that the Oz-AIN diet was associated with significantly greater fat deposition than the AIN93-M diet was not surprising. The Oz-AIN diet is 33.00% (kcal) fat (Figure 3), while the AIN93-M diet is only 10% (kcal) fat [266]. Furthermore the Oz-AIN diet is 24% higher in zinc, which promotes adipose tissue growth [275]. These properties indicate that the Oz-AIN diet led to increased fat deposition and weight gain compared to the AIN93-M diet.

However, the Oz-AIN diet is also high in niacin, which has powerful lipid lowering effects. Niacin down regulates lypolysis and generation of plasma triglycerides, and subsequently reduces production of very-low density lipoproteins and low density lipoproteins. Niacin also increases high-density lipoproteins and as such, is one of the front-line therapies for reducing cardiovascular risk [310]. The beneficial effects of niacin in managing lipid levels suggest that high levels of dietary niacin should have reduced the adiposity associated with the Oz-AIN diet.

Supplementation with niacin in doses as low as 1000 mg/day may be beneficial in treatment of diabetes and obesity [311, 312]. However, the therapeutic doses of niacin that are used to manage high-density lipoprotein and low-density lipoprotein levels are well above those that are obtained from diet (4-6 g/day compared to 53.9 mg/day) [185, 313].

Li *et al.* report that supplementing a Western-style rodent diet with 2% niacin to reduces low density lipoproteins and very-low density lipoproteins in mice [304]. However, the amount of dietary niacin Li *et al.* fed to mice is the equivalent of 18 g/day in a human diet [304]. This is over 35 times the amount of niacin in diets typically consumed by Australian adults (53.9 mg/day), and approaches toxic levels [185, 314]. The Oz-AIN diet, on the other hand, contains niacin at non-toxic levels that are commonly eaten by Australian adults, despite being 2.5 times the recommended levels [185, 261].

It is possible that the levels of niacin in the Oz-AIN diet were not high enough to effect lipid metabolism in mice. Mice metabolise niacin much faster than humans do. Therefore, greater amounts of dietary niacin equivalents are used in rodent models of obesity [269, 270, 304, 315]. Therefore, it is possible that increasing dietary niacin

2.5 times was not enough to counter the detrimental effects of the high-fat and high zinc content in the Oz-AIN diet.

2.4.3.1.2. Genotype effect on fat weight (g).

As reported on page 86, there were significant genotype effects on total fat weight collected from normal and Amy mice ($p=0.008$). Significantly more fat was collected from Amy mice that were fed the AIN93-M diet than diet-matched normal mice ($p=0.02$, Figure 9). However, there was no difference in the fat collected from normal and Amy mice that were fed the Oz-AIN diet ($p>0.99$, Figure 9). This suggested that the high-fat Oz-AIN diet has similar effects of fat deposition in normal and Amy mice, but when mice are fed an optimal diet (the AIN93-M diet), Amy mice are more susceptible to fat deposition than normal mice.

While there is plenty of evidence to suggest that obesity is a risk factor for AD and other dementias [316, 317], there is little information regarding the effect of a standard diet on adiposity or weight gain in AD patients or mice. The current results suggest that, although diet-induced obesity may be a risk factor for AD, AD-type mice may be predisposed to weight gain. This indicates that the relationship between obesity and AD-type neuropathology may be more complex, and that the AD phenotype may also be a risk factor for obesity.

Figure 9. Average weight of the total fat deposits collected from 18 month old normal and Amy mice.

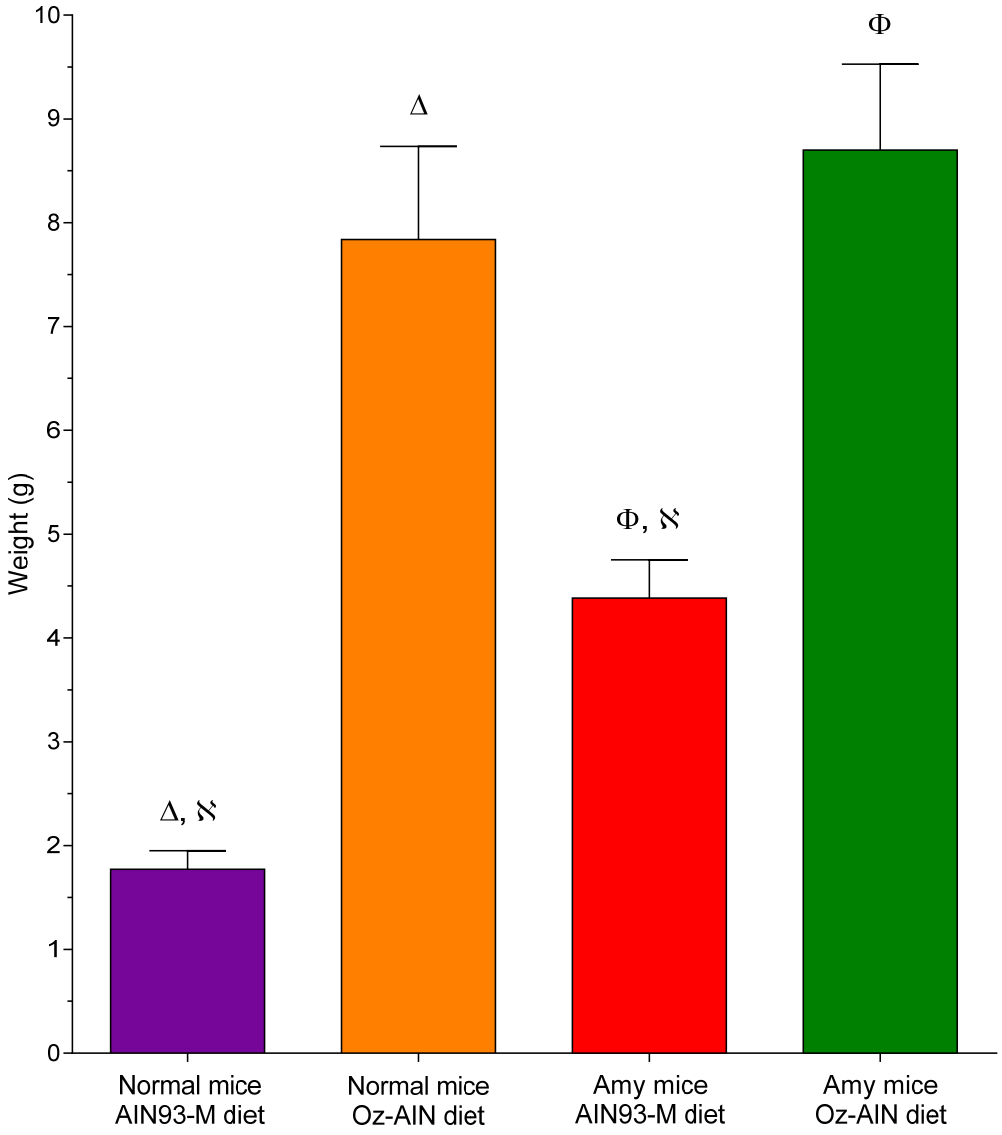


Figure 9. The weight of total fat that was collected from 18 month old normal mice fed the AIN93-M diet (purple bar, n=9), normal mice fed the Oz-AIN diet (orange bar, n=12), Amy mice fed the AIN93-M diet (red bar, n=5), and Amy mice fed the Oz-AIN diet (green bar, n=7). Bars represent mean ±SEM. A two-way ANOVA revealed significant diet-type ($p<0.0001$) and genotype ($p=0.008$) effects. Bars with matching symbols are significantly different with Bonferroni post tests. (Δ) $p<0.0001$. (Φ) $p=0.001$. (ℵ) $p=0.02$.

2.4.3.2. Diet-type and genotype effects on UTERINE FAT deposit weight (g).

A two-way ANOVA revealed significant diet-type effects on uterine fat weight that accounted for 45.29% of the overall variance of uterine fat weight ($p<0.0001$). Genotype and the genotype-diet-type interaction accounted for <1% and 5.06% respectively ($p=0.90$ and $p=0.07$ respectively). Bonferroni post tests revealed that normal mice that were fed the AIN93-M diet had significantly less uterine fat than normal mice fed the Oz-AIN diet ($p<0.0001$, Figure 10). There were trends to suggest that Amy mice that were fed the AIN93-M diet also had less uterine fat than genotype matched mice that were fed the Oz-AIN diet, however these were not significant ($p=0.11$, Figure 10). Bonferroni post tests did not detect significant differences between normal and Amy mice that were fed the AIN93-M diet ($p=0.46$, Figure 10) or between normal and Amy mice that were fed the Oz-AIN diet ($p=0.63$, Figure 10), confirming that genotype did not have an effect on uterine fat weight.

Figure 10. Average weight of uterine fat collected from 18 month old normal and Amy mice.

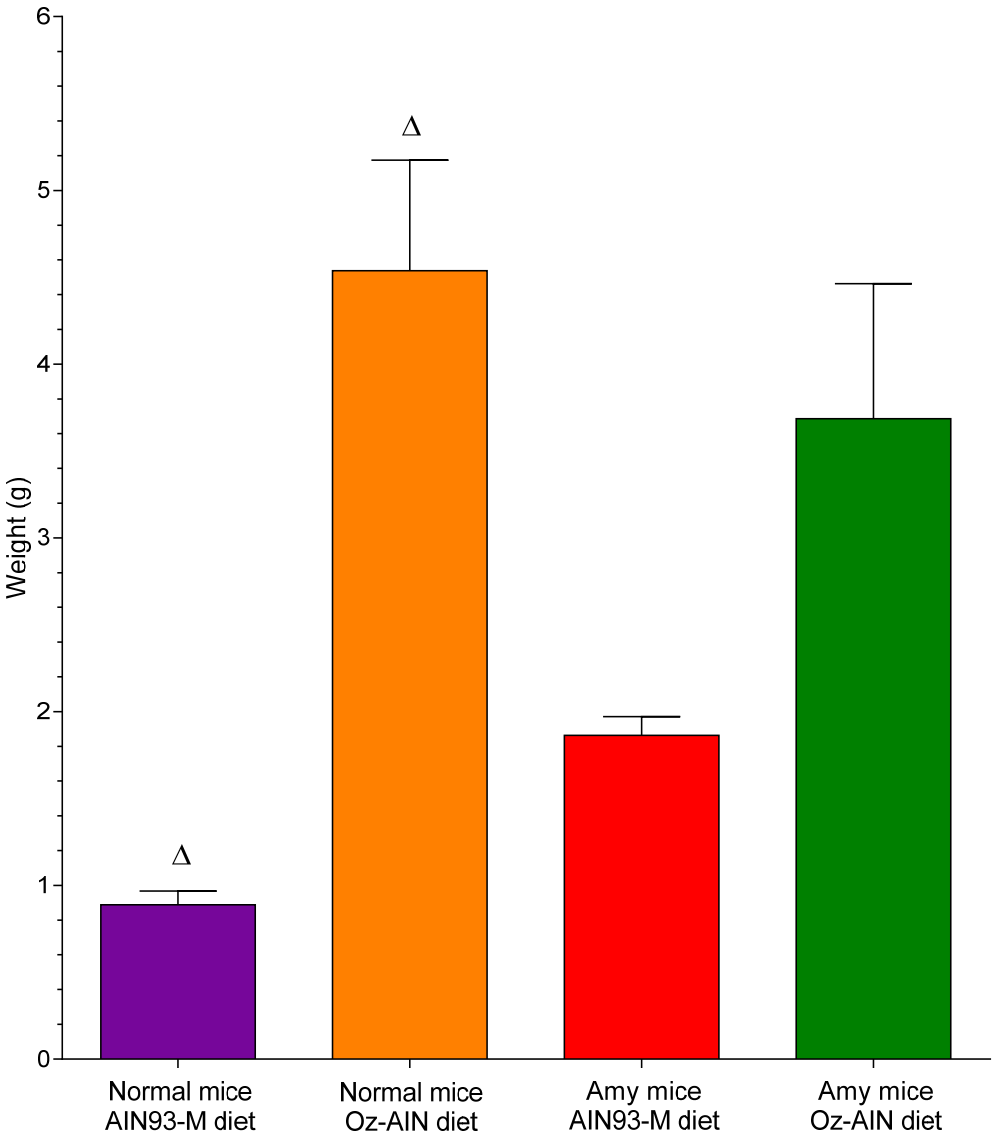


Figure 10. The average weight of uterine fat that was collected from 18 month old normal mice that were fed the AIN93-M diet (purple bar, n=9), normal mice that were fed the Oz-AIN diet (orange bar, n=12), Amy mice that were fed the AIN93-M diet (red bar, n=5), and Amy mice that were fed the Oz-AIN diet (green bar, n=7). Bars represent mean ±SEM. A two-way ANOVA revealed significant diet-type effects ($p<0.0.0001$). Bars with matching symbols are significantly different using Bonferroni post tests. (Δ) $p<0.0001$.

2.4.3.3. Diet-type and genotype effects of SUBCUTANEOUS FAT deposit weight (g).

A two-way ANOVA revealed that there were significant effects of diet-type ($p<0.0001$) and genotype ($p=0.0004$) on the weight of subcutaneous fat deposits in 18 month old mice (Figure 11). Diet-type accounted for 48.29% of the overall variation of subcutaneous fat deposit weight, and genotype accounted for 18.62% of the variation. There was no diet-type-genotype interaction (0.67% of the variation, $p=0.45$, Figure 11). Bonferroni post tests revealed that diet-type effects were between normal mice that were fed the AIN93-M diet or the Oz-AIN diet ($p<0.0001$, Figure 11), and Amy mice that were fed either the AIN93-M diet or the Oz-AIN diet ($p=0.009$, Figure 11). Genotype effects were between normal and Amy mice that were fed the AIN93-M diet ($p=0.008$, Figure 11). There was no genotype effect on the amount of subcutaneous fat in normal and Amy mice that were fed the Oz-AIN diet (Figure 11).

As suggested earlier in this chapter (pp. 78-79), diet-type effects on the weight of fat deposits in mice may be attributed to the high-fat and high-zinc content of the Oz-AIN diet [275]. These differences in total weight of fat deposits collected may explain the diet-type effects observed in the weights of subcutaneous fat collected from 18 month old mice.

The finding that there were genotype effects on subcutaneous fat deposits collected from normal and Amy mice that were fed an optimal diet (AIN93-M diet), but not between mice that were fed the Oz-AIN diet reflects that genotype effects on total weight of fat deposits collected from mice. As was suggested on page 88, this indicates that Amy mice may be more susceptible to weight gain than normal mice.

Figure 11. Average weight of subcutaneous fat collected from 18 month old normal and Amy mice.

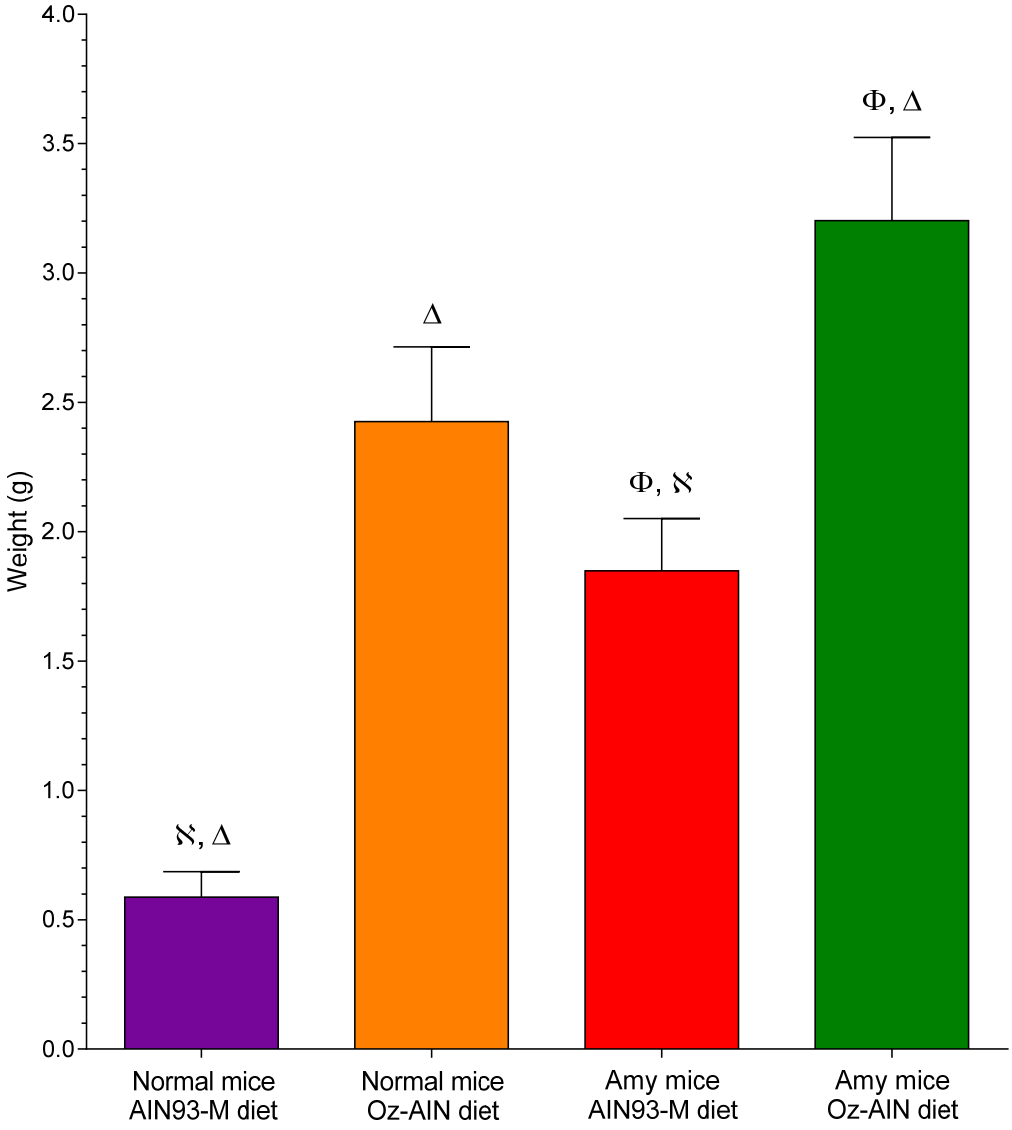


Figure 11. The average weight of fat collected from the beneath the skin (skin fat) that was collected from 18 month old normal mice fed the AIN93-M diet (purple bar, n=9), normal mice fed the Oz-AIN diet (orange bar, n=12), Amy mice fed the AIN93-M diet (red bar, n=5), and Amy mice fed the Oz-AIN diet (green bar, n=7). Bars represent mean ±SEM. Bars with matching symbols are significantly different with Bonferroni post tests. (Δ) $p<0.0001$. (ℵ) $p=0.008$. (Φ) $p=0.009$.

2.4.3.4. Diet-type and genotype effects on RENAL FAT deposit weight (g).

A two-way ANOVA revealed significant diet-type ($p<0.0001$) and genotype ($p=0.0008$) effects on the weight of renal fat collected from 18 month old mice (Figure 12). Diet-type accounted for 37.88% of the overall variation of renal fat deposit weight, and genotype accounted for 18.88% of the overall variation. There was also a genotype-diet-type interaction that accounted for 5.71% of the overall variation of renal fat weight collected from mice ($p=0.048$, Figure 12).

Amy mice that were fed the Oz-AIN diet had significantly more renal fat than Amy mice that were fed the AIN93-M diet ($p=0.0004$, Figure 12). While normal mice that were fed the Oz-AIN diet had more renal fat than normal mice that were fed the AIN93-M diet, this did not achieve significance ($p=0.07$, Figure 12).

There were significant genotype effects on the weight of renal fat collected from normal and Amy mice that were fed the Oz-AIN diet ($p=0.004$, Figure 12). However, genotype did not affect the weight of renal fat collected from normal and Amy mice that were fed the AIN93-M diet ($p>0.99$, Figure 12).

Figure 12. Average weight of renal fat collected from 18 month old normal and Amy mice.

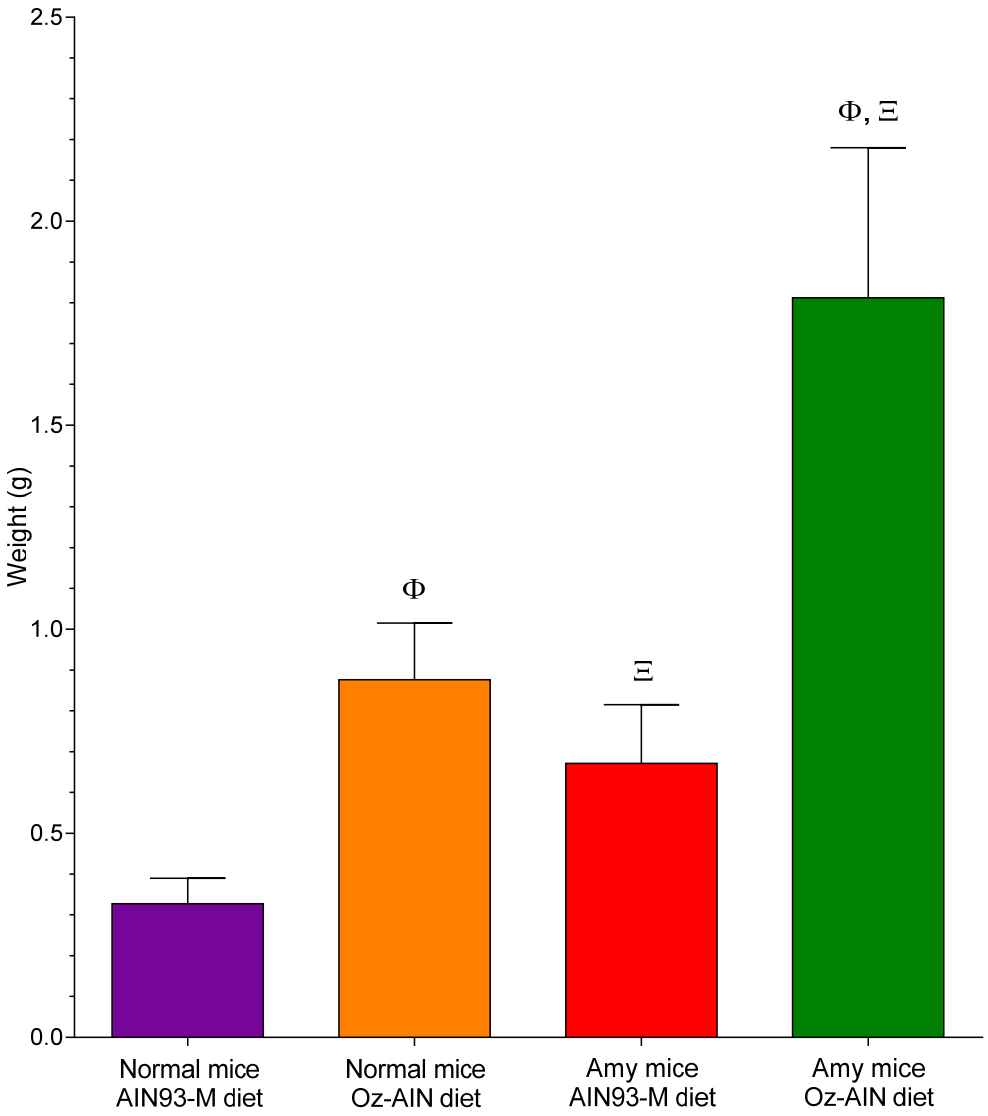


Figure 12 The average weight of renal fat that was collected from 18 month old normal mice fed the AIN93-M diet (purple bar, n=9), normal mice fed the Oz-AIN diet (orange bar, n=12), Amy mice fed the AIN93-M diet (red bar, n=5), and Amy mice fed the Oz-AIN diet (green bar, n=7). Bars represent mean ±SEM. Bars with matching symbols are significantly different. (Φ) p=0.0004. (Ξ) p=0.004.

2.4.3.5. Summary of the effect of genotype and diet-type on fat weight collected from 18 month old normal and Amy mice.

The Oz-AIN diet increased fat deposit size (g) in normal and Amy mice. However, the location of the fat deposits was different between genotypes. In normal mice, the Oz-AIN diet increased fat deposition in the subcutaneous and uterine fat deposits. Whilst the Oz-AIN diet also increased subcutaneous and uterine deposits, the renal fat deposits were affected by diet and genotype in Amy mice, but not in normal mice. These differences may be due to an increased susceptibility of fat deposition in Amy compared to normal mice. Obesity has been linked to AD development, progression and risk, and may therefore have been enhanced by the high-fat nature of the Oz-AIN diet.

2.4.4. The effect of diet and genotype on heart weight (g).

A two-way ANOVA revealed that there were significant diet-type effects ($p=0.004$) and a significant diet-type-genotype interaction ($p=0.05$), but no genotype effects ($p=0.21$) on the weight of hearts collected from 18 month old normal and Amy mice (Figure 13). Diet-type accounted for 21.50% of the overall variation of heart weight, and the diet-type-genotype interaction accounted for 9.55% of the overall variation (Figure 13).

Bonferroni post tests revealed that Amy mice that were fed the AIN93-M diet had significantly lighter hearts than Amy mice that were fed the Oz-AIN diet ($p=0.019$, Figure 13). However, heart weight was not different between normal mice that were fed the AIN93-M diet or the Oz-AIN diet ($p>0.99$, Figure 13). There were no genotype effects on the weights of hearts collected from normal and Amy mice that

were fed the AIN93-M diet ($p>0.99$, Figure 13) or between normal and Amy mice that were fed the Oz-AIN diet ($p=0.22$, Figure 13).

This is similar to the findings from measurements of fat weight in the current study that indicated that Amy mice were more susceptible to the effects of a high-fat diet than normal mice. This adds to those findings to suggest that the Oz-AIN diet does not only increase fat deposition in Amy mice, but that it also alters the size of major organs such as the heart.

2.4.5. The effect of diet and genotype on liver weight (g).

A two-way ANOVA revealed that there were significant diet-type effects on the weights of livers collected from 18 month old normal and Amy mice ($p=0.03$, Figure 14). Diet-type accounted for 15.12% of the overall variation of liver weight. There were no significant effects of genotype ($p=0.69$) or diet-type-genotype interaction ($p=0.18$) on liver weight (Figure 14).

Bonferroni post tests did not detect where diet-type effects lay. There were no significant differences in liver weights of normal mice that were fed the AIN93-M diet or the Oz-AIN diet ($p=0.25$), or between Amy mice that were fed the AIN93-M or the Oz-AIN diet ($p=0.36$). However, irrespective of genotype, mice that were fed the Oz-AIN diet tended to have heavier livers than genotype matched mice that were fed the AIN93-M diet. This suggests that the diet-type effects that were detected by the two-way ANOVA were due to the Oz-AIN diet causing slightly heavier livers.

2.4.6. The effect of diet and genotype on spleen weight (g).

There were no diet-type ($p>0.99$), genotype ($p=0.18$) or interaction ($p=0.36$) effects on spleen weight of 18 month old normal and Amy mice.

Figure 13. Average heart weight (g) of normal and Amy mice.

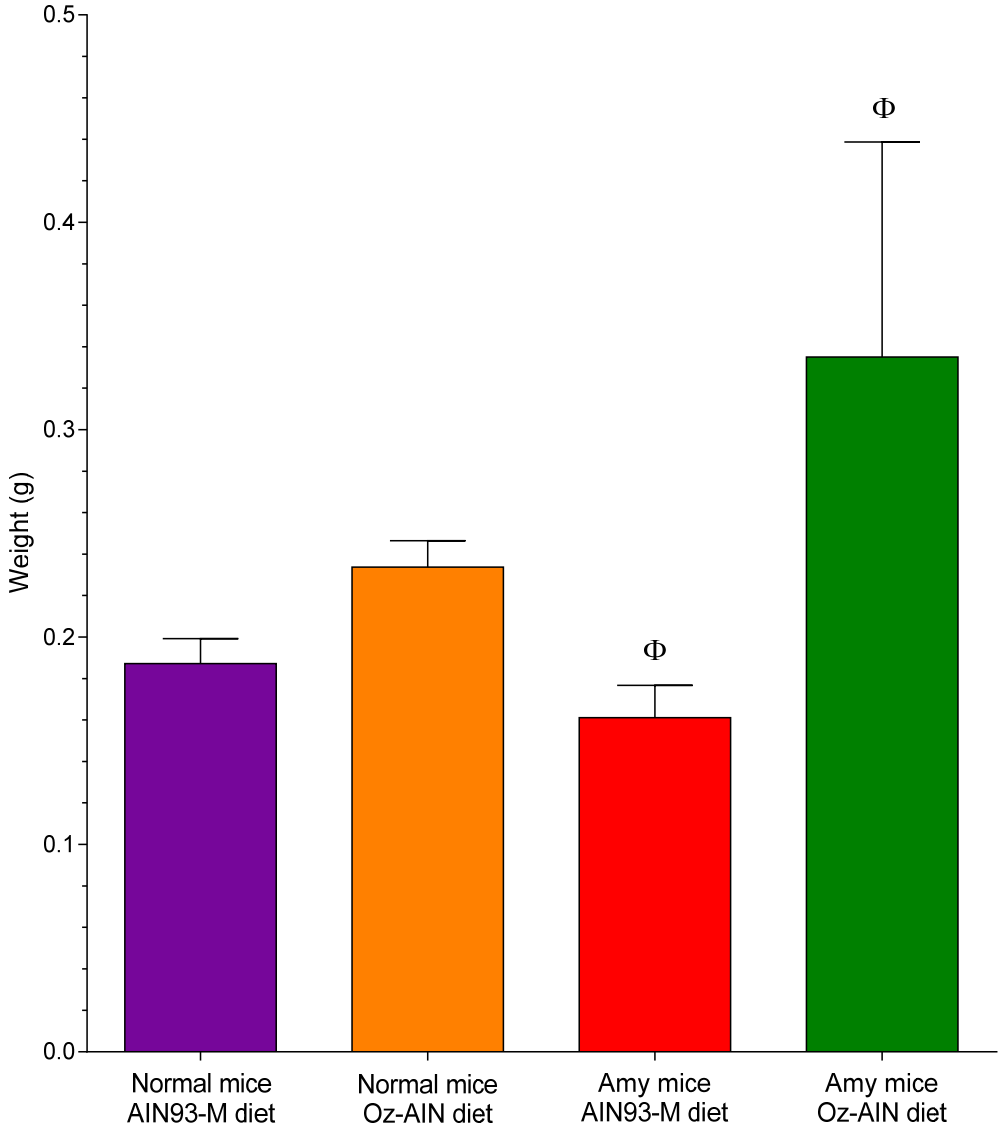


Figure 13. The weight (g) of hearts that were collected from 18 month old normal mice fed the AIN93-M diet (purple bar, n=9), normal mice that were fed the Oz-AIN diet (orange bar, n=12), Amy mice fed the AIN93-M diet (red bar, n=5), and Amy mice fed the Oz-AIN diet (green bar, n=7). Bars represent mean ±SEM. A two-way ANOVA detected significant giet-type effects (p=0.004) and a significant genotype-diet-type interaction (p=0.05). Bars with matching symbols are significantly different with Bonferroni post tests. (Φ) p=0.019.

Figure 14. Average liver weight (g) of normal and Amy mice.

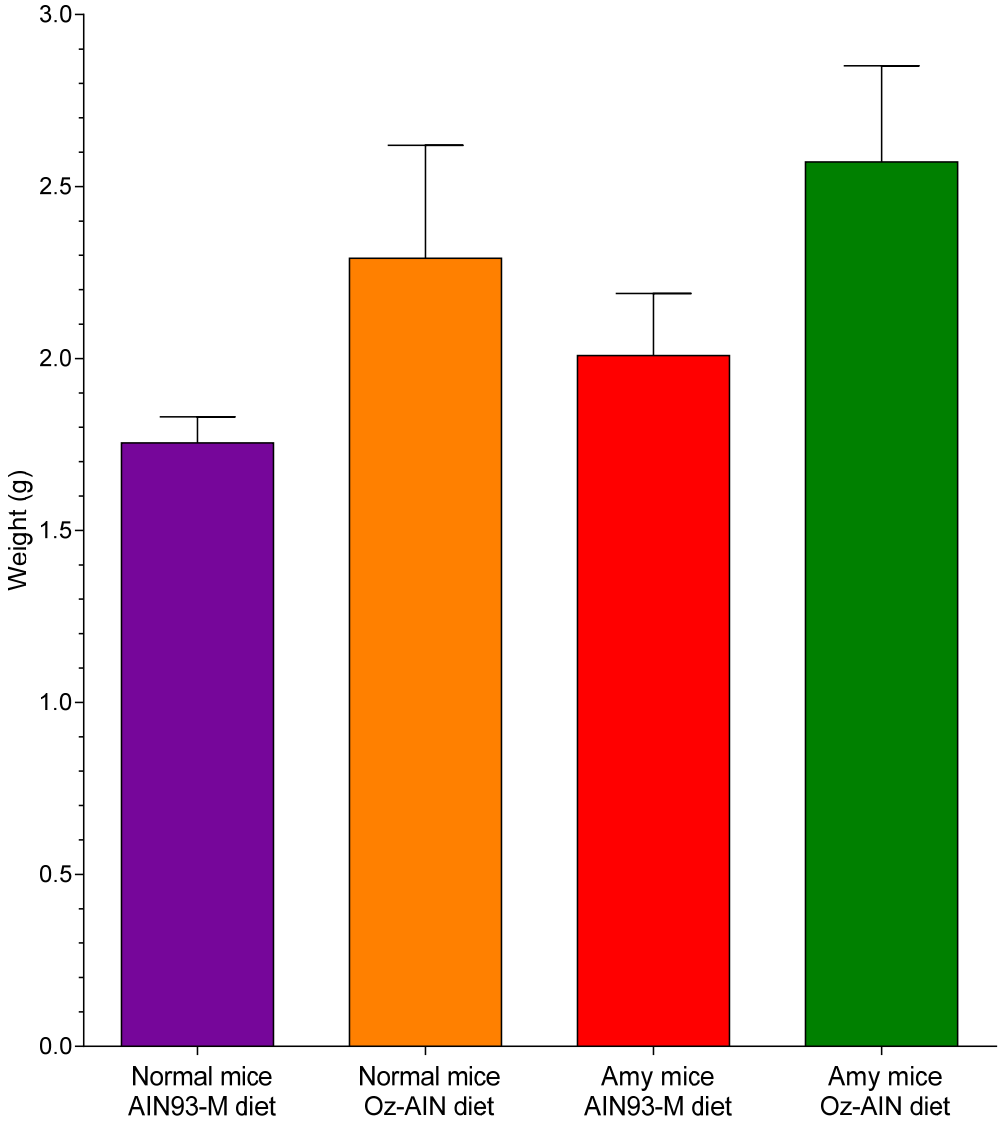


Figure 14. The weight (g) of livers that were collected from 18 month old normal mice fed the AIN93-M diet (purple bar, n=9), normal mice fed the Oz-AIN diet (orange bar, n=12), Amy mice fed the AIN93-M diet (red bar, n=5), and Amy mice fed the Oz-AIN diet (green bar, n=7). Bars represent mean ±SEM. A two-way ANOVA detected significant diet-type effects (p=0.03). However, Bonferroni post tests did not detect significant differences.

Figure 15. Average spleen weight (g) of normal and Amy mice.

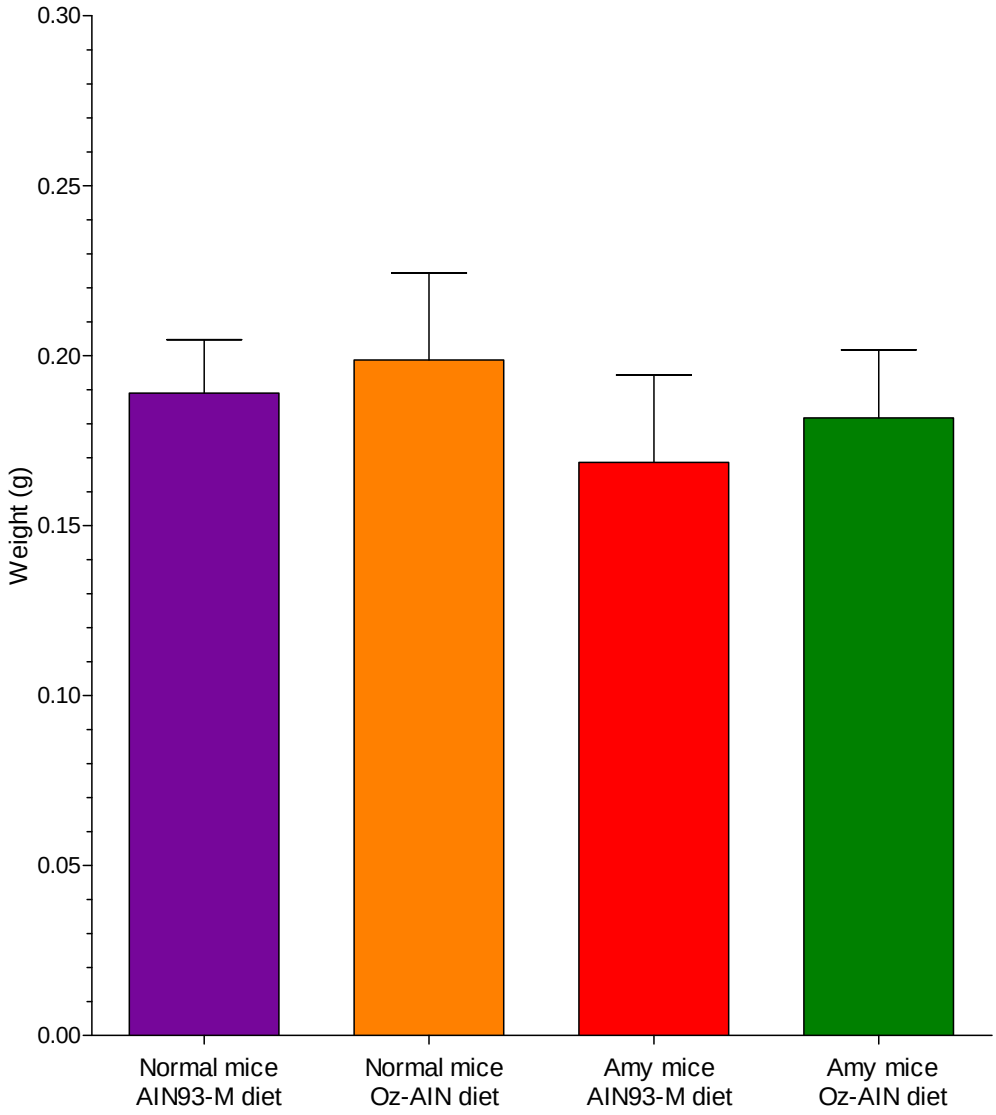


Figure 15. The weight (g) of spleens that were collected from 18 month old normal mice fed the AIN93-M diet (purple bar, n=9), normal mice fed the Oz-AIN diet (orange bar, n=12), Amy mice fed the AIN93-M diet (red bar, n=5), and Amy mice fed the Oz-AIN diet (green bar, n=7). Bars represent mean ±SEM.

2.4.7. The effect of diet and genotype on kidney weight (g).

A two-way ANOVA revealed that there were significant effects of diet-type ($p=0.02$) on the weight of kidneys collected from 18 month old normal and Amy mice (Figure 16). Diet-type accounted for 17.49% of the overall variance of kidney weight. There were no significant genotype effects ($p=0.75$) or diet-type-genotype interaction effects ($p=0.35$) on kidney weight (Figure 16).

Bonferroni post tests revealed that the normal mice fed the Oz-AIN diet had significantly heavier kidneys than those fed the AIN93-M diet ($p=0.04$, Figure 16). This is consistent with reports from others that a high-fat diet induces renal damage and can induce renal hypertrophy [318, 319]. The weights of kidneys that were collected from Amy mice that were fed the Oz-AIN diet were also higher than those of Amy mice that were fed the AIN93-M diet, this did not achieve significance ($p>0.99$, Figure 16). This suggests that Amy mice are less susceptible to kidney weight gain than control mice when fed a high-fat diet.

Figure 16. Average kidney weight (g) of normal and Amy mice.

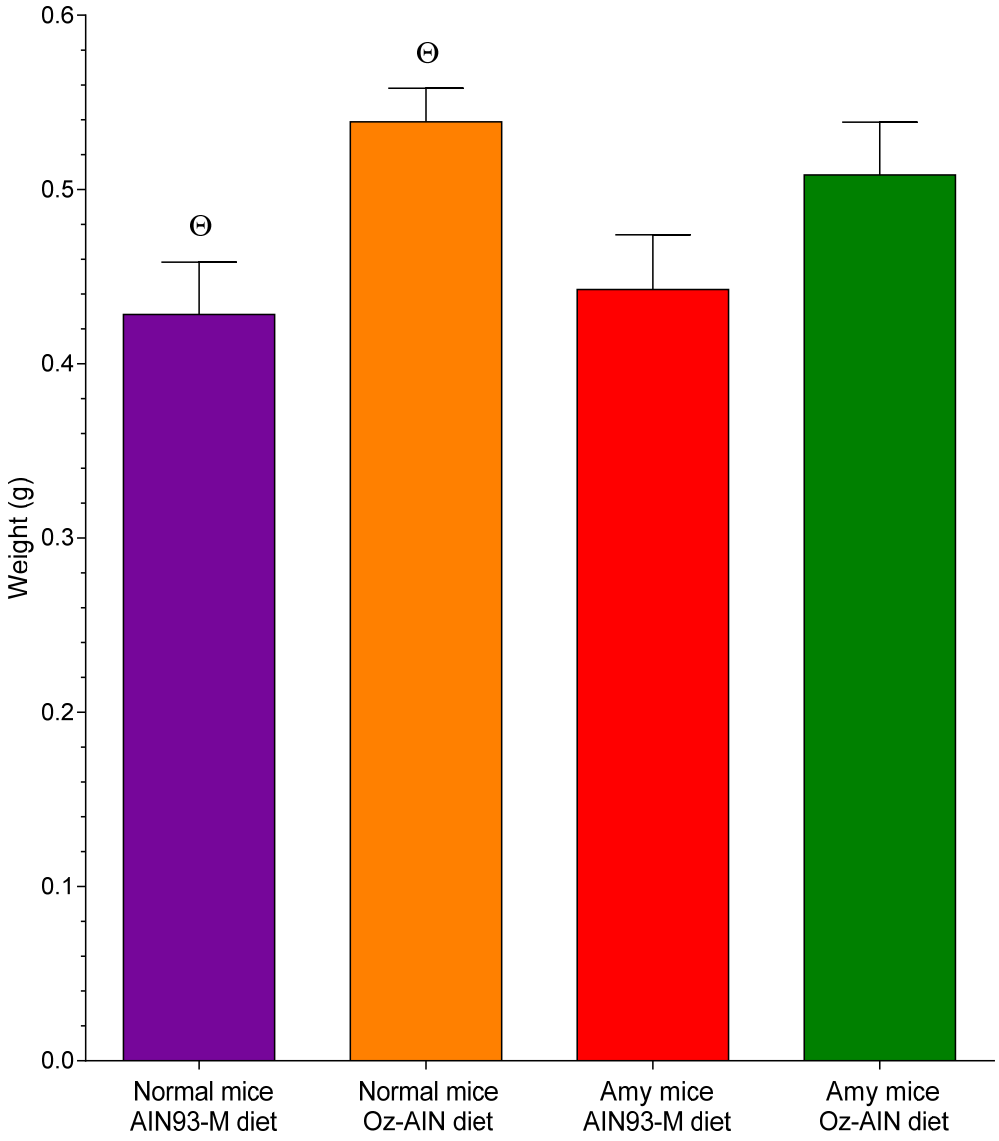


Figure 16. The weight (g) of kidneys that were collected from 18 month old normal mice fed the AIN93-M diet (purple bar, n=9), normal mice fed the Oz-AIN diet (orange bar, n=12), Amy mice fed the AIN93-M diet (red bar, n=5), and Amy mice fed the Oz-AIN diet (green bar, n=7). Bars represent mean ±SEM. Bars with matching symbols are significantly different. (Θ) p=0.04.

2.5. Conclusion.

The Oz-AIN diet that has been formulated as a part of this study is a diet that is designed to reflect the nutrient content of a diet that is typically eaten by Australian women. This diet is high in fat (33.0% kcal), with a P:M:S ratio that is 1.0: 2.4: 2.7 [185]. The altered P:M:S ratio is unique to other high-fat westernised rodent diets which often adjust saturated fat content without also increasing monounsaturated fats [280, 281, 320]. Other high-fat westernized rodent diets may not capture the true effect of an Australian-type diet on behavioural deficits of neuropathology in AD because dietary fat type, rather than total fat content, has been demonstrated to effect cognitive performance in older women [321]. The other important alteration ω-3: ω-6 ratio of the Oz-AIN diet is 1:10, which reflects the ω-3: ω-6 ratio in diets typically consumed by Australian women. This has been achieved through careful manipulation of oil and fat content in the diet design.

The micronutrient content of the Oz-AIN diet was designed by adjusting ideal micronutrient intake for rodents by a ratio of optimal to actual micronutrient intake by Australian women. Folate levels are half of optimal intake levels for rodents, whilst niacin is over 2.5 times that of optimal intake levels.

The normal and Amy mice that were fed the Oz-AIN diet gained weight much faster than mice that were fed the AIN93-M diet. This was expected as the Oz-AIN diet has a higher fat content than the AIN93-M diet (33% kcal and 10% kcal, respectively) and has higher levels of minerals such as zinc, which increase adiposity and weight gain. The effects of diet-type on weight gain were more pronounced in Amy mice who consistently weighed more than other mice through-out the study. This is consistent with reports of others that Amy mice are prone to develop obesity, and that diet-induced obesity up regulates inflammation in adipose and brain tissue of AD

mice, and therefore accelerates AD phenotype [305, 322]. While there is a lot of literature that reports that AD patients are malnourished and at risk of weight loss [323, 324], there are also reports that patients with dementia or AD are at risk of weight gain [325], suggesting that both ends of the spectrum may be equally detrimental.

The Oz-AIN diet was associated with larger fat deposits in normal and Amy mice than the AIN93-M diet. Interestingly, there were genotype and diet-type effects on fat deposition around the kidneys in Amy mice, but not on the weights of the kidneys themselves. This suggests that the fat deposition may not have had an effect on organ size and growth.

Normal and Amy mice that were fed the Oz-AIN diet had larger fat deposits than mice that were fed the AIN93-M diet. This demonstrates that the Oz-AIN diet induces adiposity in mice. While there were no genotype effects on overall adiposity, Amy mice tended to have more fat around their kidneys than normal mice, and this was significant between normal and Amy mice that were fed the Oz-AIN diet.

Genotype did not have an effect on organ size. However diet-type affected kidney weight. The Oz-AIN diet was associated with heavier kidneys in Amy and normal mice, compared to genotype matched mice that were fed the AIN93-M diet. A possible explanation for this is that high-fat diets have been demonstrated previously to have a deleterious effect on renal function and can induce renal hypertrophy [318, 319].

In conclusion, the Oz-AIN diet that is described here is a rodent diet that has been designed to reflect the sub-optimal nutrient content in diets that are typically consumed by Australian women. Interestingly, diet-induced weight gain and

adiposity, which may both play a role in AD, were accelerated in Amy mice that were fed the Oz-AIN diet. This indicates that the Oz-AIN diet has been able to modify risk factors for AD.

Chapter 3: The design of the Oz-AIN Supp rodent diet and characterisation of its effects on food consumption, weight gain and obesity in normal and Amy mice.

3. Background.

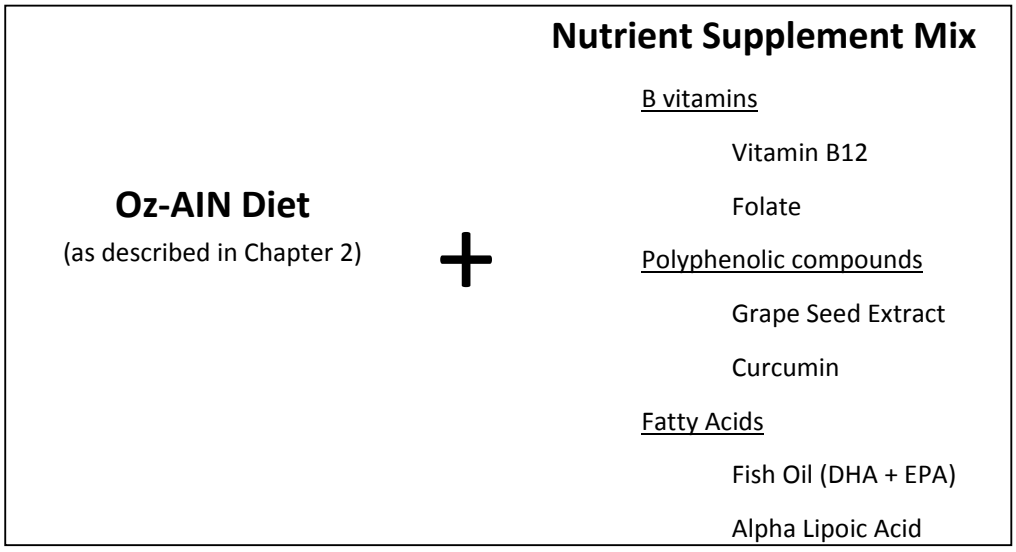
Chapter 2 presented the development of the first diet that has been designed in preparation of this thesis, which is called the Oz-AIN diet. The current chapter presents the design and development of the second diet that has been created in preparation of this thesis, which is called the Oz-AIN Supp diet. The Oz-AIN Supp diet has been designed to demonstrate the effect of supplementing an Australian-type diet with a mixture of nutrient supplements that have previously been demonstrated to be beneficial in AD prevention.

The unique features of the Oz-AIN diet, which was described in Chapter 2, are that it reflects the macronutrient and micronutrient content of diets consumed by Australian women. The P:M:S values have been carefully adjusted to 1.0: 2.4: 2.7, and the ω-3: ω-6 ratio has been adjusted to 1:10, which matches the P:M:S ratio and ω-3: ω-6 ratio in the diets typically consumed by Australian women [185]. Furthermore, micronutrient content of the Oz-AIN diet has been adjusted to reflect the degree by which essential vitamins and minerals differ from recommended levels. This has created a diet that is low in calcium and anti-oxidants such as folate, but high in vitamins such as niacin. Thus, the Oz-AIN diet is an ideal platform on which to test the effects of supplementing a diet that is typically consumed by a population at risk for AD with neuroprotective dietary nutrient supplements on the neuropathology and behavioural deficits observed in AD mice.

Dietary vitamins and minerals can influence development of the behavioural and neuropathological features of AD. Folate deficiency or iron deficiency accelerate cognitive decline and enhance β-amyloid neuropathology in AD-type mice [296, 326]. Supplementation with anti-oxidants and ω-3 fatty acids can delay the onset of learning impairments and reduce formation of β-amyloid deposits in the brains of AD-type mice [160, 202, 327]. However, whether or not these supplements have an effect when added to a diet that is typically eaten by a population at high risk of developing AD has not been determined.

The Oz-AIN Supp diet that is described here has been designed to investigate the effect of supplementing an Australian-type diet with nutrient supplements that have either been demonstrated to be protective against AD progression in other dietary models, or whose deficiency can accelerate the onset of cognitive decline or β-amyloid neuropathology [157, 296, 328, 329] (Figure 1).

Figure 1. Design of the Oz-AIN Supp diet.



Abbreviations: DHA (docosahexaenoic acid). EPA (eicosapentaenoic acid)

The chapter starts with a description of the design of the Oz-AIN Supp diet. B vitamins (vitamin B12 and folate), polyphenolic compounds (grape seed extract and curcumin) and fatty acids (α-lipoic acid and docosahexaenoic acid and eicosapentaenoic acid from fish oil) have been combined and added to the Oz-AIN diet. The selection and reasoning for each of these nutrient supplements is presented.

Chapter 2 demonstrated that both diet-type and genotype had effects on body weight, fat deposition or organs weight. Therefore the current chapter set out to determine whether or not nutrient supplements can prevent genotype or diet-type effects on weight gain, food consumption and organ size of Amy mice. This is presented in two sections (i) the potential for nutrient supplements to prevent *genotype* induced effects on weight gain, body fat and organ size in Amy mice that are fed the Oz-AIN diet, and (ii) the potential ability of nutrient supplements to prevent *diet-type* effects on weight gain, body fat and organ size in Amy mice that have been fed the Oz-AIN diet.

One of the primary findings of the study described in this chapter is that while Amy mice that are fed the Oz-AIN diet undergo rapid weight gain throughout life, the mice that are fed the Oz-AIN Supp diet gain weight at a slower rate. This suggests that the total fat content of rodent diets is not the sole contributor to weight gain in Amy mice. The finding that weight gain of Amy mice that were fed the Oz-AIN Supp diet occurred at a rate that was similar to normal mice that are fed the Oz-AIN diet suggests that an element of the Oz-AIN Supp diet mix altered lipid metabolism in Amy mice so that it was more similar to that of normal mice. Owing to the anti-obesogenic effects of ω-3 fatty acids, the reduced weight gain of the Amy mice that were fed the Oz-AIN Supp diet may be mostly attributed to docosahexaenoic acid and eicosapentaenoic acid, which were added to the Oz-AIN Supp diet as fish oil.

It is concluded that supplementing an Australian-type diet with B vitamins, polyphenolic compounds and fatty acids at levels that have previously been demonstrated to reduce characteristics of AD, may also have other health benefits. In particular, this combination of nutrient supplements may also offer a means to manage diet-induced obesity.

3.1. Methods.

3.1.1. Animals.

All experiments were approved by the Commonwealth Scientific and Industrial Research Organisation (CSIRO) Animal Welfare Committee, Australia in accordance with National Health and Medical Research Council guidelines.

Female Amy (APPswe/PSEN1dE9) mice and their female normal (C57bl/6) littermates were bred at and provided by Flinders University Animal Facility, Bedford Park, South Australia. Genotype of mice was confirmed by PCR and agarose gel electrophoresis, as described in Appendix I. Amy mice were fed either the Oz-AIN diet, the AIN93-M diet, or the Oz-AIN Supp diet from weaning until 15 months of age. Normal mice were fed the Oz-AIN diet and were used as a control. Some of the mice in each treatment group died of natural causes before the end of the experiment, and therefore group sizes were smaller at 15 months of age than at the start of the study. Group sizes every 10 weeks post weaning are reported in Table 1.

Body weight was measured twice weekly. Animals were housed in cages that were lined with sawdust and had tunnels and tissues for environmental enrichment, and had free access to food and water ($n<6$ per cage). Mice had access to their respective diets from the top of the cage and in food bowls that had been placed within the cage (see

pp . 41-43). Twice a week the amount of diet remaining in the food bowls and on the top of the cage was recorded and fresh diet was returned to the cage. This was done to ensure that the fats in the Oz-AIN diet did not oxidise while they were in cages with mice.

At the end of the experiment mice were anaesthetised with isoflurane and killed by exsanguination from the abdominal aorta. Mice were perfused with PBS before fat tissue, liver, kidneys, heart, and spleen were collected and weighed. Organs were frozen in liquid nitrogen and stored in the CSIRO bio-bank. Uterine, renal, liver and subcutaneous fat deposits were stored in RNAlater Stabilization Reagent (76106, Qiagen).

Table 1. Group sizes every 10 weeks post weaning of normal mice that were fed the Oz-AIN diet and Amy mice that were fed the AIN93-M diet, the Oz-AIN diet or the Oz-AIN Supp diet for 15 months.

	Normal mice fed the Oz-AIN diet	**Amy mice fed the AIN93-M diet**	**Amy mice fed the Oz-AIN diet**	**Amy mice fed the Oz-AIN Supp diet**
5 weeks	n = 14	n = 12	n = 15	n = 17
15 weeks	n = 13	n =12	n = 15	n = 17
25 weeks	n = 13	n = 11	n = 15	n = 16
35 weeks	n = 12	n = 11	n = 15	n = 16
45 weeks	n = 11	n = 11	n = 15	n = 16
55 weeks	n = 11	n = 11	n = 14	n = 15
65 weeks	n = 8	n = 11	n = 14	n = 12

3.1.2. Study design.

Genotype Effects.

Genotype effects were determined by comparisons between normal and Amy mice that were fed the Oz-AIN diet (Figure 2A).

The potentially beneficial effect of nutrient supplements against the genotype induced effects on weight gain, fat deposit weight or organ weight were investigated. Comparisons were made between Amy mice that were fed the Oz-AIN Supp diet and either normal or Amy mice that were fed the Oz-AIN diet. It was expected that if the nutrient supplements prevented genotype induced changes, there would be no difference in body weight, food consumption or organ size between normal mice that were fed the Oz-AIN diet and Amy mice that are fed the Oz-AIN Supp diet (Figure 2A).

The effect of nutrient supplements against genotype effects in normal and Amy mice were fed the AIN93-M diet was not investigated. This is because the aims of this study were to demonstrate the effect of supplementing a diet typically consumed by Australians, rather than an ideal diet. Therefore, supplementing the AIN93-M diet would not have been appropriate for the research question.

Diet-Type Effects.

Diet-type effects of Oz-AIN diet were determined by comparisons between Amy mice that were fed either the AIN93-M diet or the Oz-AIN diet (Figure 2B).

The potential for nutrient supplements to prevent diet-induced obesity and weight gain in Amy mice was investigated through comparisons between Amy mice that were fed the Oz-AIN Supp diet and either Amy mice that were the AIN93-M diet or

the Oz-AIN diet. It was expected that if the nutrient supplements were able to prevent diet-type induced changes in body weight, fat deposits or organ weight, then there would be no differences between Amy mice that were fed the Oz-AIN Supp diet and the AIN93-M diet, but that there would be differences between Amy mice that were fed the Oz-AIN Supp diet and the Oz-AIN diet (Figure 2B).

Figure 2A. The study design to investigate genotype effects on food and energy intake, weight gain and the weight of fat deposits and major organs.

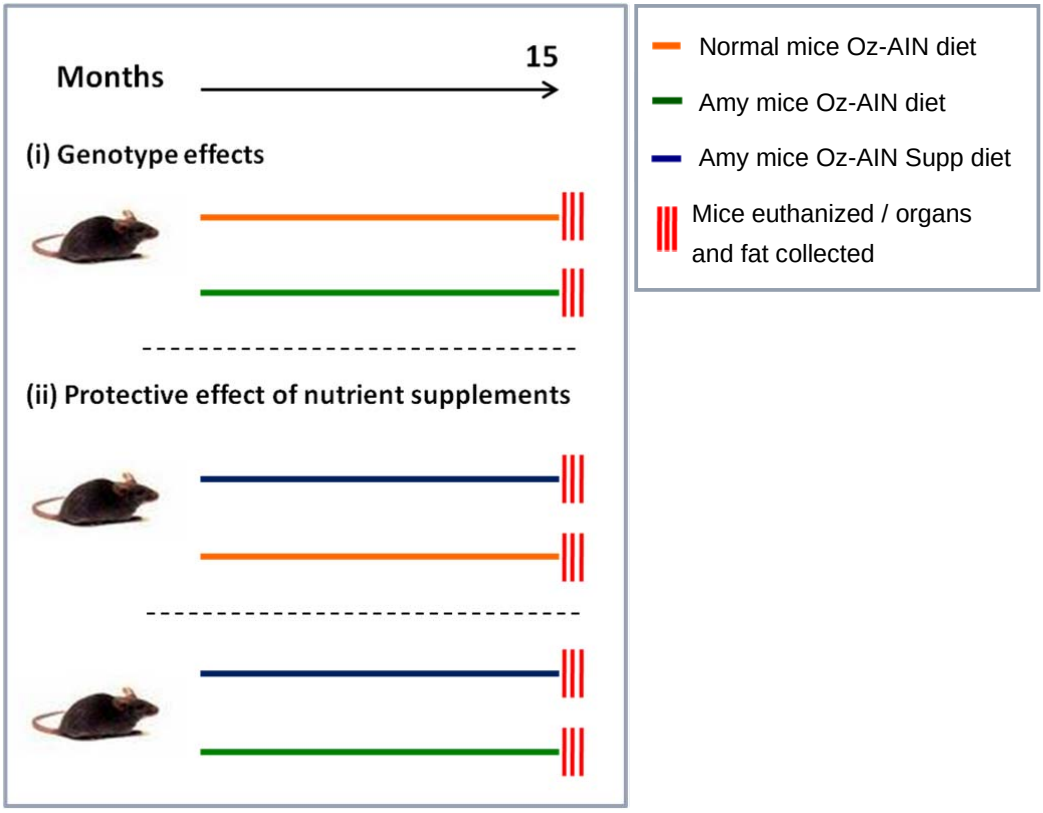


Figure 2A. Mice were fed their respective diets from weaning until they were 15 months. At the end of the study, mice were euthanized, and their organs and body fat were collected and weighed.

The ability of nutrient supplements to prevent genotype effects on food and energy intake, organ size and body weight were investigated with three comparisons:

(i) Normal mice (orange line) and Amy mice (green line) that were fed the Oz-AIN diet were compared to demonstrate the *genotype* effects on food and energy intake, body weight and organ size of Amy mice.

(ii) Normal mice and Amy mice that were fed the Oz-AIN diet were also compared with Amy mice that were fed the Oz-AIN Supp diet (blue line) to determine whether or not nutrient supplements could prevent the *genotype* induced effects on and food and energy intake, body weight, and organ size in Amy mice.

Figure 2B. The study design to investigate diet-type effects on food and energy intake, weight gain and the weight of fat deposits and major organs.

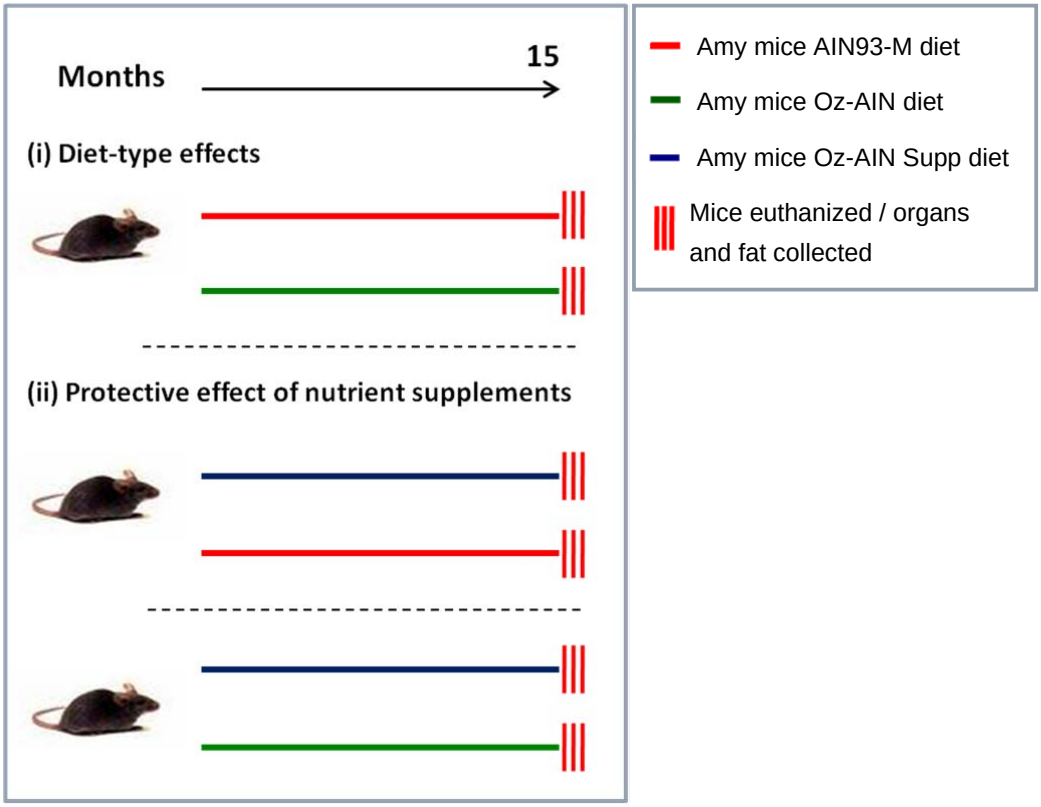


Figure 2B. Mice were fed their respective diets from weaning until they were 15 months. At the end of the study, mice were euthanized, and their organs and body fat were collected and weighed.

The ability of nutrient supplements to prevent diet-type effects on food and energy intake, organ size and body weight were investigated with three comparisons:

(i) Amy mice that were fed either the AIN93-M diet (red line) or the Oz-AIN diet (green line) were compared to demonstrate the *diet-type* effects on food and energy intake, body weight and organ size of Amy mice.

(ii) Amy mice that were fed either the AIN93-M or the Oz-AIN diet were also compared with Amy mice that were fed the Oz-AIN Supp diet (blue line) to determine whether or not nutrient supplements could prevent the *diet-type* induced effects on and energy intake, weight gain, and weight of fat deposits and major organs in Amy mice.

3.1.3. Data analysis.

To address the aims of these studies, data analysis was divided into two groups.

(i) The potentially beneficial effects of nutrient supplements against genotype effects were made through comparisons between normal mice that were fed the Oz-AIN diet, Amy mice that were fed the Oz-AIN diet and Amy mice that were fed the Oz-AIN Supp diet (Figure 2A).

(ii) The potentially beneficial effects of nutrient supplements against diet-type effects were made through comparisons between Amy mice that were fed the AIN93-M diet, Amy mice that were fed the Oz-AIN diet and Amy mice that were fed the Oz-AIN Supp diet (Figure 2B).

Food intake and estimated energy intake were measured as described in Chapter 2 (pp. 42-45). Briefly, food consumption was estimated based on the food remaining in cages at the end of each week. The energy intake was estimated by calculating food intake values by the energy content of each diet (Table 6). Every five weeks, comparisons of mean (±SEM) mouse body weight (g) and food consumption (g/day) were made using one-way ANOVA's and Bonferroni post tests. Student's *t*-tests were also used to compare normal and Amy mice that were fed the Oz-AIN diet. Overall food consumption (g) and energy intake (kJ) were calculated by area under the curves. Genotype and diet-type effects overall food consumption (g) and energy intake (kJ) were determined using one-way ANOVA's and Bonferroni post tests.

Genotype and diet-type effects on mean (±SEM) organ weight (g) and fat deposits (g) were determined using one-way ANOVA and Bonferroni post tests. Student's *t*-tests were used to compare normal and Amy mice that were fed the Oz-AIN diet. Unless otherwise indicated statistical significance was established at $p<0.05$.

3.2. Design of the Oz-AIN Supp diet.

3.2.1. Selection of nutrient supplements in the Oz-AIN Supp diet.

Nutritional supplementation with single dietary nutrients or with supplement combination mixes has been demonstrated to prevent cognitive decline and neuropathology of AD [204, 330]. The polyphenolic compounds that are found in fruits such as grape seed extract, pomegranate extract, blackcurrant extract alleviate spatial memory deficits, block β-amyloid processing and prevent aggregation of hyperphosphorylated tau [157, 331, 332].

Parachikova *et al*. supplemented a standard rodent chow (AIN73-M) with a cocktail of nutrient supplements including curcumin, piperine, epigallocatechin gallate, α-lipoic acid, N-acetylcysteine, B vitamins, vitamin C, and folate [204]. They report that transgenic mice that were fed the nutrient cocktail in small or high doses had intact spatial learning and spatial memory skills, and had reduced β-amyloid neuropathology relative to mice that did not receive supplements. While the benefits of Parachikova *et al.* are promising, their nutrient cocktail was added to a diet that already met nutritional requirements of mice. They were unable to provide information about how well supplementation with a combination of B vitamins, fatty acids and polyphenolic compounds would fair against a diet that induced oxidative stress or inflammation, which are two key events that lead to β-amyloid generation.

In the current research, the Oz-AIN diet, which is high-fat diet that is deficient in anti-oxidants such as folate has been supplemented with a nutrient supplement mixture to create the Oz-AIN Supp diet. The nutrients in the nutrient supplement mix have previously been demonstrated to be beneficial against AD, either alone or in combination, but not against a diet similar to the Oz-Ain diet (Table 2).

B Vitamin Supplements.

Folate and vitamin B12 are essential vitamins that have antioxidant capabilities and play important roles in DNA and RNA synthesis and homocysteine remethylation [287]. While the evidence for folate to reduce AD neuropathology and behavioural deficits prevention is not strong, dietary supplements may still be warranted as there is evidence to suggest that folate deficiency and vitamin B12 deficiency may lead to increased risk for developing AD [287-291]. Folate deficiency and vitamin B12 deficiency impair spatial learning and memory in aged mice [333] but not young mice [334]. This suggests that vitamins B12 and folate are important for learning and memory in adulthood. Furthermore, folate, vitamin B6 and vitamin B12 deficiency increases β-amyloid deposition in AD-type mice [296].

The detrimental effect of low vitamin B12 or low folate levels on learning and memory may be partially due to their roles in homocysteine metabolism. Elevated plasma homocysteine is associated with neurological impairments such as dementia, learning difficulties, brain atrophy, and AD [289, 297, 298], and AD patients have higher homocysteine levels and lower serum folate levels than healthy controls [335, 336]. Homocysteine requires folate and vitamin B12 for its conversion to methionine [287]. In the absence of folate and vitamin B12, the essential methyl donors and complexes that are required for conversion of homocysteine to methionine cannot be formed, and therefore homocysteine levels rise, and increases risk for AD.

Folate and vitamin B12 may be beneficial in ways other than through their roles in homocysteine remethylation. For example, vitamin B12 is required for the synthesis of succinyl CoA and subsequent production of fatty acids and neurotransmitters [295, 337]. Folate is required for *de novo* synthesis of purine and thymidylate synthase

building blocks for DNA and RNA synthesis [287, 299]. Therefore, folate may be beneficial in preventing DNA damage with aging, and reduce AD pathology.

The data from the 1995 National Nutrition Survey describe the diets typically consumed by Australian women contain half the recommended amount of folate [185]**.** It is acknowledged that since voluntary fortification of foods in 1995, dietary folate intake has increased [209, 338]. However, folate deficiency has continued to rise amongst lower socio-economic and regional communities [210], suggesting that even a modern diet may benefit from folate consumption.

Folate (6 mg/kg, F8758, Sigma-Aldrich, Aus) and vitamin B12 (1.0 mg/kg, Blackmores, Warriewood, Aus) have been added to the nutrient supplements mix in the Oz-AIN Supp diet (Table 2). The Oz-AIN diet contained low levels of each of these micronutrients. The total content of vitamin B12 and folate is 1.04 mg/kg and 7.14 mg/kg respectively. These values are well above recommended doses; however no literature could be found citing toxic effects of folate or vitamin B12 at these concentrations.

Polyphenolic Compound Supplements.

Polyphenolic compounds are small plant derived compounds that have powerful anti-oxidant capabilities and may play a role in preventing AD [339].The polyphenolic compounds found in red wine are extremely potent anti-oxidants and free radical scavengers and prevent the oxidative stress and damage that is usually associated with a high saturated-fat diet [339]. In AD, the polyphenolic compounds curcumin and grape seed extract offer protection against β-amyloid neuropathology through binding to and disaggregating β-amyloid deposits [340]. When fed to adult AD-type mice, curcumin reduces plaque burden, amyloid load, soluble and insoluble amyloid,

and plaque aggregation [214, 341]. Lower levels of dietary curcumin supplements have been demonstrated to be more successful than higher levels at reducing AD neuropathology, suggesting that there is a "saturation" effect on mechanisms used by curcumin in prevention of AD. AD-type mice fed 150 ppm curcumin have reduced astrocytosis, reduced levels of soluble and insoluble β-amyloid and reduced plaque burden, compared to mice fed 5000 ppm [214]. Wang *et al*. fed Amy mice diets containing grape seed extract (2.0%) or curcumin (0.07%) from 3 months until they were 12 months old. Both of these polyphenolic compounds reduced β-amyloid neuropathology in the Amy mouse model of AD [157]. In order to avoid the saturation effects of curcumin that were observed by Lim *et al.*, the nutrient supplement mix in the current study used the same doses of curcumin and grape seed extract as Wang *et al*. (Table 2).

Curcumin (700mg/kg, C1386, Sigma, Aus) and grape seed extract (20,000 mg/kg, Fingerprint Botanicals®, Natures Own$_{TM}$) have been added to the nutrient supplements mix in the Oz-AIN Supp diet (Table 2). Neither of these micronutrients were in the original Oz-AIN diet mixture and have been added at levels previously demonstrated to be well tolerated by mice [157].

Fatty Acid Supplements.

The ω-3 long chain fatty acid docosahexaenoic acid has been demonstrated to delay the onset of learning and memory impairments and to reduce β-amyloid neuropathology in AD-mouse models [86, 160, 218]. Whether the benefits of docosahexaenoic acid supplementation are due to the total amount of docosahexaenoic acid in a diet or the improved ω-3: ω-6 ratio after the addition of docosahexaenoic acid is still uncertain.

Docosahexaenoic acid supplements have anti-inflammatory capabilities by increasing an anti-apoptotic kinases [219] and reducing activity of pro-inflammatory cytokines [220]. The anti-inflammatory capability of docosahexaenoic acid has been demonstrated to have beneficial effects in reducing AD neuropathology and behavioural deficits in rodent models of AD [191, 219, 221].

Docosahexaenoic acid also has direct interactions with cellular membranes and alters membrane fluidity and reduce APP processing down the amyloidogenic pathway [342]. Oksman *et al.* reported that increasing the ω-3: ω-6 ratio of a diet to 1.0:1.4 reduces β-amyloid neuropathology in 9 month old Amy mice [218]. Later studies by Hooijmans *et al*. demonstrated that docosahexaenoic acid supplements restored learning abilities and reduced β-amyloid neuropathology in aged Amy mice [86]. The present study has included fish oil in the nutrient supplemented diet as a source of docosahexaenoic acid that will also enable a low ω-3: ω-6 ratio. Fish oil (OmegaSure liquid Fish Oil, BioCeuticals, NSW, Australia) was added to the diet as a source of ω-3 fatty acids with an ω-3: ω-6 ratio of 1.0:1.4 (Table 5).

One of the major characteristics of AD brains is impaired mitochondrial functioning. Alpha-lipoic acid is an essential precursor for many mitochondrial enzymes and can facilitate mitochondrial functioning in the brain [343, 344]. Furthermore, α-lipoic acid is more successful at slowing the rate of cognitive decline in AD patients than acetyl choline esterase inhibitors, which are a common pharmaceutical intervention for AD [345]. In rodent models of AD, supplementation with α-lipoic acid at concentrations as low as 1.0% (wt/wt) have been demonstrated to reduce oxidative stress and restore learning and memory [346-348]. Owing to its low toxicity at these levels, α-lipoic acid (Nutra-Life, NZ) has been added to the nutrient supplemented diet at 1.0% (wt/wt) (Table 2).

3.2.2. Production of the Oz-AIN Supp diet nutrient supplement mix.

The nutrient supplement mix that is used in the Oz-AIN Supp diet is a combination of nutrients that have previously been demonstrated to delay or prevent behavioural deficits or neuropathology of AD (Table 2). Where possible, nutrients that are commercially available for human consumption were used.

Folate, vitamin B12, α-lipoic acid, curcumin, and grape seed extract were combined and made up to 50 g/kg with commercially available sucrose (SUGW/25, FTA Food Solutions, Altona, Vic, Aus). To prevent oxidation of the nutrients within each mix, mixes were stored in airtight bags at 4°C until required. While fish oil was also added to the Oz-AIN Supp diet, it was more appropriate to add fish oil with other oils while making the diet rather than combine it with the nutrient supplement mix.

Table 2. Nutrients in the nutrient supplement mix that was used in the Oz-AIN Supp diet.

NUTRIENT	**mg / kg diet**
Vitamin B12 (cyancobalamin) (Blackmores, Warriewood, Aus)	1.0
Folate (folic acid) (F8758, Sigma-Aldrich, Aus)	6.0
Curcumin (C1386, Sigma, Aus)	700.0
Alpha Lipoic Acid (Nutra-Life, NZ)	1,000.0
Grape Seed Extract (Fingerprint Botanicals®, Natures Own$_{TM}$)	20,000.0
Sucrose (SUGW/25, FTA Food Solutions, Vic, Aus)	28,293.0
TOTAL	50,000.0

3.2.3. Production of the Oz-AIN Supp vitamin and mineral mixes.

The vitamin and mineral mixes that were used in the Oz-AIN Supp diet are the same vitamin and mineral mixes as those used in the Oz-AIN diet (Table 3, Table 4).

As described in Chapter 2, the Oz-AIN vitamin mix and Oz-AIN mineral mix were made independently and each was made up to 15 g/kg with commercially available sucrose that is used for human consumption (Table 3, Table 4). To prevent oxidation of vitamins and minerals within each mix, both mixes were stored in airtight bags at 4°C until required.

3.2.4. Production of the Oz-AIN Supp diet.

The Oz-AIN Supp diet reflects an Australian-type diet with additional nutrient supplements. Therefore, the Oz-AIN Supp diet was made by a similar process as the Oz-AIN diet (pp. 54-63, Chapter 2), with the following modifications:

(i) 50 g/kg of sucrose was replaced with 50 g/kg of the nutrient supplement mix (Table 2, Table 5), which was added at the same time as vitamin mix (Table 3) and mineral mix (Table 4).

(ii) Fish oil was added at 23.3 g/kg (Table 5) to provide a P:M:S ratio of 1.0: 1.7: 1.9, in comparison with the P:M:S ratio of the Oz-AIN diet, which is 1.0: 2.4: 2.7. The ω-3: ω-6 ratio has been adjusted from 1.0: 10.0 to 1.0: 5.2 (Table 6).

(iii) Oz-AIN Supp diet was made weekly in one kg batches and stored in sealed glass containers at 4°C for no longer than 7 days to minimize the likelihood of oxidation.

Table 3. The Vitamin Mix for the Oz-AIN Supp diet is the same as that used in the Oz-AIN diet.

VITAMINS	IU / 10 kg diet	mg / 10 kg diet
Vitamin A (Retinol) (IU) (Thompsons Auckland, NZ)	58,540.0	58,540.0
Vitamin D (Cholecalciferol) (IU) (Blackmores, NSW, Aus)	10,000.0	10,000.0
Vitamin E (α-tocopherol) (Sigma-Aldrich, Aus)		338.0
Vitamin K (Menadione) (47775, Supelco, Aus)		9.0
Choline (C7017, Sigma-Aldrich, Aus)		10,000.0
Vitamin B1 (Thiamin) (T4625, Sigma-Aldrich, Aus)		64.0
Vitamin B2 (Riboflavin) (R-4500, Sigma, Aus)		98.0
Niacin (Nicotinic acid) (N0761, Sigma, Aus)		756.0
Vitamin B6 (Pyridoxine) (P5669, Sigma, Aus)		60.0
Pantothenic acid (P5155, Sigma, Aus)		150.0
Folate (Folic acid) (F8758, Sigma-Aldrich, Aus)		11.0
Vitamin B12 (Cyancobalamin) (Blackmores, NSW, Aus)		0.4
Biotin (B4639, Sigma, Aus)		2.0
Sucrose (SUGW/25, FTA Food Solutions, Vic, Aus)		38,512.0
TOTAL		**50000.0**

Table 4. The Mineral Mix for the Oz-AIN Supp diet is the same as that used in the Oz-AIN diet.

MINERAL SALT	mg / 10 kg diet
Calcium Carbonate (10068, BDH Chemicals, Aus)	40,810.5
Potassium Phosphate (P5379, Sigma, Aus)	38,219.4
Potassium Citrate●H_2O (60153, Sigma-Aldrich, Aus)	1,903.8
Sodium Chloride (S9888, Sigma-Aldrich, Aus)	11,100.0
Potassium Sulfate (P9458, Sigma-Aldrich, Aus)	7,025.0
Magnesium Oxide (243388, Sigma-Aldrich, Aus)	3,190.5
Ferric Citrate (F-6129, Sigma-Aldrich, Aus)	606.0
Zinc Carbonate (ZL004, Chem Supply, SA, Aus)	306.3
Manganous Carbonate (29136, BDH Chemicals, Aus)	61.8
Copper (II) Carbonate (CL035, Fluka, Gilman, SA, Aus)	45.0
Potassium Iodide (60399, Fluka, Gilman, SA, Aus)	1.5
Sodium Selenate (S5261, Sigma, Aus)	1.5
Ammonium Paramolybdate●$4H_2O$ (09878, Fluka, Gilman, SA. Aus)	1.2
Sodium Metasilicate (S4392, Sigma, Aus)	217.5
Chromium Potassium Sulphate $12H_2O$ (S243361, Sigma, Aus)	41.5
Lithium Chloride (LL036, Chem Supply, SA, Aus)	2.6
Boric Acid (B6768, Sigma, Aus)	12.2
Sodium Fluoride (10246, AnalaR, British Drug Houses, Eng)	9.5
Nickel Carbonate (NL007, Chem Supply, SA, Aus)	4.8
Ammonium Vanadate (AL072, Chem Supply, SA, Aus)	1.0
Sucrose (SUGW/25, FTA Food Solutions, Vic, Aus)	46,438.4
TOTAL	**150,000.0**

Table 5. Composition of the Oz-AIN Supp diet.

Ingredient	g / kg
Sugar (SUGW/25, FTA Food Solutions, Vic, Aus)	193.8
Starch (National Starch, Lane Cove, NSW, Aus)	309.6
Lard (Conroy's Small Goods, Bowden, SA, Aus)	44.8
Canola Oil (ml) (Coles Supermarkets Australia)	22.4
Sunflower Oil (ml)(Crisco; Goodman Fielder, North Ryde, NSW, Aus)	16.8
Coconut Oil (Nui; African Pacific Pty. Ltd., Terry Hills, NSW, Aus)	61.6
Olive Oil (ml) (Coles Supermarkets Australia)	39.2
Fish Oil (ml)(OmegaSure Liquid Fish Oil, BioCeuticals®, Blackmores Limited, NSW, Aus)	23.3
Protein (acid casein 1704896-6, Fonterra Ltd., Auckland, NZ)	193.9
Fibre (α-cellulose, C-8002, Sigma-Aldrich)	38.0
Nutrient supplements mix	50.0
Vitamin mix	15.0
Mineral mix	15.0
TOTAL	**1000.0**

Table 6. Comparison of the energy content (kcal) and P:M:S ratio of the AIN93-M diet, Oz-AIN diet and Oz-AIN Supp diet.

	Total energy (kcal)	Carbohydrate (% energy)	Protein (% energy)	Fat (% energy)	P:M:S
AIN93-M diet	16.66	75.9%	14.1%	10.0%	3.7: 1.5: 1.0
Oz-AIN diet	20.11	46.7%	16.8%	33.0%	1.0: 2.4: 2.7
Oz-AIN Supp	20.98	47.4%	15.7%	36.9%	1.0: 1.7: 1.9

3.3. An investigation into the potentially beneficial effect of nutrient supplements against genotype induced changes in Amy mice.

Chapter 2 reported that there were genotype effects on food and energy intake, fat deposit weight and that Amy mice tended to be heavier than normal mice. Therefore, an investigation was made to determine whether or not nutrient supplements could alter genotype induced effects on food and energy intake, body weight gain and the weight of major organs and fat deposits in Amy mice that had been fed the Oz-AIN diet.

3.3.1. The potential effect of nutrient supplements against genotype effects on estimated food intake (g/day).

A one-way ANOVA of total food consumed by normal mice that were fed the Oz-AIN diet, Amy mice that were fed the Oz-AIN diet and Amy mice that were fed the Oz-AIN Supp diet did not reveal significant differences of food consumption over 65 weeks ($p=0.68$, Table 7, Figure 3A).

Table 7. The ability of nutrient supplements to prevent genotype effects on overall food intake (g) and energy intake (kJ) by normal and Amy mice that were fed the Oz-AIN diet.

	Normal mice fed the Oz-AIN diet	Amy mice fed the Oz-AIN diet	Amy mice fed the Oz-AIN Supp diet
Food intake over 15 months (g)	1576.0 ±124.7	1630.8 ±140.9	1757.3 ±168.5
Energy intake over 15 months (kJ)	31691 ±2510	29771 ±2495	36858 ±3537

Overall food intake (g) and energy intake (kJ) were calculated from the area under the curve of figures 3A and 3B respectively. Values are mean ±SEM.

Comparison of the amount of food that Amy mice and normal mice ate every five weeks indicated that there were significant differences in food consumption on week 20 ($p=0.0001$, Figure 3A), week 30 ($p=0.004$, Figure 3A), and week 35 ($p=0.003$, Figure 3A). However, these differences were not attributed to genotype. Bonferroni post tests revealed that Amy mice that were fed the Oz-AIN diet and normal mice that were fed the Oz-AIN diet only differed in the average amount of food eaten per day (g/day) only once (week 35, $p=0.04$, Figure 3A). The remaining weeks where there were significant differences between groups for the amount of food eaten were due to Amy mice that were fed the Oz-AIN Supp diet eating more food than either Amy mice that were fed the Oz-AIN diet (week 20, $p=0.0002$; week 30, $p=0.004$, Figure 3A) or normal mice that were fed the Oz-AIN diet (week 20, $p=0.0007$; week 35, $p=0.003$, Figure 3A). Collectively, these data indicate that while nutrient supplements effected the amount of food eaten by Amy mice, this was not due to any genotype effects.

3.3.2. The potential effect of nutrient supplements against genotype effects on estimated energy intake (kJ/day).

There was no significant effect of genotype on the energy intake over 15 months (Table 7, Figure 3B). One-way ANOVA on energy intakes every five weeks indicated that energy intakes were different between mice throughout young adulthood. These differences were significant when mice were 15 weeks ($p=0.008$, Figure 3B), 20 weeks ($p<0.0001$, Figure 3B), 25 weeks ($p=0.020$, Figure 3B), 30 weeks ($p=0.0002$, Figure 3B) and 35 weeks old ($p=0.001$, Figure 3B). However, Bonferroni post tests revealed that these differences were mostly attributed to energy intake by Amy mice that were fed the Oz-AIN Supp diet (Figure 3B). This suggests that, similar to food intake, energy intake was not affected by genotype. Possible diet-type effects on energy intake are discussed on pages 151-152.

Figure 3A. The effect of nutrient supplements on potential genotype effects on food intake (g/day) by normal and Amy mice.

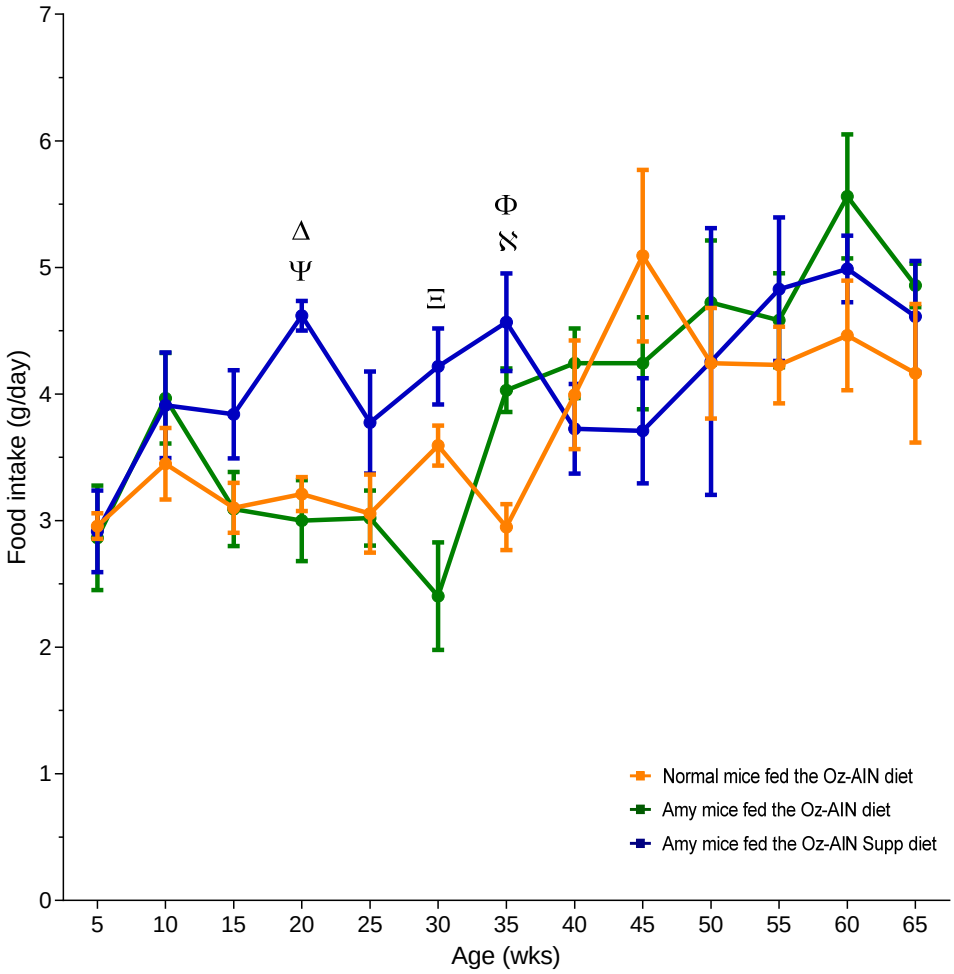


Figure 3A. The effect of nutrient supplements on potential genotype effects on food consumed (g/day). Normal mice fed the Oz-AIN diet (orange line, wk 5: n=14, wk 65: n=8), and Amy mice fed the Oz-AIN diet (green line, wk 5: n=15, wk 65: n=14). Error bars are mean ±SEM. Significant differences were detected between groups with Bonferroni post tests. (Φ) p=0.04 between normal mice that were fed the Oz-AIN diet and Amy mice that were fed the Oz-AIN diet. (Ψ) p=0.0002, (Ξ) p=0.003 between Amy mice that were fed the Oz-AIN diet and Amy mice that were fed the Oz-AIN Supp diet. (Δ) p=0.0007, (χ) p=0.003 between normal mice that were fed the Oz-AIN diet and Amy mice that were fed the Oz-AIN Supp diet.

Figure 3B. The effect of nutrient supplements on potential genotype effects on energy intake (kJ/day) by normal and Amy mice.

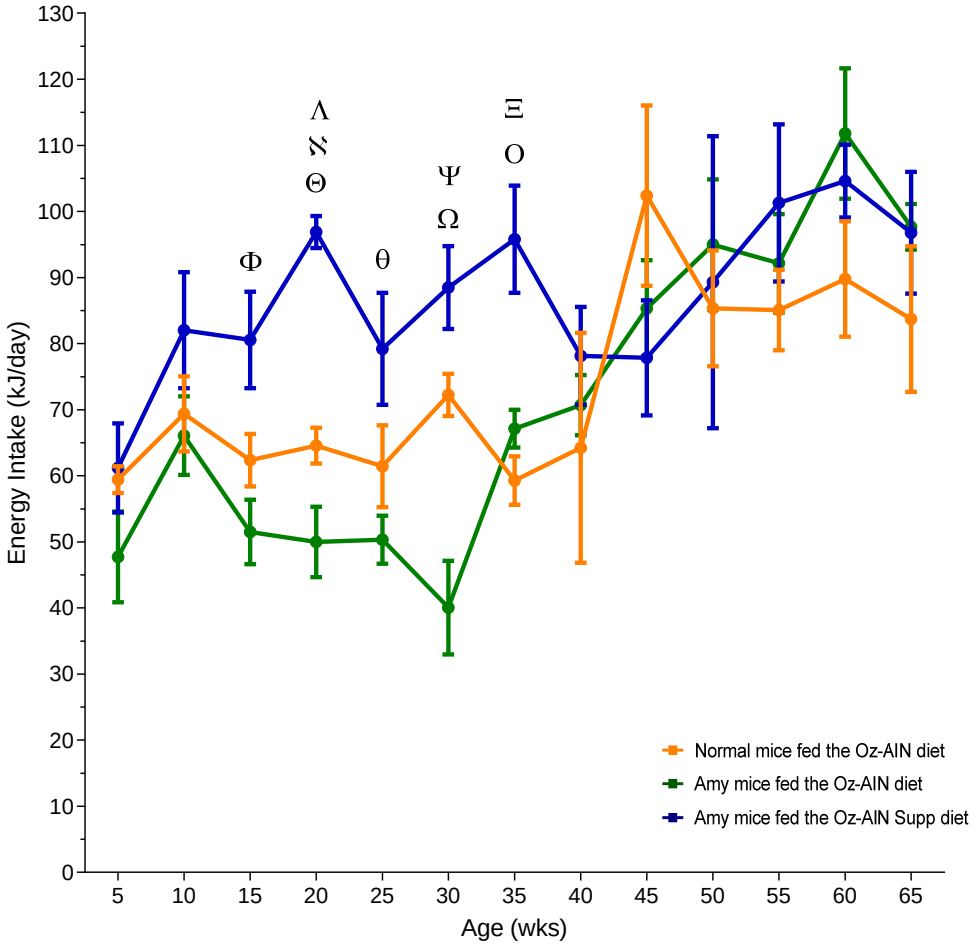


Figure 3B. Estimated energy intake (kJ/day) from the Oz-AIN diet. Normal mice fed the Oz-AIN diet (orange line, wk 5: n=14, wk 65: n=8), and Amy mice fed the Oz-AIN diet (green line, wk 5: n=15, wk 65: n=14) for 65 weeks. Error bars are mean ±SEM. (Ψ) p=0.01, (Δ) p=0.05 between normal mice that were fed the Oz-AIN diet and Amy mice that were fed the Oz-AIN diet. Significant differences were detected between groups with Bonferroni post tests. (Θ) p<0.0001, (Ω) p=0.0001, (Φ) p=0.007, (O) p=0.008, (θ) p=0.02 between Amy mice that were fed the Oz-AIN diet and Amy mice that were fed the Oz-AIN Supp diet. (χ) p<0.0001, (Ξ) p=0.001 between normal mice that were fed the Oz-AIN diet and Amy mice that were fed the Oz-AIN Supp diet.

3.3.3. The potential effect of nutrient supplements against genotype induced weight gain (g) in Amy mice.

Amy mice that were fed the Oz-AIN diet gained weight faster than normal mice that were fed the Oz-AIN diet (Figure 4). However, Bonferroni post tests did not detect significant genotype effects on body weight until mice were 35 weeks old, when Amy mice that were fed the Oz-AIN diet were significantly heavier than normal mice ($p=0.004$, Figure 4). Amy mice that were fed the Oz-AIN diet remained significantly heavier than normal mice that were fed the Oz-AIN diet for the remainder of the study (Figure 4). This indicates that there were genotype effects on body weight of normal mice and Amy mice that were fed the Oz-AIN diet.

There were no differences in body weights of normal mice that were fed the Oz-AIN diet and Amy mice that were fed the Oz-AIN Supp diet (Figure 4). This suggests that the nutrient supplements were able to prevent genotype induced differences in body weight.

As discussed in Chapter 2, AD-type mice may be more susceptible to weight gain due to increased susceptibility to insulin resistance (p.83). If this is the case, this also provides a potential mechanism through which the nutrient supplements may have been able to prevent the genotype effects on body weight. Cheng *et al.* report that insulin resistance and signalling, which are impaired in mice that have been fed an obesogenic diet, was prevented with polyunsaturated fats [349]. This suggests that the ω-3 polyunsaturated fatty acids in the Oz-AIN Supp diet may have improved insulin signalling in Amy mice, and therefore prevented diet-induced obesity. However, the current study does not have measures of insulin levels to support this.

The beneficial effects of nutrient supplements were not likely to be solely mediated through prevention of genotype-induced weight gain. Bonferroni post tests revealed

that Amy mice that were fed the Oz-AIN Supp diet were significantly lighter than Amy mice that were fed the Oz-AIN diet at 20 weeks ($p=0.03$, Figure 4) and 30 weeks ($p=0.01$, Figure 4), which is earlier than when genotype effects start to appear (Figure 4). This suggests that the beneficial effects of nutrient supplements on body weight may also have been attributed to other factors. This is discussed further on pages 154-155.

Figure 4. The potentially beneficial effect of nutrient supplements on genotype induced weight gain (g) in Amy mice.

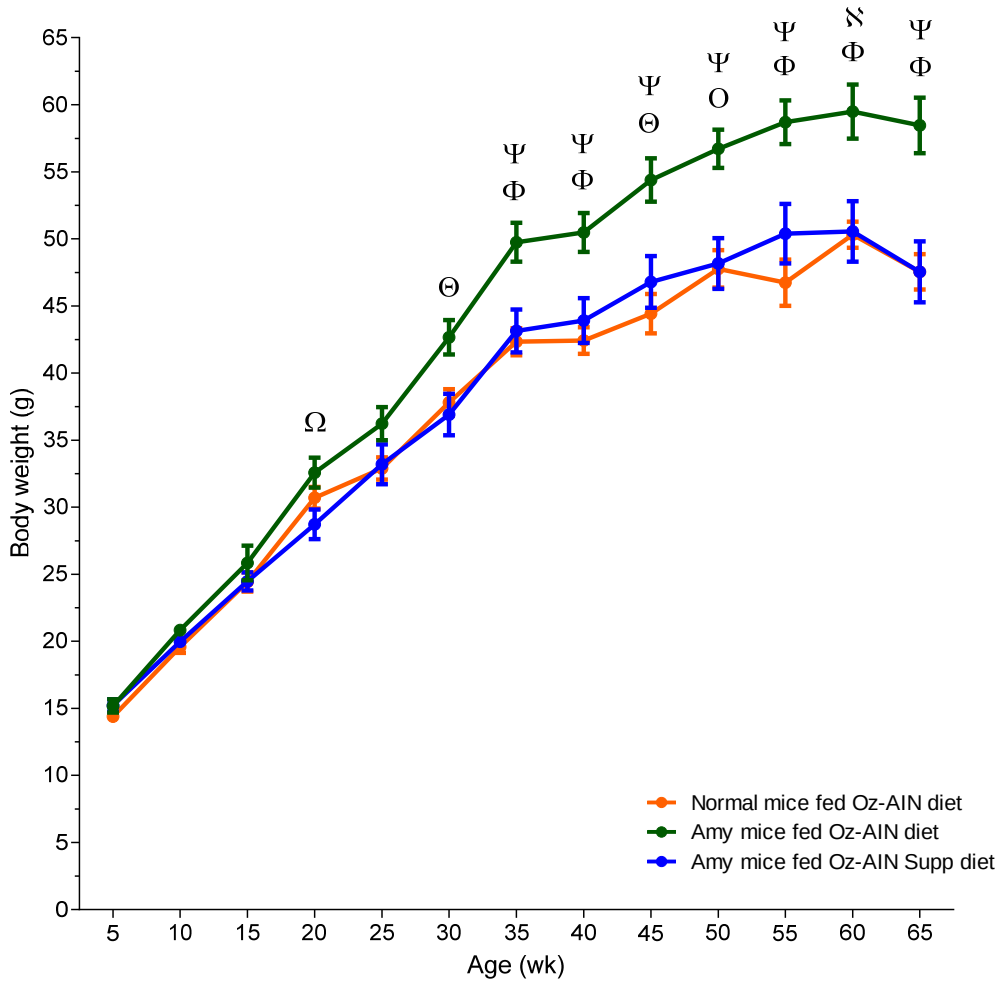


Figure 4. Weight gain (g) of normal mice fed the Oz-AIN diet (orange line, wk 5: n=14, wk 65: n=8), Amy mice fed the Oz-AIN diet (green line, wk 5: n=15, wk 65: n=14), or Amy mice fed the Oz-AIN Supp diet (blue line, week 5: n=16, wk 65: n=12). Error bars are mean ±SEM. Significant differences were detected using Bonferroni post tests. (Ψ) $p<0.01$, (ϰ) $p=0.01$ between normal mice that were fed the Oz-AIN diet and Amy mice that were fed the Oz-AIN diet. (O) $p=0.001$, (Φ) $p=0.01$, (Θ) $p=0.01$, (Ω) $p=0.03$ between Amy mice that were fed the Oz-AIN diet and Amy mice that were fed the Oz-AIN Supp diet. No differences were detected between Amy mice that were fed the Oz-AIN Supp diet and normal mice that were fed the Oz-AIN diet.

3.3.4. The potential effect of nutrient supplements against genotype effects on the weight (g) of fat deposits in Amy mice.

3.3.4.1. Weight (g) of TOTAL FAT deposits collected.

A one-way ANOVA detected significant differences of the weight of fat deposits collected from normal mice that were fed the Oz-AIN diet, Amy mice that were fed the Oz-AIN diet and Amy mice that were fed the Oz-AIN Supp diet (p=0.009, Figure 5). Bonferroni post tests suggested that genotype did not affect the weight of fat collected (p=0.08, Figure 5). However, a Student's *t*-test between the weight of fat collected from normal and Amy mice that were fed the Oz-AIN diet indicated that Amy mice had significantly more fat than normal mice (p=0.02, Figure 5). This is indicative of a genotype effect, and suggests that Amy mice have more fat than normal mice.

Bonferroni post tests revealed that Amy mice that were fed the Oz-AIN Supp diet had significantly less fat than Amy mice that were fed the Oz-AIN diet (p=0.01, Figure 5). This was also found with Student's *t*-test (p=0.006, Figure 5). Neither Bonferroni post tests nor Student's *t*-tests detected differences in the weight of fat collected from Amy mice that were fed the Oz-AIN Supp diet or the normal mice that were fed the Oz-AIN diet. This suggests that the nutrient supplements were able to prevent genotype-induced effects on the weight of fat deposits collected from Amy mice. These data indicate that there is a genotype effect on total fat deposition in Amy mice that are fed the Oz-AIN diet, and this is prevented with nutrient supplements.

Figure 5. The protective effect of nutrient supplements against genotype-induced increase in the weight (g) of fat deposits in 15 month old Amy mice.

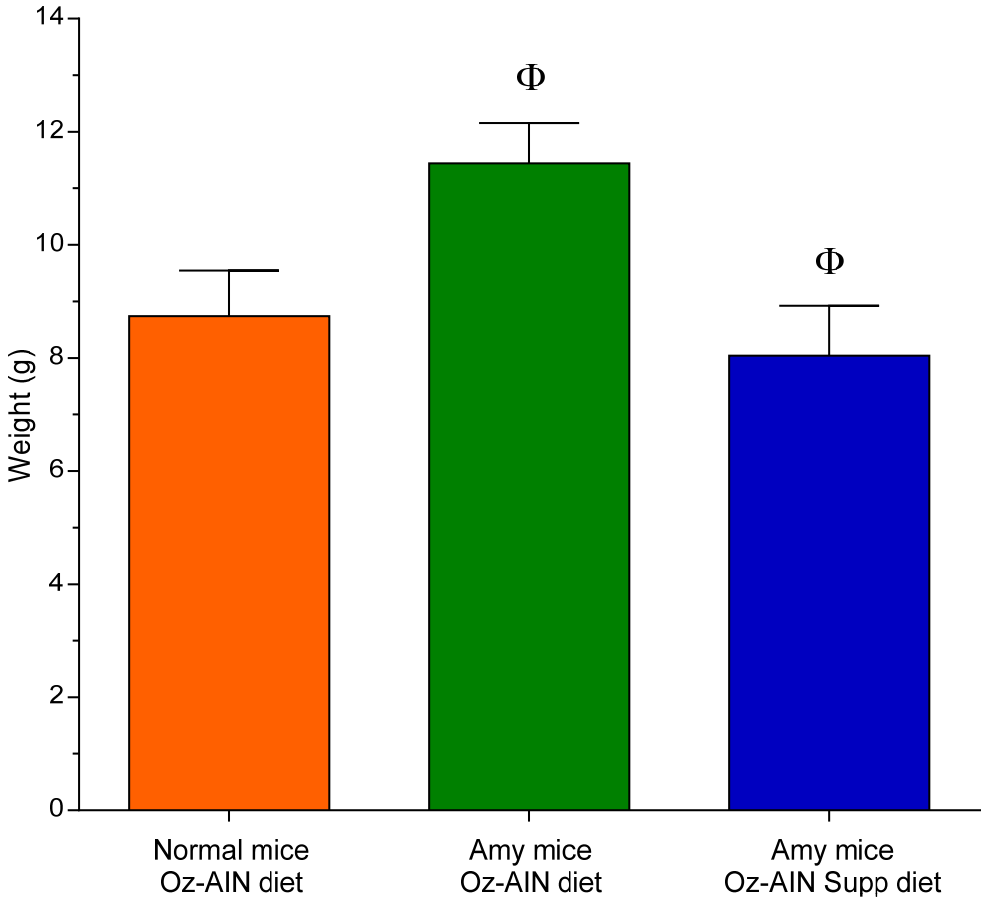


Figure 5. The average weight (g) of total fat that was collected from 15 month old normal mice fed the Oz-AIN diet (n=8, orange bar), Amy mice fed the Oz-AIN diet (n=14, green bar), and Amy mice fed the Oz-AIN Supp diet (n=12, blue bar). Bars represent mean ±SEM. Bars with common symbols are significantly different using Bonferroni post tests. (Φ) p=0.01.

3.3.4.2. Weight (g) of UTERINE FAT deposits.

A one-way ANOVA detected significant differences in the weight of uterine fat collected from normal mice that were fed the Oz-AIN diet, Amy mice that were fed the Oz-AIN diet and Amy mice that were fed the Oz-AIN Supp diet ($p=0.05$, Figure 6). Bonferroni post tests did not detect significant differences between groups. Student *t*-tests revealed that Amy mice that were fed the Oz-AIN diet had significantly more uterine fat than normal mice that were fed the same diet ($p=0.04$, Figure 6).

There was no difference in the weight of uterine fat collected from Amy mice that were fed the Oz-AIN Supp diet and normal mice that were fed the Oz-AIN diet ($p>0.99$, Figure 6), suggesting that the nutrient supplements were able to prevent the genotype effects detected by the one-way ANOVA and Student's *t*-test.

The Amy mice that were fed the Oz-AIN Supp diet had less uterine fat than Amy mice that were fed the Oz-AIN diet ($p=0.06$, Figure 6). While this was not significant at $p<0.05$, it would be significant at $p=0.10$, suggesting a weak trend for the Amy mice that were fed the Oz-AIN Supp diet to have less uterine fat than Amy mice that were fed the Oz-AIN diet. This indicates that nutrient supplements may partially reduce the amount of uterine fat in Amy mice, and that this is mediated through prevention of genotype effects.

Figure 6. The potentially beneficial effect of nutrient supplements against genotype induced increase of uterine fat deposit weight (g) from 15 month old Amy mice.

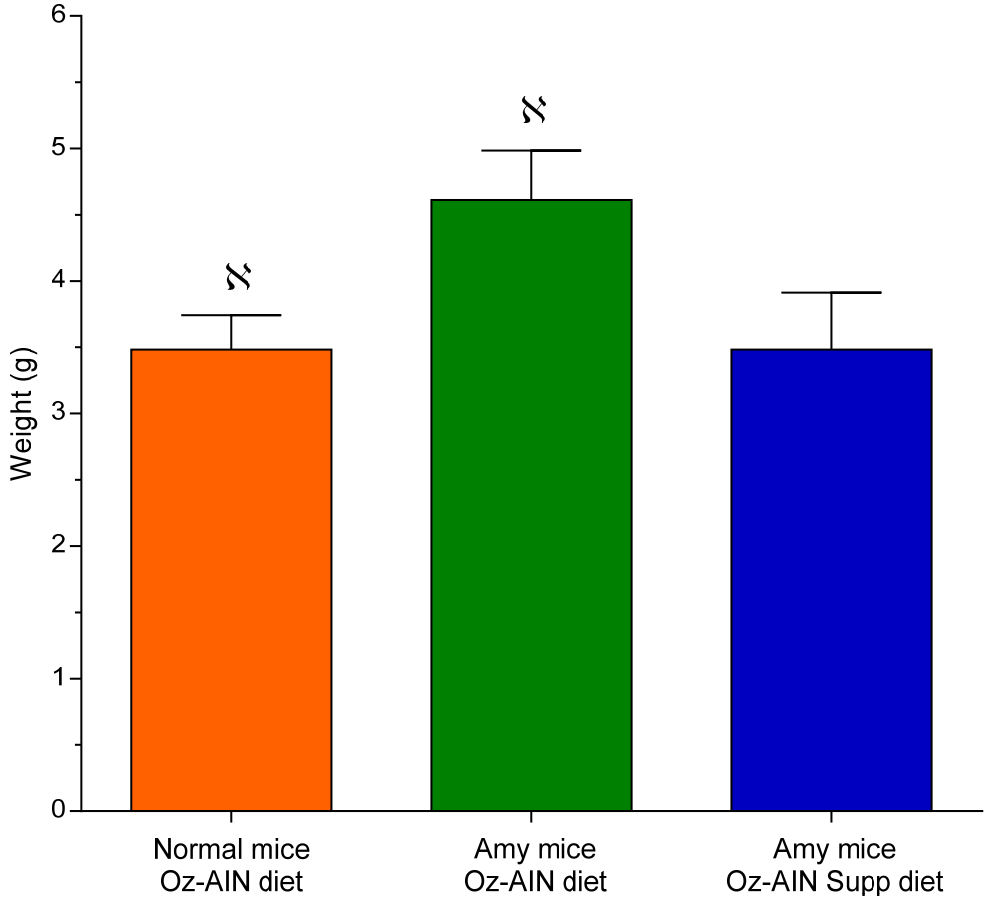


Figure 6. The average weight (g) of uterine fat that was collected from 15 month old normal and Amy mice. Normal mice fed the Oz-AIN diet (n=8, orange bar), Amy mice fed the Oz-AIN diet (n=14, green bar), and Amy mice fed the Oz-AIN Supp diet (n=12, blue bar). Bars represent mean ±SEM. No differences were detected with Bonferroni post tests. Bars with matching symbols are significantly different with Student's *t*-tests. (ℵ) p=0.04.

3.3.4.3. Weight (g) of SUBCUTANEOUS FAT deposits.

A one-way ANOVA detected significant differences on subcutaneous fat deposits in normal mice that were fed the Oz-AIN diet, Amy mice that were fed the Oz-AIN diet and Amy mice that were fed the Oz-AIN Supp diet (p=0.002, Figure 7). Bonferroni post tests revealed that these differences could be attributed to genotype effects, because normal mice that were fed the Oz-AIN diet had significantly less fat than Amy mice that were fed the Oz-AIN diet (p=0.03, Figure 7). These significant genotype effects were also detected with Student's *t*-tests (p=0.019, Figure 7).

Bonferroni post tests also revealed that Amy mice that were fed the Oz-AIN Supp diet had significantly less subcutaneous fat than Amy mice that were fed the Oz-AIN diet (p=0.002, Figure 7). This indicates that nutrient supplements were able to prevent the genotype-induced increase in subcutaneous fat deposits in Amy mice. There was no significant difference between the amount of subcutaneous fat in normal mice that were fed the Oz-AIN diet or the Amy mice that were fed the Oz-AIN Supp diet (p=0.41, Figure 7), which further indicates that nutrient supplements were able to prevent genotype effects on Amy mice.

Figure 7. The protective effect of nutrient supplements against genotype induced increase of subcutaneous fat deposit weight (g) from 15 month old Amy mice.

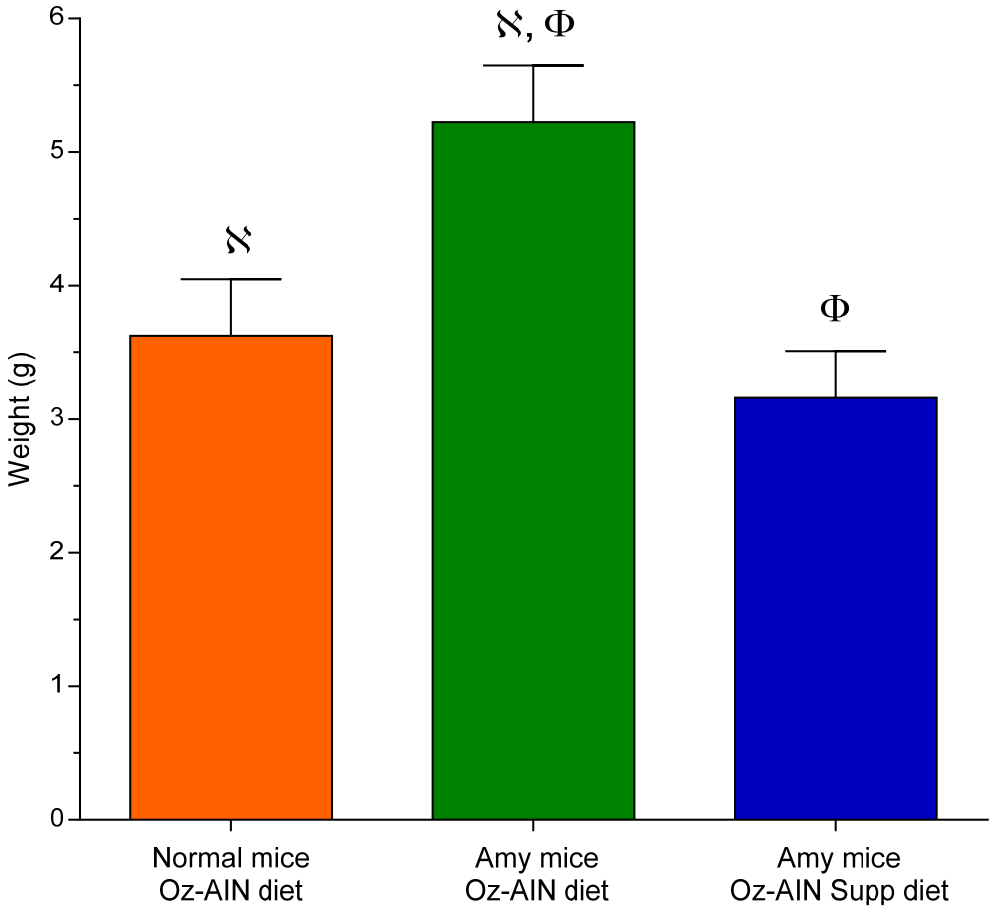


Figure 7. The average weight (g) of subcutaneous fat that was collected from 15 month old normal and Amy mice. Normal mice fed the Oz-AIN diet (n=8, orange bar), Amy mice fed the Oz-AIN diet (n=14, green bar), and Amy mice fed the Oz-AIN Supp diet (n=12, blue bar). Bars represent mean ±SEM. Bars with matching symbols are significantly different with Bonferroni post tests. (א) p=0.03, (Φ) p=0.002.

3.3.4.4. Weight (g) of RENAL FAT deposits.

A one-way ANOVA did not detect significant differences on the weight of renal fat collected from normal mice that were fed the Oz-AIN diet, Amy mice that were fed the Oz-AIN diet and Amy mice that were fed the Oz-AIN Supp diet ($p=0.34$, Figure 8). Student's *t*-tests did not detect significant differences between normal mice that were fed the Oz-AIN diet and Amy mice that were fed the Oz-AIN diet ($p=0.42$, Figure 8). Collectively, this suggests that there were no genotype effects on the weight of renal fat that was collected from normal and Amy mice. Furthermore, these data suggest that nutrient supplements do not affect the weight of renal fat in Amy mice.

Figure 8. The potentially beneficial effect of nutrient supplements against genotype induced increase of renal fat deposit weight (g) from 15 month old Amy mice.

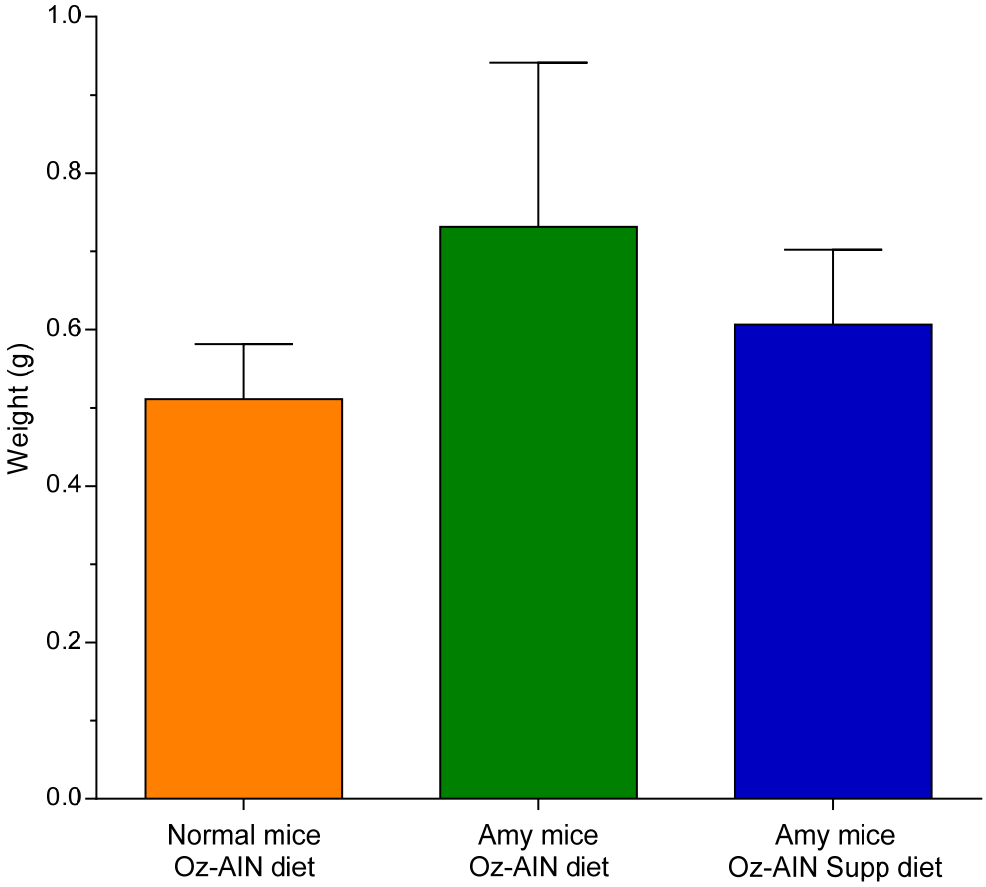


Figure 8. The average weight (g) of renal fat that was collected from 15 month old normal and Amy mice. Normal mice fed the Oz-AIN diet (n=8, orange bar), Amy mice fed the Oz-AIN diet (n=14, green bar), and Amy mice fed the Oz-AIN Supp diet (n=12, blue bar). Bars represent mean ±SEM

3.3.5. The preventative effects of nutrient supplements against genotype induced increase of heart weight (g) in Amy mice.

A one-way ANOVA detected significant differences of the weight of hearts collected from normal mice that were fed the Oz-AIN diet, Amy mice that were fed the Oz-AIN diet, or Amy mice that were fed the Oz-AIN Supp diet ($p=0.01$, Figure 9). Bonferroni post tests revealed that the hearts from 15 month old Amy mice that were fed the Oz-AIN diet were significantly heavier than hearts from normal mice that were fed the Oz-AIN diet ($p=0.02$, Figure 9). This indicates that there were significant genotype effects on the weight of hearts from 15 month old Amy mice.

The genotype effects were prevented with nutrient supplements. Bonferroni post tests revealed that Amy mice that were fed the Oz-AIN Supp diet had hearts that were significantly lighter than the hearts from Amy mice that had been fed the Oz-AIN diet ($p=0.05$, Figure 9). Student's *t*-tests also indicated that the mice that had received the nutrient supplements had lighter hearts than mice that were fed the Oz-AIN diet (there $p=0.008$, Figure 9). There was no difference in the weight of hearts from normal mice that were fed the Oz-AIN diet and hearts from Amy mice that were fed the Oz-AIN Supp diet with either Bonferroni post tests ($p>0.99$) or Student's *t*-tests ($p=0.65$, Figure 9). This adds further support for the finding that nutrient supplements may prevent genotype-induced effects on weight of hearts from 15 month old Amy mice that were fed the Oz-AIN diet.

Figure 9. The preventative effect of nutrient supplements against genotype induced increase of heart weight (g) in 15 month old Amy mice.

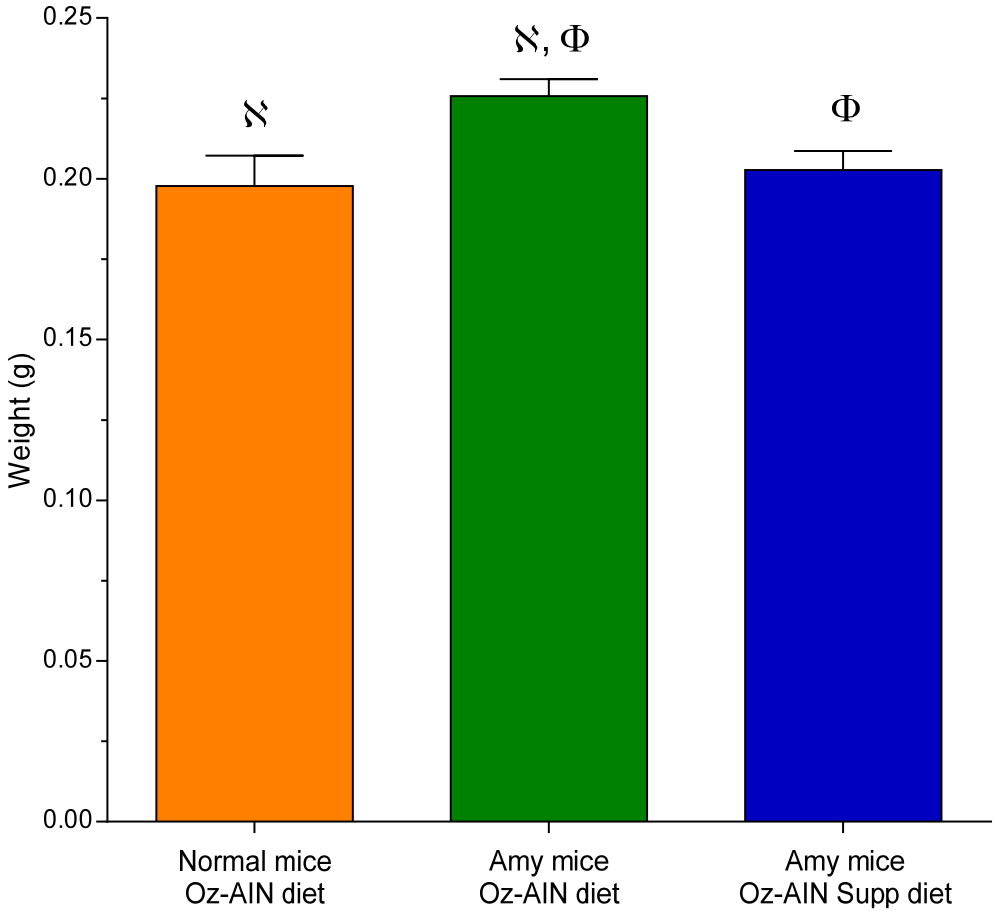


Figure 9. The average weight (g) of hearts from 15 month old normal and Amy mice. Normal mice fed the Oz-AIN diet (n=8, orange bar), Amy mice fed the Oz-AIN diet (n=14, green bar), and Amy mice fed the Oz-AIN Supp diet (n=12, blue bar). Bars represent mean ±SEM. Bars with matching symbols are significantly different with Bonferroni post tests. (ϰ) p=0.02, (Φ) p=0.05.

3.3.6. The preventative effects of nutrient supplements against genotype induced increase of liver weight (g) in Amy mice.

A one-way ANOVA detected significant differences in the weights of livers collected from normal mice that were fed the Oz-AIN diet, Amy mice that were fed the Oz-AIN diet and Amy mice that were fed the Oz-AIN Supp diet ($p=0.005$, Figure 10). Bonferroni post tests revealed that livers from Amy mice that were fed the Oz-AIN diet were significantly heavier than those from normal mice that were fed the same diet ($p=0.02$, Figure 10). Student's *t*-tests also found significant differences in the weight of hearts from normal and Amy mice that were fed the Oz-AIN diet ($p=0.008$, Figure 10).

The genotype effect on weight of livers of Amy mice was prevented by nutrient supplements. Bonferroni post tests and Student's *t*-tests revealed that Amy mice that were fed the Oz-AIN Supp diet had livers that were significantly lighter than those of Amy mice that were fed the Oz-AIN diet ($p=0.02$ and $p=0.013$ respectively, Figure 10). Furthermore, neither Bonferroni post tests nor Student's *t*-tests were able to detect significant differences between the weights of livers from Amy mice that were fed the Oz-AIN Supp diet and normal mice that were fed the Oz-AIN diet ($p>0.99$ and $p=0.88$, Figure 10).

Collectively, these data suggest that there are genotype effects on the weight of livers in Amy mice, and that these genotype effects can be prevented with nutrient supplements.

Figure 10. The preventative effect of nutrient supplements against genotype induced increase of liver weight (g) in 15 month old Amy mice.

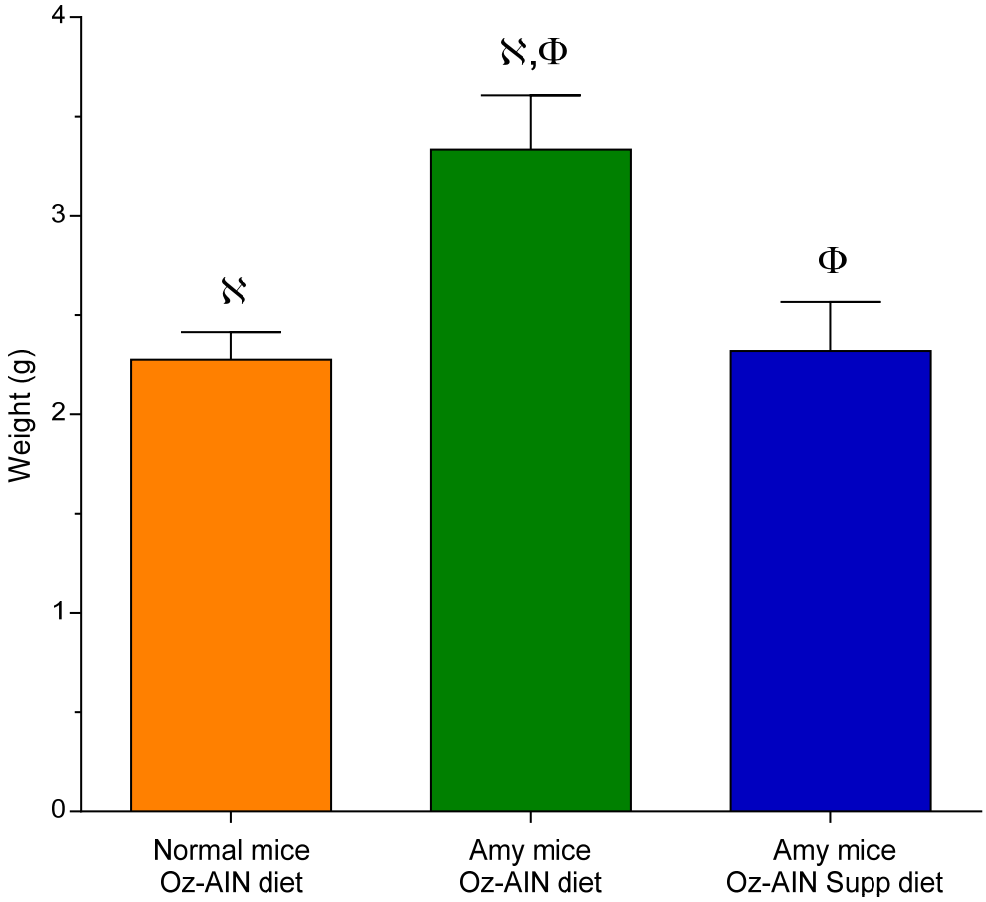


Figure 10. The average weight (g) of livers from 15 month old normal and Amy mice. Normal mice fed the Oz-AIN diet (n=8, orange bar), Amy mice fed the Oz-AIN diet (n=14, green bar), and Amy mice fed the Oz-AIN Supp diet (n=12, blue bar). Bars represent mean ±SEM. Bars with matching symbols are significantly different using Bonferroni post tests. (א) p=0.02. (Φ) p=0.02.

3.3.7. The potentially beneficial effects of nutrient supplements against genotype induced increase of kidney weight (g) in Amy mice.

A one-way ANOVA did not detect significant differences in the weight of kidneys collected from normal mice that were fed the Oz-AIN diet, Amy mice that were fed the Oz-AIN diet or Amy mice that were fed the Oz-AIN Supp diet (p=0.64, Figure 11). This suggests that genotype does not affect kidney weights in Amy mice.

Student's *t*-tests did not detect significant differences between the kidney weights from 15 month old Amy mice that were fed the Oz-AIN Supp diet and either normal mice that were fed the Oz-AIN diet (p=0.45, Figure 11) or the Amy mice that were fed the Oz-AIN diet (p=0.63, Figure 11). This indicates that nutrient supplements do not affect kidney weight in mice.

3.3.8. The preventative effects of nutrient supplements against genotype induced increase of spleen weight (g) in Amy mice.

A one-way ANOVA did not detect significant differences in the weight of spleens collected from normal mice that were fed the Oz-AIN diet, Amy mice that were fed the Oz-AIN diet or Amy mice that were fed the Oz-AIN Supp diet (p=0.76, Figure 12). Student's *t*-tests did not detect significant differences spleens from normal or Amy mice that were fed the Oz-AIN diet (p=0.76, Figure 12).This suggests that genotype does not affect spleen weight in 15 month old normal and Amy mice.

Nutrient supplements did not have an effect on the weights of spleens collected from mice. Student's *t*-tests did not detect significant differences in the weights of spleen collected from Amy mice that were fed the Oz-AIN Supp diet and either normal mice that were fed the Oz-AIN diet (p=0.57, Figure 12) or Amy mice that were fed the Oz-AIN diet (p=0.34, Figure 12).

Figure 11. The potentially beneficial effect of nutrient supplements against genotype induced increase of kidney weight (g) in 15 month old Amy mice.

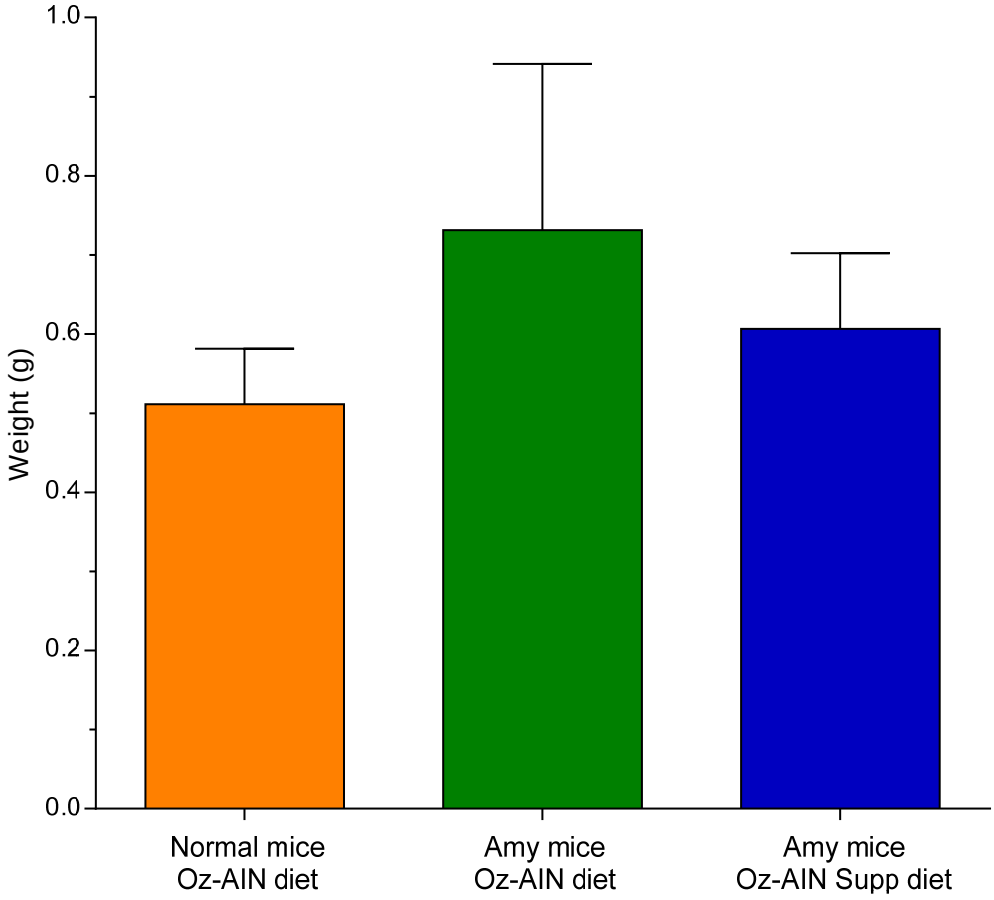


Figure 11. The average weight (g) of kidneys from 15 month old normal and Amy mice. Normal mice fed the Oz-AIN diet (n=8, orange bar), Amy mice fed the Oz-AIN diet (n=14, green bar), and Amy mice fed the Oz-AIN Supp diet (n=12, blue bar). Bars represent mean ±SEM.

Figure 12. The preventative effect of nutrient supplements against genotype induced increase of spleen weight (g) in 15 month old Amy mice.

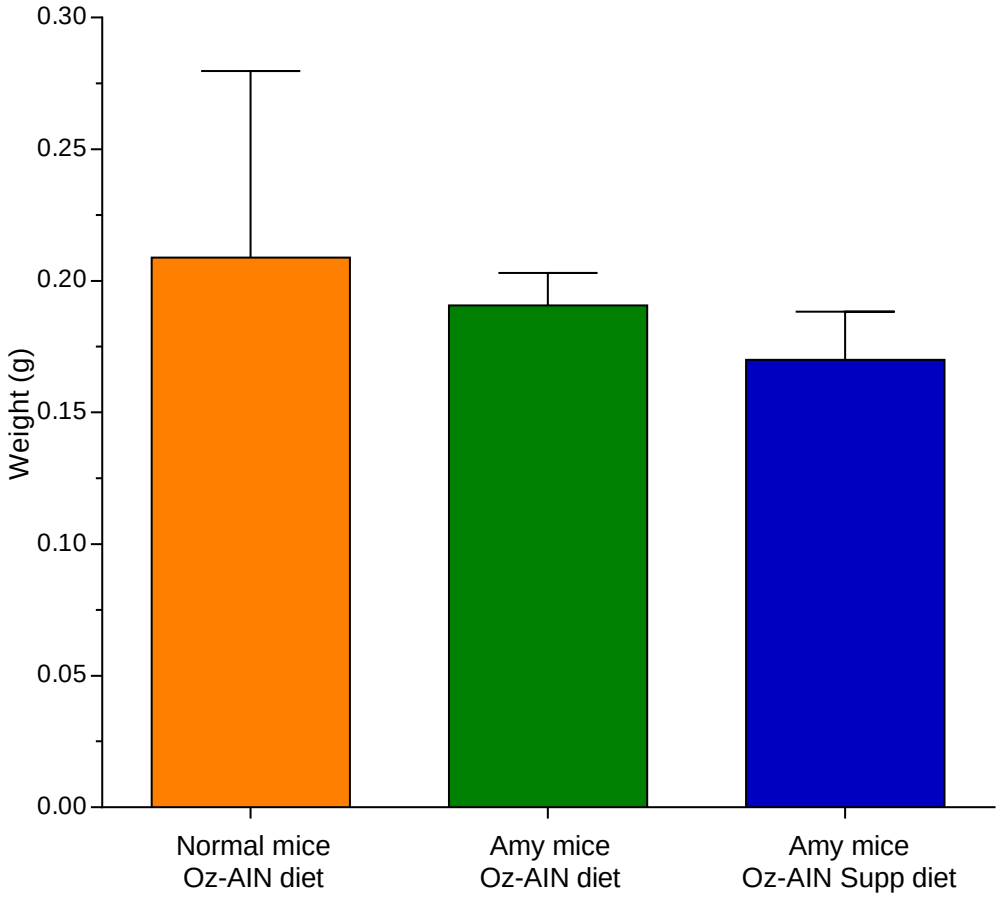


Figure 12. The average weight (g) of spleens from 15 month old normal and Amy mice. Normal mice fed the Oz-AIN diet (n=8, orange bar), Amy mice fed the Oz-AIN diet (n=14, green bar), and Amy mice fed the Oz-AIN Supp diet (n=12, blue bar). Bars represent mean ±SEM.

3.4. An investigation into the potentially beneficial effect of nutrient supplements against diet-type induced changes in Amy mice.

An investigation was made to determine whether or not nutrient supplements could alter diet-type induced effects on food and energy intake, body weight gain and the weight of major organs and fat deposits in Amy mice.

3.4.1. The effect of nutrient supplements against diet-type effects on estimated food intake (g/day).

A one-way ANOVA did not detect significant diet-type effects on the amount of food eaten by Amy mice over 15 months (Table 8, Figure 13). However, one-way ANOVA's on food eaten every five weeks suggested that diet-type affected food intake in young Amy mice.

Table 8. The ability of nutrient supplements to prevent diet-type effects on overall food intake (g) and energy intake (kJ) by Amy mice that were fed the Oz-AIN diet.

	Amy mice fed the AIN93-M diet	**Amy mice fed the Oz-AIN diet**	**Amy mice fed the Oz-AIN Supp diet**
Food intake over 15 months (g)	1328.5 ±94.3	1630.8 ±140.9	1757.3 ±168.5
Energy intake over 15 months (kJ)	22132 ±1571$^{\Theta}$	29771 ±2495	36858 ±3537$^{\Theta}$

Overall food intake (g) and energy intake (kJ) were calculated from the area under the curve of figures 13A and 13B respectively. Values are mean ±SEM. Numbers with matching symbols are significantly different using Bonferroni post tests. (Θ) p=0.01.

One-way ANOVA's detected significant differences in the amount of food eaten by Amy mice that were fed either the AIN93-M, Oz-AIN or Oz-AIN Supp diet at 20 weeks ($p=0.002$, Figure 13), 30 weeks ($p=0.003$, Figure 13), 35 weeks ($p=0.005$, Figure 13) and 40 weeks ($p=0.030$, Figure 13). Bonferroni post tests revealed that Amy mice that were fed the AIN93-M diet ate significantly less than Amy mice that were fed the Oz-AIN diet at 40 weeks ($p=0.03$, Figure 13). All other differences were attributed to Amy mice that were fed the Oz-AIN Supp diet eating more than mice that were fed either the AIN93-M diet (20 weeks, $p=0.01$; 35 weeks, $p=0.004$, Figure 13A) or the Oz-AIN diet (20 weeks, $p=0.003$; 30 weeks , $p=0.003$, Figure 13). This suggests that the nutrient supplements may have led to increased food intake in young mice.

After 35 weeks of age, there was a trend for the mice that were fed the AIN93-M to eat less than the Oz-AIN or the Oz-AIN Supp diet (Figure 13). While this did not achieve significance, it suggests that in late adulthood and old age, Amy mice may prefer high-fat diets over low-fat optimal diets.

Collectively, these data suggest that diet-type may affect food intake at different stages of life in Amy mice. Younger mice that were fed the nutrient supplements ate more than mice receiving either an optimal or sub-optimal diet. In old age, these preferences changed, and mice that were fed the optimal diet ate less than mice that were fed either of the high-fat diets (Figure 13).

Figure 13. The effect of nutrient supplements on diet-type effects on food consumed (g/day) by Amy mice.

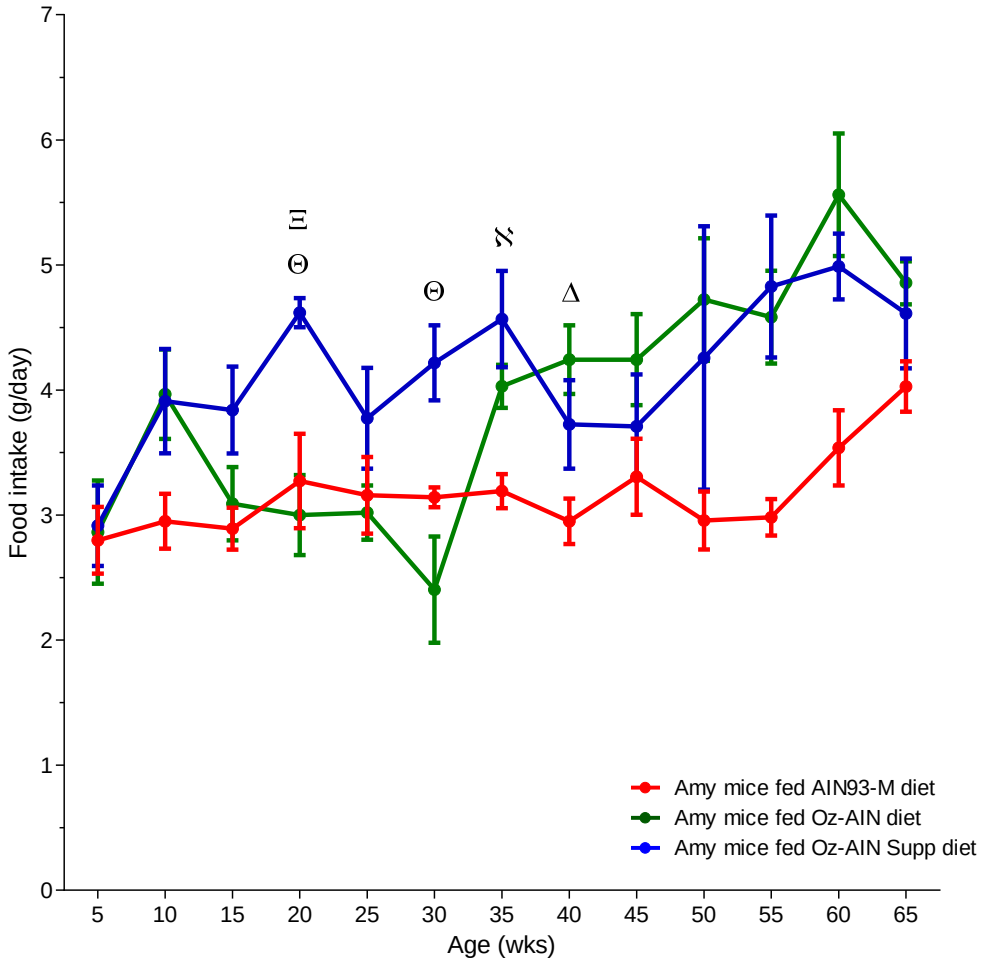


Figure 13. Estimated food intake (g/day) by Amy mice fed the AIN93-M diet (red line, wk 5: n=11, wk 65: n=11), the Oz-AIN diet (green line, wk 5: n=15, wk 65: n=14), or the Oz-AIN Supp diet (blue line, wk 5: n=16, wk 65: n=12). Error bars are mean ±SEM. Significant differences were detected with Bonferroni post tests. (Δ) p=0.03 between Amy mice that were fed either the AIN93-M diet or the Oz-AIN diet. (Θ) p=0.003 between Amy mice that were fed either the Oz-AIN diet or the Oz-AIN Supp diet. (χ) p=0.004, (Ξ) p=0.01 between Amy mice that were fed the AIN93-M diet or the Oz-AIN Supp diet.

3.4.2. The effect of nutrient supplements against diet-type effects on estimated energy intake (kJ/day).

One-way ANOVA detected significant differences in energy intake of Amy mice that were fed either the AIN93-M diet, the Oz-AIN diet or the Oz-AIN Supp diet ($p=0.01$, Figure 14). Bonferroni post tests revealed that these differences were due to Amy mice that were fed the Oz-AIN Supp diet having higher energy intakes Amy mice that were fed the AIN93-M diet ($p=0.01$, Table 8, Figure 14).

The Oz-AIN Supp diet contains a higher amount of energy than the Oz-AIN or the AIN93-M diet (20.98 kcal, 20.11 kcal and 16.66 kcal respectively, Table 6). This may partially explain the significantly higher energy intakes of Amy mice that were fed the Oz-AIN Supp diet as compared to those fed the AIN93-M or the Oz-AIN diet (Figure 14).

However, the significant differences in energy intake are not solely due to the varying energy content of each diet. Comparison of energy intake every five weeks showed that from 10 to 35 weeks old, Amy mice that were fed the Oz-AIN Supp diet had significantly greater energy intakes than Amy mice that were fed either the AIN93-M diet ($p<0.05$ each comparison, Figure 14) or Oz-AIN diet ($p<0.05$ each comparison, Figure 14). This may be a consequence of increased food intake throughout this period (see page 150, Figure 13).

Despite the different energy contents of the Oz-AIN and the AIN93-M diets (Table 6), there was only one occasion when Amy mice that were fed the Oz-AIN diet consumed significantly more energy than those fed the AIN93-M diet (55 weeks, $p=0.03$, Figure 14). However, there were trends for mice that were fed the Oz-AIN diet to have higher energy intakes than those fed the AIN93-M diet in later life (Figure 14).

These data suggest that young Amy mice had a higher preference for the Oz-AIN Supp diet, and as a result Amy mice that were fed the Oz-AIN Supp diet ate more than Amy mice that were fed other diets. There was only one occasion when there was a significant difference between the Oz-AIN diet and the AIN93-M diet, suggesting that energy content of diet was not the only reason that Amy mice that were fed different diets had different energy intakes throughout life.

Figure 14. The effect of nutrient supplements on diet-type effects on estimated energy intake (kJ/day) by Amy mice.

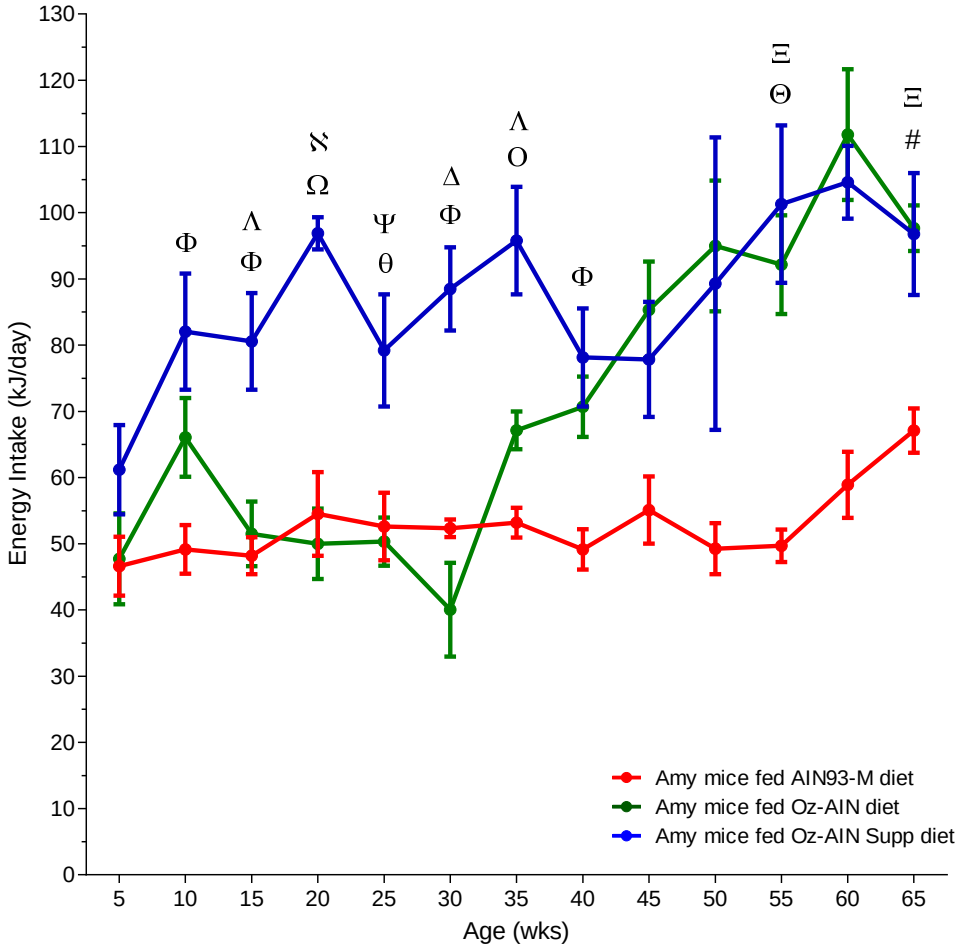


Figure 14. Estimated energy intake (kJ/day) by Amy mice fed the AIN93-M diet (red line, wk 5: n=11, wk 65: n=11), the Oz-AIN diet (green line, wk 5: n=15, wk 65: n=14), or the Oz-AIN Supp diet (blue line, wk 5: n=16, wk 65: n=12). Error bars are mean ±SEM. Significant differences were detected with Bonferroni post tests. (Ξ) p=0.03 between Amy mice that were fed either the AIN93-M diet or the Oz-AIN diet. (Δ) p=0.0001, (ϰ) p<0.001, (Λ) p<0.01, (Ψ) p=0.01 between Amy mice that were fed either the Oz-AIN diet or the Oz-AIN Supp diet. (O) p<0.0001, (Ω) p<0.001, (Φ) p<0.01, (Θ) p=0.01, (θ) p=0.03, (#) p=0.04 between Amy mice that were fed either the AIN93-M diet or the Oz-AIN Supp diet.

3.4.3. The potentially beneficial effect of nutrient supplements against diet-type induced weight gain (g) in Amy mice.

A one-way ANOVA detected significant differences in the body weight of Amy mice that were fed the AIN93-M diet, the Oz-AIN diet or the Oz-AIN Supp diet as young as 15 weeks ($p=0.04$, Figure 15). Bonferroni post tests revealed that the 15 week old Amy mice that were fed the AIN93-M diet were significantly lighter than age-matched Amy mice that were fed the Oz-AIN diet ($p=0.04$, Figure 15). The significant differences in body weight were even more apparent at 20 weeks of age ($p=0.0003$, Figure 15). The 20 week old Amy mice that were fed the Oz-AIN diet were significantly heavier than either the Amy mice that were fed the AIN93-M diet (respectively, $p=0.0002$, Figure 15) or the Amy mice that were fed the Oz-AIN Supp diet ($p=0.03$, Figure 15). Amy mice that were fed the Oz-AIN diet remained the heaviest group of mice for the remainder of the study (Figure 15).

The nutrient supplements reduced weight gain in Amy mice, but were unable to entirely prevent diet-type effects on body weight. Amy mice that were fed the Oz-AIN Supp diet were significantly heavier than those fed the AIN93-M diet at 25 weeks of age ($p=0.004$, Figure 15). For the remainder of the study, Amy mice that were fed the Oz-AIN Supp diet were significantly heavier than Amy mice that were fed the AIN93-M diet, but were also significantly lighter than Amy mice that were fed the Oz-AIN diet (Figure 15). This suggests that the nutrient supplements were able to at least partially prevent the diet-induced weight gain in Amy mice.

The long chain ω-3 fatty acids docosahexaenoic acid and eicosapentaenoic acid may have played a role in the reduced weight gain of the Oz-AIN Supp diet fed mice. Supplementing a high-fat diet with fatty acids reduces high-fat diet induced weight gain in mice [350-353]. The ω-3 fatty acids enhance lipid catabolism and reduce

lipogenesis in high-fat diet fed mice, and prevents obesity through regulation of adipose cell turnover [350, 352, 354]. Supplementing a high-fat diet with ω-3 fatty acids reduces oxidative stress that is associated with long term high-fat feeding [350-352, 355]. Cui *et al.* report that feeding mice dietary supplements of lipoic acid prevented diet-induced weight gain and upregulated expression of genes involved in anti-oxidant defence systems, such as super oxide dismutase, peroxiredoxin-4 and glutathione peroxidise [351]. The current study has not assessed oxidative state of adipose tissue in normal or Amy mice. Nonetheless, the reduced weight gain in Amy mice that were fed the Oz-AIN Supp diet may be due to ω-3 fatty acid supplements reducing oxidative stress and adipose tissue inflammation and preventing lipogenesis.

Polyphenolic compounds and B vitamins also prevent high-fat diet-induced oxidative stress and can also alter lipid metabolism, and may therefore have also played a role in the anti-obesogenic effects of the Oz-AIN Supp diet [339, 356, 357]. Polyphenolic compounds scavenge free radicals and prevent lipid peroxidation and oxidation of low-density lipoproteins [339]. Similarly, folate reduces high-fat diet induced NADPH oxidase activity and expression [356]. However, Sarna *et al.* report that although folate reduced high-fat diet induced increase in NADPH expression, folate did not prevent diet induced weight gain [356]. This suggests that folate did not prevent weight gain in the Amy mice that were used in the current study.

Park *et al*. fed mice high fat diets that had been supplemented with *Sophora japonica L*, and report not only a dose-dependent decrease in weight gain, but also a reduction in serum cholesterol and low density lipoprotein levels [358]. *Sophora japonica L*. contains flavonoids, a class of polyphenolic compounds [359]. This indicates that polyphenolic compounds can protect against high-fat diet induced weight gain, through altered lipid metabolism. This is consistent with reports from others [360].

Figure 15. Effect of nutrient supplements on diet-type induced weight gain in Amy mice.

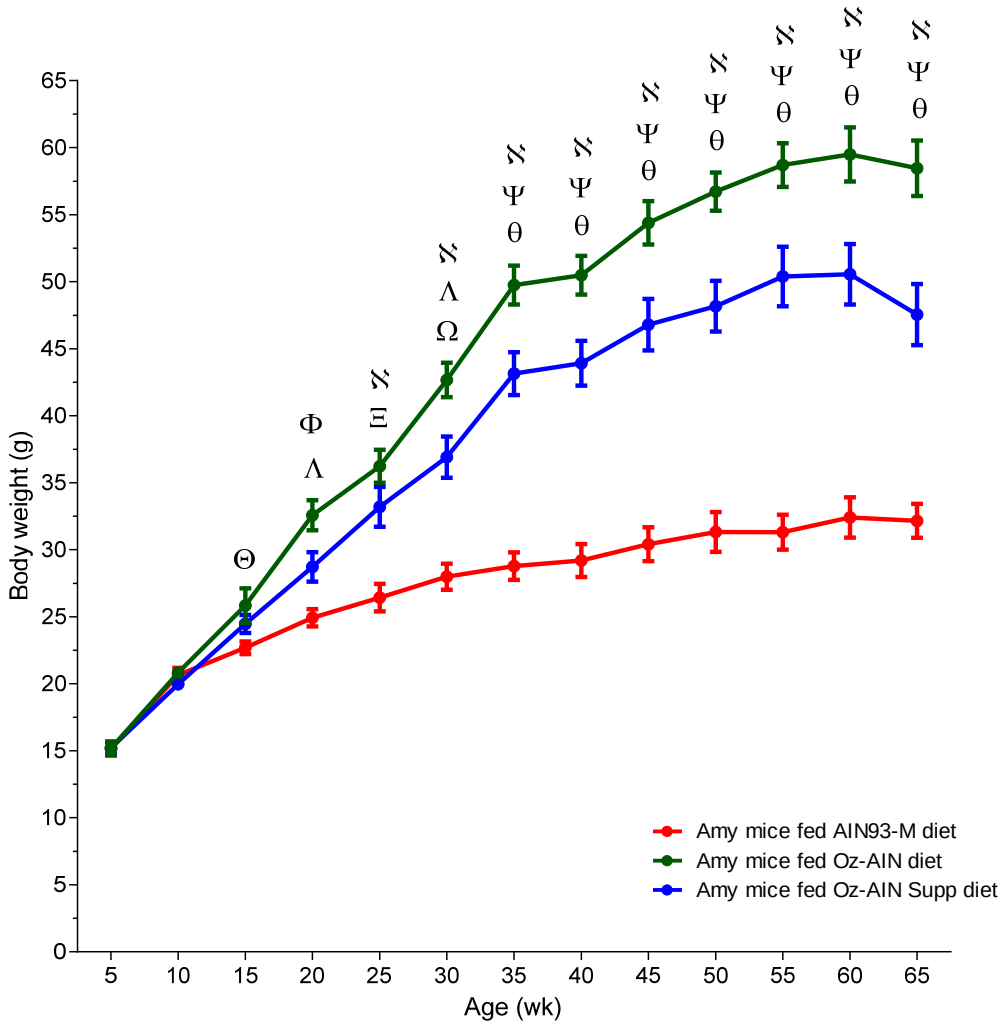


Figure 15. Weight gain (g) of Amy mice that were fed the AIN93-M diet (red line, wk 5: n=11, wk 65: n=11), the Oz-AIN diet (green line, wk 5: n=15, wk 65: n=14), or the Oz-AIN Supp diet (blue line, wk 5: n=16, wk 65: n=12). Error bars are mean ±SEM. Significant differences were detected with Bonferroni post tests.
(Θ) $p<0.05$, (Φ) $p<0.001$, (χ) $p<0.0001$ between Amy mice that were fed either the AIN93-M diet or the Oz-AIN diet. (Λ) $p<0.05$, (Ψ) $p<0.01$, between Amy mice that are fed the Oz-AIN diet or the Oz-AIN Supp diet. (Ξ) $p<0.01$, (Ω) $p<0.001$, (θ) $p<0.0001$ between Amy mice that were fed the AIN93-M diet and the Oz-AIN Supp diet.

3.4.4. The potentially beneficial effect of nutrient supplements against diet-type induced increased fat deposit weight (g) in Amy mice.

3.4.4.1. Weight (g) of TOTAL FAT deposits.

A one-way ANOVA revealed that diet-type had a significant effect on the total amount of fat collected from Amy mice that were fed either the AIN93-M diet, the Oz-AIN diet or the Oz-AIN Supp diet ($p<0.0001$, Figure 16). Amy mice that were fed the Oz-AIN diet significantly more total fat collected than the Amy mice that were fed the AIN93-M diet, demonstrating that the sub-optimal Oz-AIN diet increases fat deposition ($p<0.0001$, Figure 16). This was expected, owing to the well established links between high-fat diets and obesity [361].

The diet-induced increase in total fat was partially alleviated by nutrient supplements. The total amount of fat was significantly less from Amy mice that were fed the Oz-AIN Supp diet than Amy mice that were fed the Oz-AIN diet ($p=0.009$, Figure 16). However, nutrient supplementation was not able to completely reverse the effect of the Oz-AIN diet, as Amy mice that were fed the Oz-AIN Supp diet had significantly more total fat than Amy mice that were fed the AIN93-M diet ($p=0.004$, Figure 16).

These data suggest that the high-fat content of the Oz-AIN and Oz-AIN Supp diets significantly increased the total amount of fat collected from 15 month old Amy mice, and that nutrient supplements were able to reduce the total amount of fat deposition.

Figure 16. The preventative effect of nutrient supplements against diet-type induced increase of fat deposits in 15 month old Amy mice.

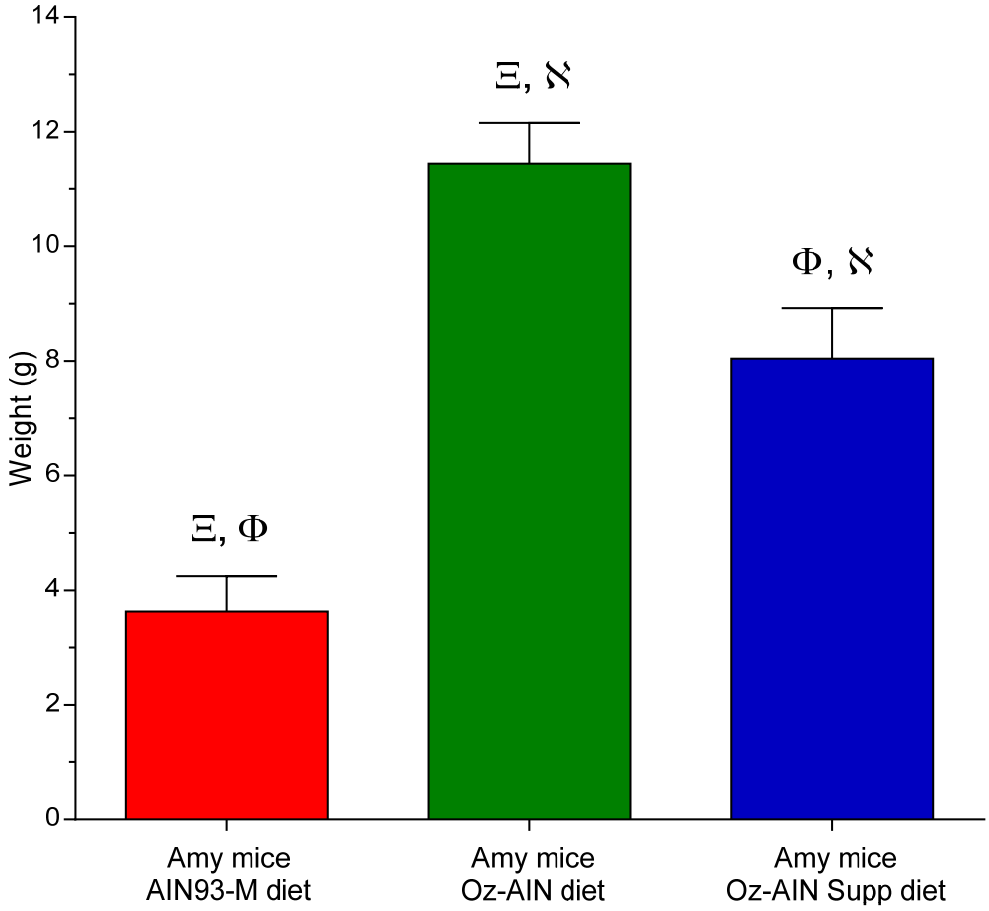


Figure 16. The average weight (g) of total fat that was collected from 15 month old Amy mice. Amy mice fed the AIN93-M diet (n=11, red bar), Amy mice fed the Oz-AIN diet (n=14, green bar), and Amy mice fed the Oz-AIN Supp diet (n=12, blue bar). Bars represent mean ±SEM. Bars with matching symbols are significantly different using Bonferroni post tests. (Ξ) $p<0.0001$. (Φ) $p=0.004$. (χ) $p=0.009$.

3.4.4.2. Weight (g) of UTERINE FAT deposits.

A one-way ANOVA detected that the amount of fat that was collected from uterine tissue was significantly affected by diet-type ($p<0.0001$, Figure 17). Bonferroni post tests indicated that Amy mice that were fed the Oz-AIN diet significantly more fat around the uterus than the Amy mice that were fed the AIN93-M diet. This suggests that the sub-optimal Oz-AIN diet increased fat deposition in uterine tissue ($p<0.0001$, Figure 17). The diet-type effect on fat deposition around the uterus in 15 month old Amy mice was not prevented by nutrient supplements. Amy mice that were fed the Oz-AIN Supp diet had significantly more fat around the uterus than Amy mice that were fed the AIN93-M diet ($p=0.005$, Figure 17). Furthermore, there was no significant differences in the weight of fat around the uterus that was collected from Amy mice that were fed the Oz-AIN diet or the Oz-AIN Supp diet ($p=0.11$, Figure 17).

These results suggest that diet-type has an effect on fat deposition around the uterus, and that this is not prevented by nutrient supplementation.

Figure 17. The preventative effect of nutrient supplements against diet-type induced increase of uterine fat deposits in 15 month old Amy mice.

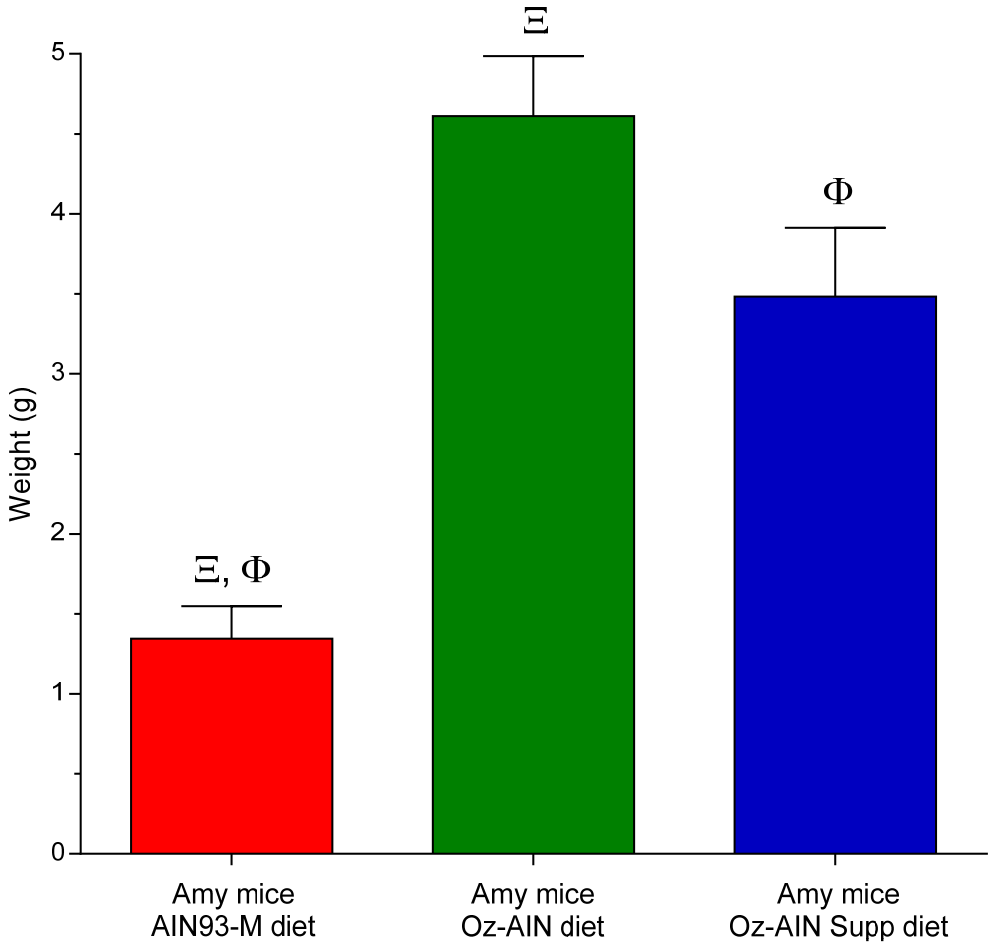


Figure 17. The average weight (g) of uterine fat that was collected from 15 month old Amy mice. Amy mice fed the AIN93-M diet (n=11, red bar), Amy mice fed the Oz-AIN diet (n=14, green bar), and Amy mice fed the Oz-AIN Supp diet (n=12, blue bar). Bars represent mean ±SEM. Bars with matching symbols are significantly different using Bonferroni post tests. (Ξ) $p<0.0001$. (Φ) $p=0.005$.

3.4.4.3. Weight (g) of SUBCUTANEOUS FAT deposits.

A one-way ANOVA detected that diet-type had a significant effect on the amount of subcutaneous fat that was collected from 15 month old Amy mice ($p<0.0001$, Figure 18). Bonferroni post tests indicated that Amy mice that were fed the Oz-AIN diet significantly more subcutaneous fat than the Amy mice that were fed the AIN93-M diet, demonstrating that the sub-optimal Oz-AIN diet increases subcutaneous fat deposition ($p<0.0001$, Figure 18).

The diet-type effect on subcutaneous fat deposition was reduced by nutrient supplements. Amy mice that were fed the Oz-AIN Supp diet had significantly less subcutaneous fat than Amy mice that were fed the Oz-AIN diet ($p=0.002$, Figure 18). There was no significant difference detected between the amount of subcutaneous fat collected from Amy mice that were fed the AIN93-M diet and the Amy mice that were fed the Oz-AIN Supp diet ($p=0.11$, Figure 18).

This data suggests that diet type has a significant effect on the amount of subcutaneous fat in Amy mice, and that subcutaneous fat deposition was reduced with nutrient supplements.

Figure 18. The preventative effects of nutrient supplements against diet-type induced increase of subcutaneous fat deposit weight (g) in 15 month old Amy mice.

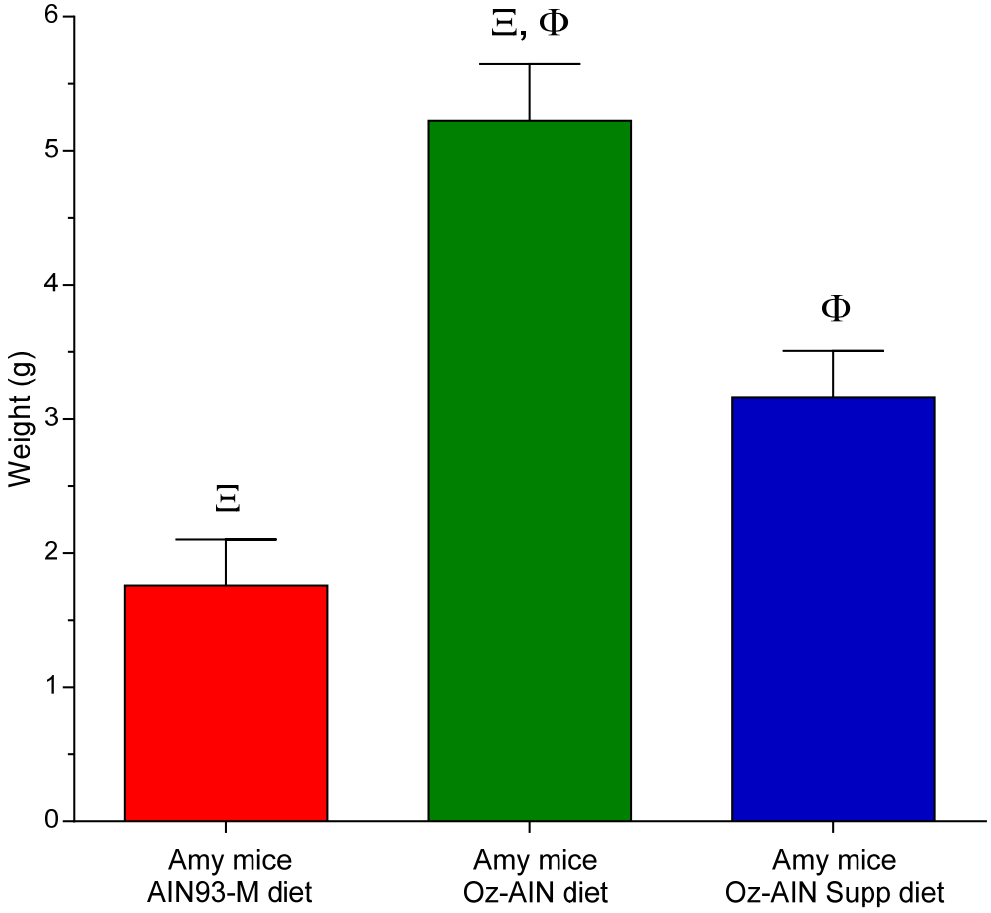


Figure 18. The average weight (g) of subcutaneous fat that was collected from 15 month old Amy mice. Amy mice fed the AIN93-M diet (n=11, red bar), Amy mice fed the Oz-AIN diet (n=14, green bar), and Amy mice fed the Oz-AIN Supp diet (n=12, blue bar). Bars represent mean ±SEM. Bars with matching symbols are significantly different using Bonferroni post tests. (#) $p<0.0001$. (Φ) $p<0.01$.

3.4.4.4. Weight (g) of RENAL FAT deposits.

A one-way ANOVA detected that diet-type had a significant effect on the amount of fat collected from around the kidneys of 15 month old Amy mice ($p=0.0007$, Figure 19). Amy mice that were fed the Oz-AIN diet had significantly more fat than Amy mice that were fed the AIN93-M diet ($p=0.0005$, Figure 19). Amy mice that were fed the Oz-AIN Supp diet also had significantly more fat than Amy mice that were fed the AIN93-M diet ($p=0.008$, Figure 19). There were no differences detected between the amount of fat collected from the kidneys of Amy mice that were fed either the Oz-AIN diet or the Oz-AIN Supp diet ($p>0.99$, Figure 19). This suggests that the fat deposition around the kidneys of Amy mice was increased by a high-fat diet, and was not prevented by nutrient supplements.

3.4.4.5. Summary of the potentially beneficial effects of nutrient supplements on diet-type induced fat deposition in Amy mice.

These results indicate that diet-type has different effects on fat deposition around different organs in 15 month old Amy mice. The high-fat nature of the Oz-AIN diet increased subcutaneous fat, uterine fat and renal fat in Amy mice. However, the nutrient supplements were only able to significantly reduce fat deposits that lined the skin.

Figure 19. The potentially beneficial effect of nutrient supplements against diet-type induced increase of renal fat deposit weight (g) in 15 month old Amy mice.

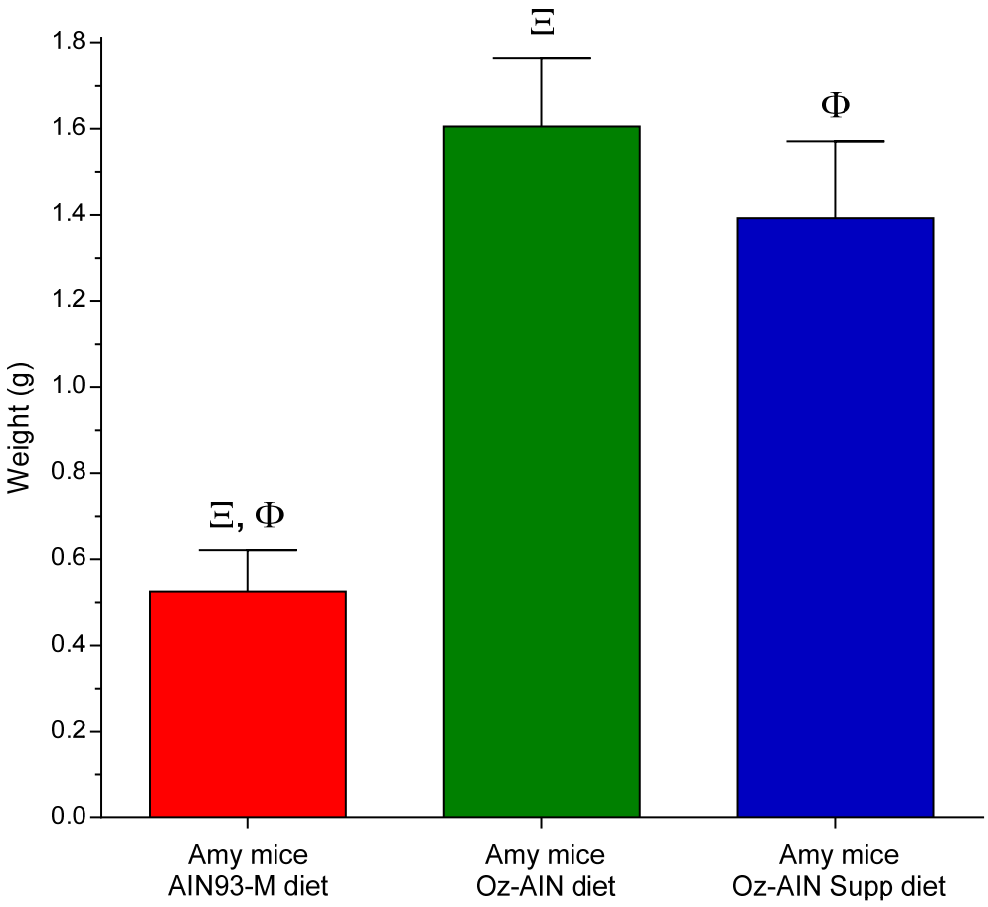


Figure 17. The average weight (g) of subcutaneous fat that was collected from 15 month old Amy mice. Amy mice fed the AIN93-M diet (n=11, red bar), Amy mice fed the Oz-AIN diet (n=14, green bar), and Amy mice fed the Oz-AIN Supp diet (n=12, blue bar). Bars represent mean ±SEM. Bars with matching symbols are significantly different using Bonferroni post tests. (Ξ) p=0.005. (Φ) p=0.008.

3.4.5. The preventative effects of nutrient supplements against diet-type induced increase of heart weight (g) in Amy mice.

A one-way ANOVA detected that diet-type had a significant effect on heart weight of 15 month old Amy mice ($p<0.001$, Figure 20). Bonferroni post tests indicated that Amy mice that were fed the Oz-AIN diet had significantly heavier hearts than Amy mice that were fed the AIN93-M diet ($p<0.0001$, Figure 20). This suggests that the Oz-AIN diet increased heart weight of Amy mice.

Amy mice that were fed the Oz-AIN Supp had significantly lighter hearts than Amy mice that had been fed the Oz-AIN diet, suggesting that nutrient supplements reduced the diet-type effect on heart weight ($p=0.03$, Figure 20). However, the hearts that were collected from Amy mice that were fed the Oz-AIN Supp diet were also significantly heavier than those of Amy mice that were fed the AIN93-M diet ($p=0.0004$, Figure 20). This suggests that nutrient supplements were unable to completely prevent the Oz-AIN diet induced effects on heart weight in Amy mice

Figure 20. The preventative effect of nutrient supplements against diet-induced increase of heart weight (g) in 15 month old Amy mice.

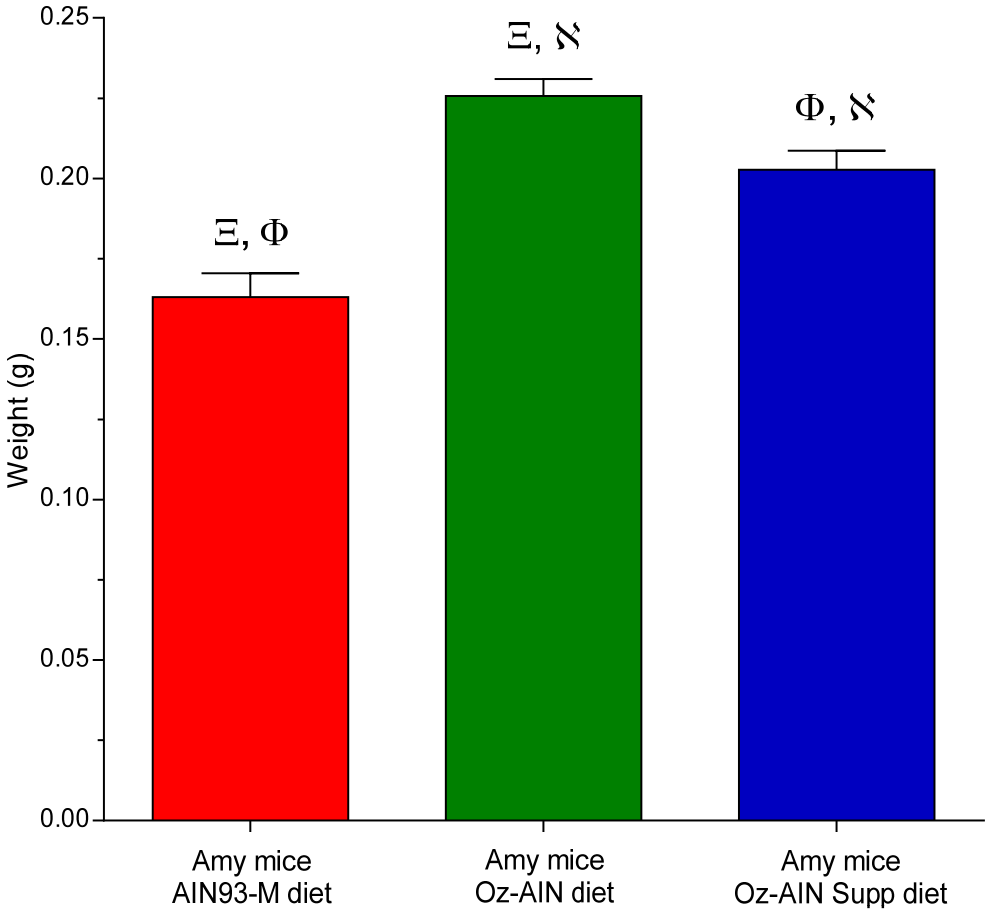


Figure 20. The average weight (g) of hearts from 15 month old Amy mice. Amy mice fed the AIN93-M diet (n=11, red bar), Amy mice fed the Oz-AIN diet (n=14, green bar), and Amy mice fed the Oz-AIN Supp diet (n=12, blue bar). Bars represent mean ±SEM. Bars with matching symbols are significantly different. (Ξ) $p<0.0001$. (Φ) $p=0.0004$. (ϰ) $p=0.03$.

3.4.6. The preventative effects of nutrient supplements against diet-type induced increase of liver weight (g) in Amy mice.

A one-way ANOVA detected significant diet-type effects on the weight of livers collected from 15 month old Amy mice that were fed either the AIN93-M diet, the Oz-AIN diet or the Oz-AIN Supp diet ($p<0.0001$, Figure 21). Bonferroni post tests revealed that Amy mice that were fed the Oz-AIN diet had significantly heavier livers than Amy mice that were fed the AIN93-M diet ($p<0.0001$, Figure 21). This suggests that the Oz-AIN diet increased liver weight in Amy mice.

The livers from Amy mice that were fed the Oz-AIN Supp diet were significantly lighter than livers that were collected from Amy mice that were fed the Oz-AIN diet ($p=0.01$, Figure 21). This suggests that the nutrient supplements were able to prevent diet induced increase in liver weight. In further support of this, there was no difference in the weights of livers of mice that were fed the Oz-AIN Supp diet and the AIN93-M diet ($p=0.09$, Figure 21).

Figure 21. The preventative effects of nutrient supplements against diet-type induced increase of liver weight (g) in 15 month old Amy mice.

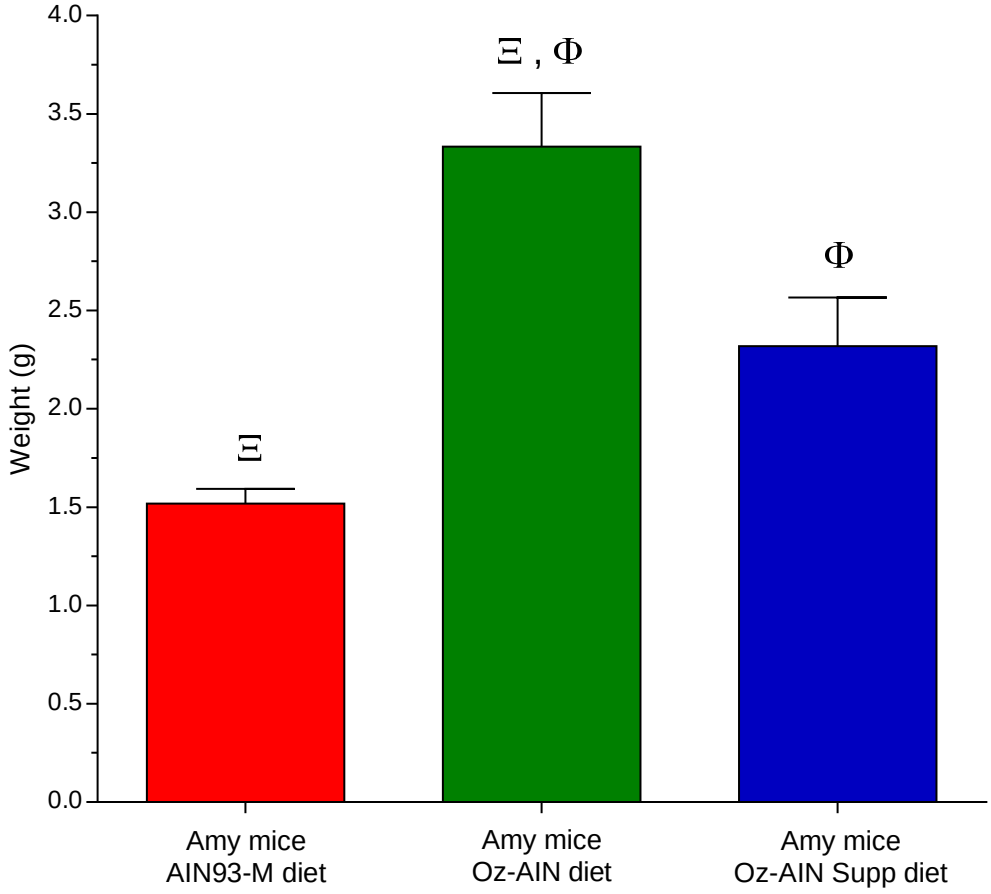


Figure 21. The average weight (g) of livers from 15 month old Amy mice. Amy mice fed the AIN93-M diet (n=11, red bar), Amy mice fed the Oz-AIN diet (n=14, green bar), and Amy mice fed the Oz-AIN Supp diet (n=12, blue bar). Bars represent mean ±SEM. Bars with matching symbols are significantly different using Bonferroni post tests. (Ξ) $p<0.0001$. (Φ) $p=0.01$.

3.4.7. The preventative effects of nutrient supplements against diet-type induced increase of kidney weight (g) in Amy mice.

A one-way ANOVA detected significant diet-type effects on the weight of kidneys collected from 15 month old Amy mice that were fed either the AIN93-M diet, the Oz-AIN diet, or the Oz-AIN Supp diet ($p<0.0001$, Figure 22). Bonferroni post tests indicated that Amy mice that were fed the Oz-AIN diet had significantly heavier kidneys than Amy mice that were fed the AIN93-M diet ($p<0.0001$, Figure 22). This suggests that the Oz-AIN diet increases kidney weight in Amy mice.

These diet-type effects were not prevented by nutrient supplements. Amy mice that were fed the Oz-AIN Supp diet also had heavier kidneys than Amy mice that were fed the AIN93-M diet ($p=0.0003$, Figure 22). Furthermore, no differences were detected between the weight of kidneys collected from Amy mice that were fed either the Oz-AIN diet or the Oz-AIN Supp diet ($p>0.99$, Figure 22).

This suggests that the high-fat nature of the Oz-AIN diet and the Oz-AIN Supp diet increased the weights of kidneys of Amy mice. The supplementation of a high-fat, sub-optimal diets with nutrients that have been demonstrated to reduce weight gain in other organs, was unable to prevent or reduce weight gain in kidneys of Amy mice.

Figure 23. The preventative effect of nutrient supplements against diet-type induced increase of kidney weights (g) in 15 month old Amy mice.

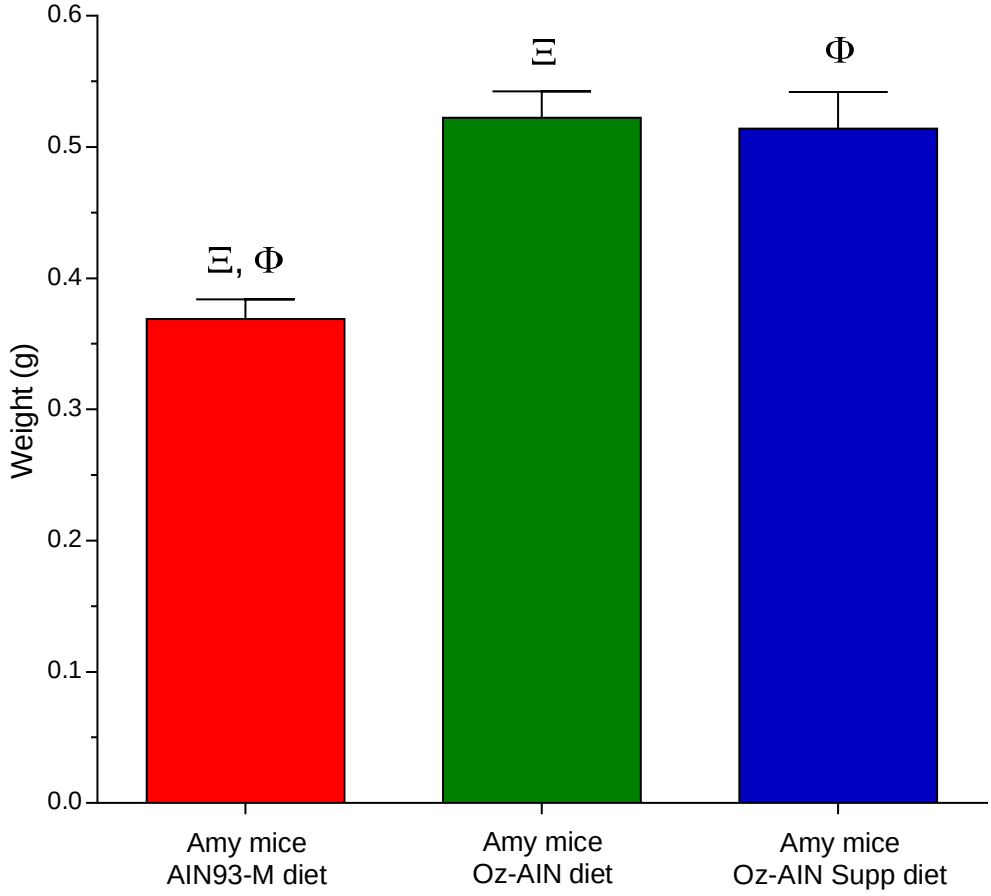


Figure 22. The average weight (g) of kidneys from 15 month old Amy mice. Amy mice fed the AIN93-M diet (n=11, red bar), Amy mice fed the Oz-AIN diet (n=14, green bar), and Amy mice fed the Oz-AIN Supp diet (n=12, blue bar). Bars represent mean ±SEM. Bars with matching symbols are significantly different using Bonferroni post tests. (Ξ) $p<0.0001$. (Φ) $p=0.0003$.

3.4.8. The preventative effects of nutrient supplements against diet-type induced increase of spleen weight (g) in Amy mice.

A one-way ANOVA detected significant diet-type effects on the weight of spleens collected from 15 month old Amy mice ($p=0.006$, Figure 23). Bonferroni post tests indicated that Amy mice that were fed the Oz-AIN diet had significantly heavier spleens than Amy mice that were fed the AIN93-M diet ($p=0.005$, Figure 23). This indicates that the Oz-AIN diet increases the weight of spleens in Amy mice.

There was no significant difference detected for the weights of spleens that were collected from Amy mice that were fed either the Oz-AIN diet or the Oz-AIN Supp diet ($p=0.90$, Figure 23). While this may suggest that the nutrient supplements did not affect the weight of spleens in Amy mice, there were also no differences in the weights of spleens from Amy mice that were fed the Oz-AIN Supp diet or the AIN93-M diet ($p=0.08$, Figure 23). This suggests that the nutrient supplements may have marginally decreased weight of spleens from Amy mice that were fed the high-fat, sub-optimal Oz-AIN diet so that they were more similar to those of mice that were fed an optimal rodent diet. However, the nutrient supplements were not able to completely overcome the effects of the Oz-AIN diet on weight of spleens from 15 month old Amy mice.

Figure 23. The preventative effect of nutrient supplements against diet-type induced increase of spleen weight (g) in 15 month old normal and Amy mice.

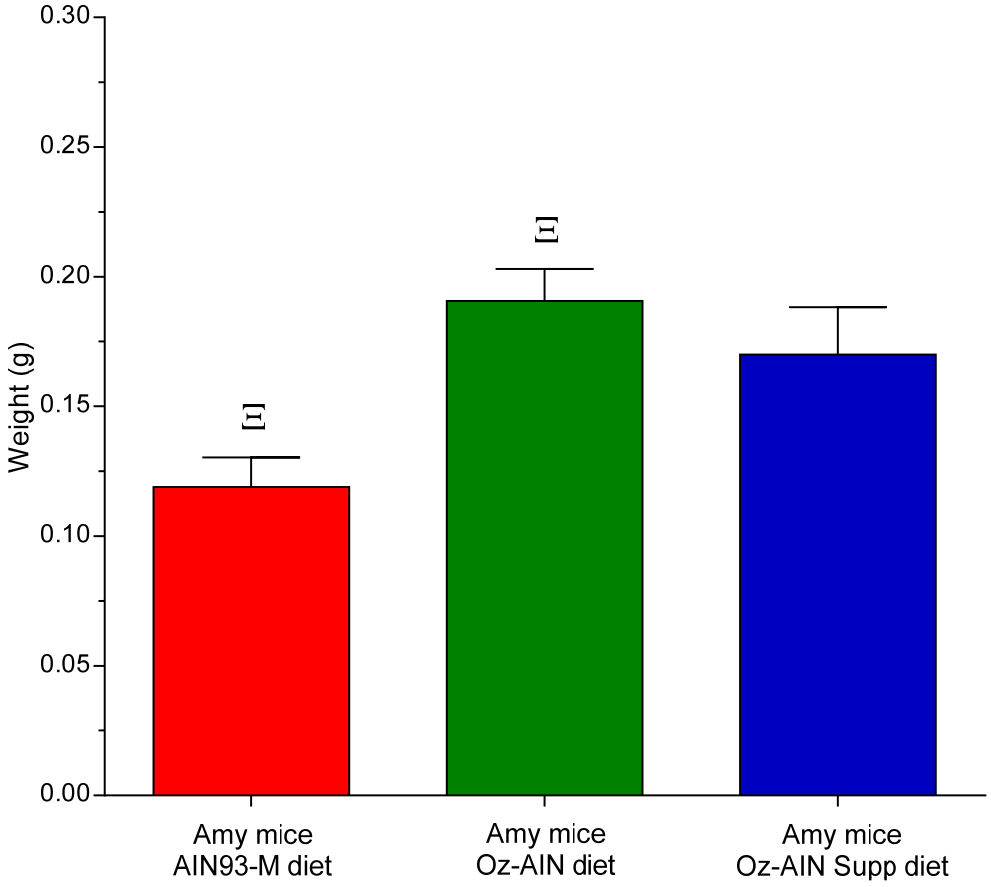


Figure 23. The average weight (g) of spleens from 15 month old Amy mice. Amy mice fed the AIN93-M diet (n=11, red bar), Amy mice fed the Oz-AIN diet (n=14, green bar), and Amy mice fed the Oz-AIN Supp diet (n=12, blue bar). Bars represent mean ±SEM. Bars with matching symbols are significantly different. (Ξ) p=0.005.

3.5. Conclusion.

Lifestyle modifications, such as dietary intervention, could offer a means to delay or prevent the onset of AD. To this end, much research has focussed on the benefit of nutrient supplements either alone or in combination against the neuropathology or behavioural deficits that are observed in AD. However, to date, no such research exists that explores the effect of supplementing a diet that is unique to Australia with a combination of nutrients that have potential benefits against AD-type neuropathology or cognitive decline. The Oz-AIN Supp diet that has been created as a component of the current thesis has been designed to fill this gap.

The Oz-AIN Supp diet was made by supplementing the Oz-AIN diet with a combination of nutrients that have been demonstrated to have potential benefits against AD. As such, the Oz-AIN Supp diet is high in fat, with a PMS ratio of 1.0: 1.7: 1.9. The nutrient supplements included in the Oz-AIN Supp diet were folate, vitamin B12, curcumin, grape seed extract, α-lipoic-acid and fish oil, which was used as a source of the ω-3 fatty acid docosahexaenoic acid.

The Oz-AIN Supp diet was able to prevent genotype effects on weight gain, fat deposition and organ size in Amy mice. This was apparent when comparing weight gain of normal mice that were fed the Oz-AIN diet, Amy mice that were fed the Oz-AIN diet and Amy mice that were fed the Oz-AIN Supp diet. The nutrient supplements were so effective at preventing genotype effects that the weight gain of Amy mice that were fed the Oz-AIN Supp diet was almost exactly the same as that of normal mice.

Diet-type effects were also observed on weight gain of Amy mice. Amy mice

fed the Oz-AIN Supp diet were significantly lighter than Amy mice fed the Oz-AIN Supp diet. This may be attributed to the hypolipidemic properties of the ω-3 fatty acids docosahexaenoic acid and eicosapentaenoic acid, which were included in the Oz-AIN Supp diet, but were not present in the Oz-AIN diet.

The Oz-AIN Supp diet described here is safe for use with rodents. The current results suggest that supplementing a diet that reflects a diet typically consumed by Australian women with B vitamins, polyphenolic compounds and fatty acids may prevent or delay genotype and diet-type effects on weight gain and fat deposition.

Chapter 4: Characterisation of the β-amyloid neuropathology in the brains of 15 and 18 month old Amy mice.

4. Background.

The two defining neuropathological features of AD are the presence of extracellular deposits of aggregated β-amyloid protein (plaques), and intracellular build up of hyperphosphorylated tau (tangles) [362, 363].

The primary hypothesis regarding the development and progression of AD is the "Amyloid Cascade Hypothesis". This hypothesis proposes that β-amyloid is cleaved from the membrane bound amyloid precursor protein and triggers a cascade of events leading to oxidative damage, neuroinflammation and cell death [29, 44, 364]. Neurodegeneration occurs in regions of the brain that are important for cognitive function and memory, both of which decline as AD progresses. However, the number and size of β-amyloid deposits does not correlate as well with the behavioural deficits that are associated with AD. Regardless, over the past 30 years, much of the research of AD has gone into understanding the role of β-amyloid in AD progression, which has cumulated in the "Amyloid Cascade Hypothesis" [44].

The Amy mouse model that is used in the current study is a genetic mouse model that over-expresses amyloid precursor protein and presenilin 1. The increased expression of presenilin 1 drives amyloid precursor protein processing down the amyloidogenic pathway, leading to increased neuronal β-amyloid in Amy mouse brains [132, 365]. By six months of age, Amy mice develop extracellular β-amyloid deposits, which

under low magnification are representative of the β-amyloid plaques that develop in the brains of AD patients [132, 160, 366, 367].

Like many β-amyloid mouse models, these mice do not develop the intracellular deposits of tau that are observed in AD. Therefore, the Amy mouse model does not truly reflect AD *per se,* but enables a better understanding β-amyloid over-expression in the brain [137, 368].

Dietary manipulation can alter the formation of β-amyloid deposits [160, 218]. High-fat diets enhance β-amyloid neuropathology in the brains of AD-type mice [86, 167, 369]. Pro-oxidant diets that are folate and vitamin E deficient and high in iron increase β-amyloid levels in normal and AD-type mice [370]. The ω-3 fatty acid docosahexaenoic acid, on the other hand, reduces β-amyloid deposition and in conjunction with the polyphenolic compound curcumin, reverses high-fat diet induced β-amyloid deposition in mouse brains [217]. However, the effect of nutritional supplementation on β-amyloid neuropathology has not been demonstrated in the context of an Australian-type diet which has an imbalanced poly-unsaturated: mono-unsaturated: saturated fat ratio (P:M:S) and suboptimal micronutrients levels.

The aims of the study described in this chapter are to:

1. **Examine and classify the types of β-amyloid neuropathology in the Amy mouse model.**

   This aim was achieved using immunohistochemical and immunofluorescent techniques. Immunohistochemical techniques were used to view β-amyloid deposits with low power bright field microscopy. Immunofluorescent techniques were used to characterise β-amyloid deposit co-localisation with DAPI nuclear staining, neurons and glial cells with confocal microscopy.

2. **Demonstrate the effect of an Australian-type diet on β-amyloid neuropathology in the brains of 15 and 18 month old Amy mice.**

   This aim was achieved using immunohistochemical and immunofluorescent techniques. Immunohistochemical techniques were used to compare the number and size of β-amyloid deposits in the brains of age-matched Amy mice that had been fed either an optimal diet (the AIN93-M diet) or an Australian-type rodent diet (the Oz-AIN diet) for 15 or 18 months under low power microscopy. Immunofluorescent techniques were used to compare β-amyloid co-localisation with DAPI nuclear staining, neurons or glial cells in the brains of age-matched Amy mice that had been fed either the AIN93-M diet or the Oz-AIN diet.

3. **Demonstrate the effect of supplementing an Australian-type diet with dietary nutrients that delay or prevent AD pathology, on the β-amyloid deposits in the brains of 15 month old Amy mice.**

   This aim was achieved using immunohistochemical and immunofluorescent techniques. Immunohistochemical techniques were used to compare the number and size of β-amyloid deposits in the brains of 15 month old Amy mice that had been fed a nutrient supplemented diet (the Oz-AIN Supp diet) with age-matched Amy mice that had been fed either the AIN93-M diet or the Oz-AIN diet, using low power microscopy. Immunofluorescent techniques were used compare β-amyloid co-localisation with DAPI nuclear staining, neurons or glial cells in the brains of 15 month old Amy mice that had been fed the Oz-AIN Supp diet, the AIN93-M diet or the Oz-AIN diet, using confocal microscopy.

The exploratory study that is described in this chapter makes two conclusions about the effect of diet on β-amyloid deposition in the brains of Amy mice. The first

conclusion is that diet may have an effect on β-amyloid aggregation. High total-fat content appeared to increase aggregation of β-amyloid into larger deposits. However, this was not ameliorated by dietary nutrient supplements. Although the nutrient supplements in the Oz-AIN Supp diet have been demonstrated to prevent or reduce β-amyloid deposition in other AD mouse models [160, 204, 348, 371], they did not prevent the increased β-amyloid aggregation that was associated with a high-fat diet with imbalanced P:M:S ratio and deficient in essential micronutrients.

The second conclusion is that there were three separate and distinct β-amyloid pathologies in the Amy mouse brain: (i) Intracellular β-amyloid that was associated with necrosis; (ii) Large diffuse deposits of β-amyloid that contained small, intact nuclei; and (iii) Small diffuse deposits that co-localised with astrocyte processes that line the blood brain barrier. These three pathologies were present in the same ratios in the brains of mice that were fed the AIN93-M diet or the Oz-AIN diet, suggesting that diet type did not affect β-amyloid deposition. However, nutrient supplements normalised the profile of β-amyloid deposits so that they occurred with similar frequencies. This may be due to interactions between nutrient supplements and the blood brain barrier. However, whether this is reflective of β-amyloid invasion or clearance was undetermined.

4.1. Methods.

4.1.1. Animals.

All experiments were approved by the Commonwealth Scientific and Industrial Research Organisation (CSIRO) Animal Welfare Committee, Australia in accordance with National Health and Medical Research Council guidelines.

Female Amy (APPswe/PSEN1dE9) mice and their female normal (C57bl/6) littermates were bred at and provided by Flinders University Animal Facility, Bedford Park, South Australia. Genotype was confirmed by PCR and agarose gel electrophoresis, as described in Appendix I. Mice were housed (n<6) in cages lined with sawdust, and had free access to food and water.

In order to clearly demonstrate the second and third aim of this chapter, mice were separated into two studies (Table 1, Figure 1). The first study was designed to demonstrate the potentially detrimental effects of an Australian-type diet on β-amyloid neuropathology in Amy mice. Amy mice were randomly allocated to one of two groups and fed either the AIN93-M diet or the Oz-AIN diet from weaning until they were 18 months old. Six normal mice were fed the Oz-AIN diet from weaning until they were 18 months and were used as controls (Table 1, Figure 1A).

The second study was designed to demonstrate the effects of supplementing the Australian diet with nutritional supplements that have previously been demonstrated to have a beneficial effect against β-amyloid neuropathology in Amy mice. Amy mice were randomly allocated to one of three groups and fed the AIN93-M diet, the Oz-AIN diet or the Oz-AIN Supp diet until the end of the study. Six normal mice that were fed the Oz-AIN diet from weaning and served as controls in the second study (Table 1, Figure 1B). There were difficulties managing mice over grooming each other after 15 months of age. Therefore, the second study only ran for 15 months.

While it was not the original intention to have first and the second studies end at different ages, this created an opportunity to compare β-amyloid neuropathology between 15 and 18 month old Amy mice. This may not appear to be a large enough window to observe changes in the Amy mouse brain. However, β-amyloid deposition in the Amy mouse brain starts at 6 months and increases exponentially with age [132,

160, 366, 367, 372, 373]. Therefore, comparison of 15 and 18 month old mice enabled the opportunity to compared neuropathology of Amy mice during different stages of adulthood after β-amyloid neuropathology is well established.

Table 1 outlines the total numbers of brains that were analysed in each study. There were no mice that were fed the Oz-AIN Supp diet for 18 months. This is because the study that ran for 18 months was designed to demonstrate the effect of an Australian-type diet (Oz-AIN diet) on β-amyloid neuropathology, rather than demonstrate the potential benefits of nutrient supplementation.

Table 1. The numbers of mice that were used to investigate the effects of an Australian-type diet, with or without nutritional supplements, on β-amyloid pathology in Amy mouse brains.

	Normal mice Oz-AIN diet	**Amy mice AIN93-M diet**	**Amy mice Oz-AIN diet**	**Amy mice Oz-AIN Supp diet**
Study 1: The effect of an Australian-type diet (18 months)	n = 6	n = 6	n = 6	- - N/A - -
Study 2: The effect of nutrient supplements (15 months)	n = 6	n =7	n = 12	n = 12

Figure 1A. The design of Study 1: The effect of an Australian-type diet on β-amyloid pathology in Amy mouse brains.

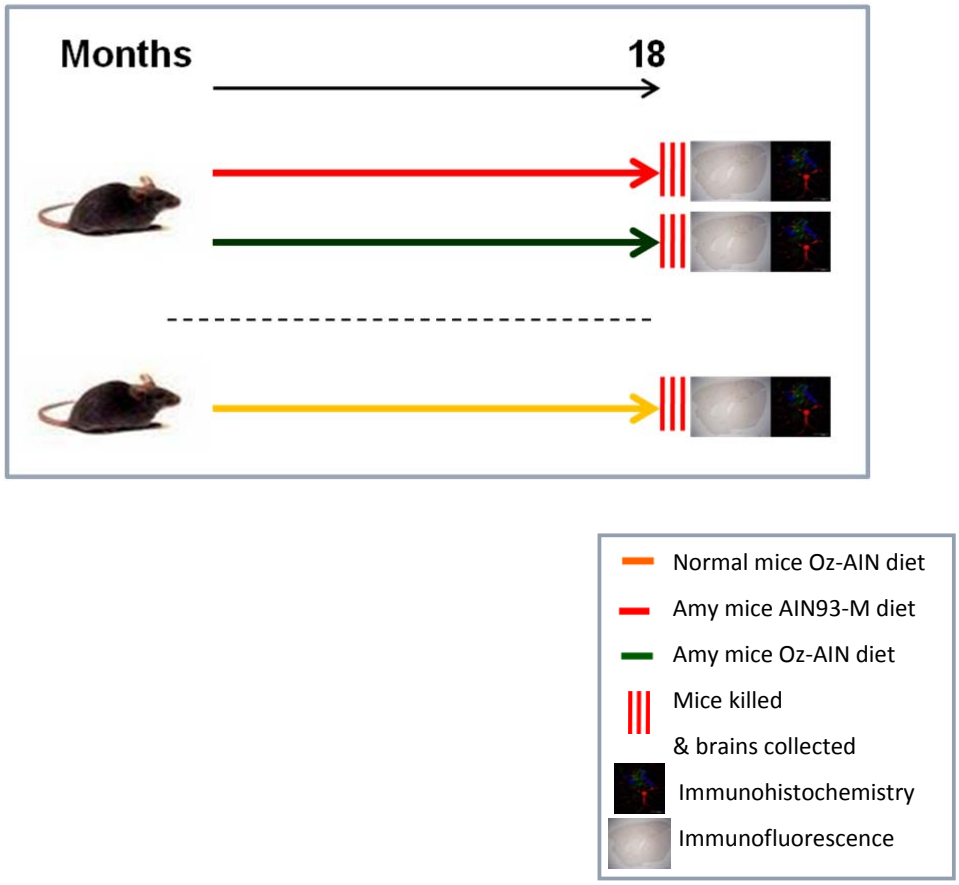


Figure 1A. Mice received their respective diets for 18 months and then were killed and their brains removed. Immunohistochemistry and immunofluorescent techniques were used to investigate β-amyloid neuropathology.
The effect of an Australian-type diet on β-amyloid neuropathology was investigated through comparisons of brains from 18 month old Amy mice that had been fed the AIN93-M diet (red arrow, n=6) or the Oz-AIN diet (green arrow, n=6).
Normal mice that were fed the Oz-AIN diet (yellow line, n=6) were used as controls.

Figure 1B. The design of Study 2: The effect of nutrient supplements on β-amyloid pathology in Amy mouse brains.

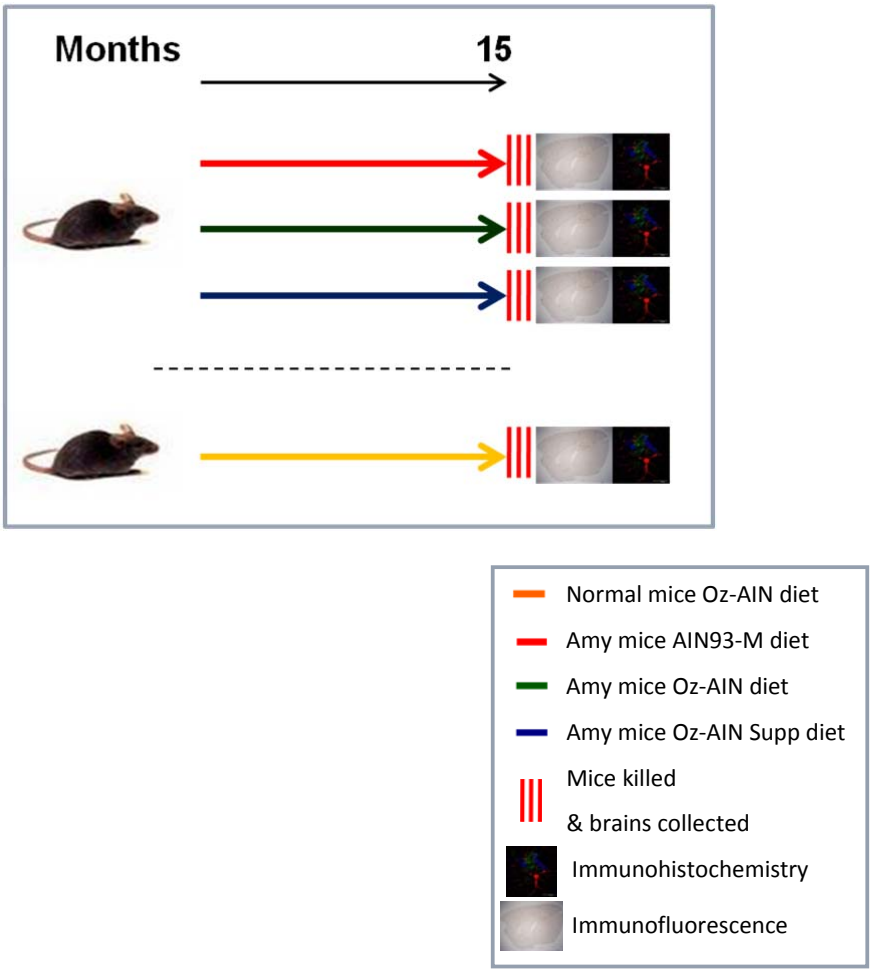


Figure 1B. Mice received their respective diets for 15 months and then were killed and their brains removed. Immunohistochemistry and immunofluorescent techniques were used to investigate β-amyloid neuropathology.
The effects of nutritional supplementation were made by comparing Amy mice that had been fed the Oz-AIN Supp diet (blue arrow, n=12) with Amy mice that had been fed either the AIN93-M diet (red arrow, n=7) or the Oz-AIN diet (green arrow, n=12). Normal mice that were fed the Oz-AIN diet (yellow line, n=6) were used as controls.

4.1.2. Tissue collection and storage.

Mice that were in the first and second studies were sacrificed at 18 and 15 months respectively. Mice were anaesthetised with isoflurane and killed by exsanguination from the abdominal aorta. Mice were perfused with PBS before brains were removed, weighed and halved. The right brain hemisphere was snap frozen in liquid nitrogen and stored for DNA analysis (Chapter 9). The left brain hemisphere was placed in formalin (Formalin solution, neutral buffered, HT501640, Sigma, Australia) to be used in immunohistochemical and immunofluorescent analysis. Left brain hemispheres were embedded in paraffin and cut in 5μm sagittal sections parallel to the midline of the brain, and mounted onto coated slides at the Histology Core Laboratory (Melbourne Brain Centre, University of Melbourne, Parkville, VIC, Australia).

Liver, kidneys, heart, spleen, and ovaries were also collected, weighed, and frozen in liquid nitrogen and stored in the CSIRO bio-bank. Uterine fat tissue, renal fat tissue, and fat tissue lining the skin were collected, weighed and stored in RNAlater Stabilization Reagent (76106, Qiagen).

4.1.3. De-paraffinisation and rehydration.

4.1.3.1. Protocol.

Sagittal sections were de-paraffinised in a xylene bath (Xylene, low in sulphur, 4.10234, Kilsyth, Victoria) for 15 min at room temperature, followed by another 15 min in a fresh xylene bath at room temperature.

Slides were re-hydrated in a series of four (100% to 70%) ethanol baths (EA043-P, Ethanol 100% Undenatured, Chem Supply, SA) for 3 min each at room temperature and then washed in dH_2O for 5 min.

4.1.4. Antigen retrieval.

4.1.4.1. Protocol.

The slides were transferred to a plastic rack with a lid containing EDTA•NaOH pH 8.0, and heated in a microwave until the solution started to boil (2 min, high power). Slides were heated for another 10 min (low power) and then cooled under running dH_2O, displacing the EDTA•NAOH PH 8.0.

After antigen retrieval, slides may be used for either immunohistochemical analysis (4.1.5.) or immunofluorescence (4.1.6.).

4.1.5. Immunohistochemistry.

4.1.5.1. Protocol.

After antigen retrieval, water was shaken away and excess water was wiped off with a clean tissue.

A circle was drawn around each sample with a wax pen. Slides were placed flat in the humidifier box and H_2O_2 (30%) was dropped into the centre of each circle. Enough H_2O_2 was used to cover each sample completely (100 – 200 μL), to block endogenous peroxidase. Samples incubated in the humidifier box for 5 min at room temperature.

H_2O_2 was washed away with PBS. Slides were rinsed in a bath of PBS (5 – 10 min, room temperature). The slides were removed from PBS, excess PBS was shaken away and the back and sides of slides were dried with a clean tissue.

4.1.5.1.1. Blocking non-specific binding sites.

All slides were returned to the humidifier box and 100 – 200 μL blocking solution (20% NHS in PBS) was dropped into the centre of each circle to block non-specific binding sites. Slides incubated in the humidifier box for 60 min at room temperature.

4.1.5.1.2. Addition of primary antibody.

Primary antibody, 6E10 (Sig 39320, beta-amyloid 1:16, monoclonal, Covance, Covance research products, Dedham, MA) was made up to a 1:500 solution in antibody buffer (1% NHS in PBS).

The blocking solution (20% NHS in PBS) was shaken from slides, and a clean tissue was used to dry the back and side of each slide.

The slides were returned to the humidifier box and 100 – 200 μL of primary antibody solution was gently dropped onto each sample. Slides incubated in the humidifier box overnight at room temperature. While the slides were incubating, enough water was kept in the humidifier to keep air moist and to prevent the slides from drying.

Primary antibody solution was washed away by dribbling PBS across the top of each slide. Slides were rinsed in a bath of PBS (5 – 10 min at room temperature).

4.1.5.1.3. Addition of secondary antibody.

While slides were in the bath of PBS, the secondary antibody (biotinylated α-mouse secondary antibody) was made up to a 1:2000 solution in antibody buffer (1% NHS in PBS).

The slides were removed from PBS, excess PBS was shaken away and the back and sides of each slide was dried with a clean tissue.

The slides were returned to the humidifier box and 100 – 200 µL of the biotinylated secondary antibody was added to each sample. Slides incubated in the humidifier box for 120 min at room temperature. Enough water was kept in the humidifier to keep air moist and to prevent the slides from drying.

The biotinylated secondary antibody was washed away by dribbling PBS across the top of each slide. Slides were rinsed in a bath of PBS (5 – 10 min, room temperature).

4.1.5.1.4. Stepavidin peroxidase staining (ABC kit).

Six µL/mL of Solution A and 6 µL/mL of Solution B from the VECTASTAIN® ABC kit (Vector Laboratories, Burlinghame, CA) were combined in PBS to make an ABC solution, and incubated for 30 min at room temperature before use.

Slides were removed from the PBS bath, excess PBS was shaken away and a clean tissue was used to dry the back and side of each slide.

Slides were returned to the humidifier box and 100 – 200 µL of the ABC solution was dropped onto to each sample. Slides incubated in the humidifier box for 60 min at room temperature.

The ABC solution was washed away by dribbling PBS across the top of each slide. Slides were rinsed in a bath of PBS (5 – 10 min, room temperature) and then transferred to a fresh bath of PBS for 15 min at room temperature.

4.1.5.1.5. DAB staining.

DAB mixture (6 mg DAB in 10 mL PBS, 10 μL 30% H_2O_2) was made and incubated for 15 – 20 min at room temperature.

Slides were removed from PBS, excess PBS was shaken away and the back and sides of each slide was dried with a clean tissue.

Slides were returned to the humidifier box, and 100 – 200 μL of DAB solution was dropped onto to slides. Slides incubated in the humidifier for 5 min at room temperature and then rinsed with tap water to stop the reaction.

4.1.5.1.6. Counterstaining slides with haematoxylin.

Slides were placed in haematoxylin for 10 sec at room temperature, and rinsed in dH_2O until the water ran clear. To remove any excess haematoxylin, slides were briefly dipped in acid alcohol, and then rinsed in dH_2O. Slides were placed in lithium carbonate for 2 min at room temperature to fix the colour, and washed in a fresh dH_2O bath for 10 sec at room temperature.

Slides were dehydrated in a series of two baths of absolute alcohol for 10 min at room temperature. Dehydration was completed by placing slides in a series of two xylene baths for 2 min at room temperature.

4.1.5.1.7. Coverslip slides.

One drop of DPX was placed over each sample on the slide.

Coverslips were gently placed on the top of the slide, and gentle pressure was applied to remove air bubbles. Excess DPX was wiped away with a tissue, with care not to allow the cover slip to slide around.

Analysis of slides was carried out under bright field microscopy using an Olympus BX50 upright microscope (brightfield) (Olympus®, UK) with camera attachment.

4.1.5.2. Slide analysis using brightfield microscopy.

All representative samples were taken from 5µm sagittal sections that were cut parallel to the midbrain and represent similar brain regions.

Slides were re-coded using the coding system used by Histology Core Laboratory (Melbourne Brain Centre) which was completely blinded to treatment group.

A trained experimenter who was blinded to the treatment groups for each of the slides took images of representative brain samples using a BX50 microscope (Olympus®) at 2x magnification. Image J analysis software (Image J 1.46r, National Institutes of Health, USA) was used to measure amyloid load, integrated density, deposit size and to count the total number of deposits.

Amyloid load and integrated density were both calculated automatically by Image J analysis software (Image J 1.46r, National Institutes of Health). Amyloid load was measured as the percentage of 6E10 positive staining per brain section. Three representative sections per brain were calculated and averaged to represent amyloid load in each brain. Integrated density is a calculation of the product of mean grey value and the total area of a section. Similar to amyloid load, the average integrated density measured from three representative brain sections was used to reflect integrated density of each mouse brain.

The total number of β-amyloid deposits was scored manually in three representative samples per brain using Image J analysis software (Image J 1.46r, National Institutes of Health). The 2x images were dense, and it was possible to increase magnification and maintain clarity of images using the Image J software. Therefore, β-amyloid deposits were counted and measured at 45x magnification using Image J software.

Averages of these counts were used to represent the total β-amyloid deposit number per section of mouse brain. The β-amyloid deposits were then scored by size. The distance across the centre of the widest point of the deposit was used to classify the deposit as large (>35μm), medium (15μm – 35μm), or small (<15μm). Deposits that were smaller than 5μm and did not contain a well defined core were classified as debris and were not included in counts. Counts of each deposit size were made in three representative sections per brain and an average of these counts was used to reflect β-amyloid deposit population within that brain. Accuracy of total counts and counts based on size was confirmed through comparison of counts of between multiple experimenters.

Data was stored in an Excel spreadsheet (Microsoft, 2007). All statistical analysis was carried out using GraphPad Prism® (Prism 5 for windows, version 5.4, GraphPad Prism Software). Age-diet-type interactions were investigated using two-way ANOVA and Bonferroni post tests. All other analysis were carried out with one-way ANOVA with Bonferroni post tests. When there were too few groups to compare using one-way ANOVA, data was analysed using Student's *t*-test. All data is reported as mean ±SEM. All differences where $p<0.05$ were considered significant.

4.1.6. Immunofluorescence.

4.1.6.1. Protocol.

After antigen retrieval, water was shaken away and excess water was wiped off with a clean tissue.

4.1.6.1.1. Blocking non-specific binding sites.

A circle was drawn around each sample with a wax pen. Slides were placed flat in the humidifier box and 100 – 200 μL blocking solution (20% NHS in PBS) was dropped into the centre of each circle. Slides incubated in the humidifier box for 60 min at room temperature.

4.1.6.1.2. Addition of primary antibody.

Primary antibodies were made up as per Table 2. Blocking solution was shaken off slides, and the back and sides of each slide was dried with a clean tissue.

Slides were placed in the humidifier and 100 – 200 μL of the appropriate primary antibody (Table 2), diluted in antibody buffer, was added to each sample.

Slides incubated with primary antibody overnight at room temperature. While the slides were incubating, enough water was kept in the humidifier to keep air moist and to prevent the slides from drying.

Primary antibody solution was washed away by dribbling PBS across the top of each slide. Slides were rinsed in a PBS bath (5 – 10 min, room temperature).

Table 2. Primary antibodies used for immunofluorescence.

Antigen	Type	Dilution	Source	Catalogue number
6E10 (beta-amyloid 1:16)	Ms pAb	1:100	Covance, Covance research products, MA.	Sig 39320
P25 (Oligodendrocytes)	RbpAb	1: 100	Gift from colleague.	-- N/A --
MAP2 (Neurons)	RbpAb	1:2000		366-380, Osc-212.
GFAP (Astrocytes)	RbpAb	1:1000	Promega, WI.	G5601
GPX1 (Microglia)	ShpAb	1:100	Abcam	Ab21966
Cleaved Caspase 3	RbpAb	1:600	Cell Signalling Technology, Arundel, Queensland.	9661 Asp175

All antibodies were diluted in antibody buffer (1% NHS in PBS).

4.1.6.1.3. Addition of secondary antibody.

Secondary antibodies were made up as per Table 3.

Slides were removed from PBS. Excess PBS was shaken away and the back and sides of each slide was dried with a clean tissue. Slides were returned to the humidifier box and 100 – 200 μL of the appropriate antibody (Table 3), which had been diluted in antibody buffer was added to each sample.

Table 3. Secondary antibodies used for immunofluorescence.

Antibody	Type	Dilution	Catalogue number
488 (green)	(α-mo)	1:50	715-225-150
Cy3 (red)	(α-sh)	1:100	713-165-147
Cy3 (red)	(α-rb)	1:50	711-165-152
Cy3 (red)	(α-rb)	1:100	711-165-152
Cy5 (blue)	(α-rb)	1:100	711-495-152

All antibodies were diluted in antibody buffer (1% NHS in PBS).
All secondary antibodies were from Jackson ImmunoResearch Laboratories, West Grove, PA.

Slides incubated in the humidifier box for 120 min at room temperature. Enough water was kept in the humidifier to keep air moist and to prevent the slides from drying.

Secondary antibodies were washed away by dribbling PBS across the top of each slide. Slides were rinsed in PBS (5 – 10 min, room temperature).

4.1.6.1.4. DAPI staining.

Slides were removed from PBS and excess PBS was shaken away and the back and sides of each slide were dried with a clean tissue.

Slides were placed in the humidifier box and 100 – 200 µL DAPI (0.04 % Thimersal) (50 µL / 50 mL in PBS) was dropped over each section. Slides incubated for 5 min at room temperature.

DAPI (0.04 % Thimersal) was washed away by dribbling PBS across the top of each slide. Slides were rinsed in PBS (15 min, room temperature). This was repeated in a fresh bath of PBS.

4.1.6.1.5. Coverslip slides.

A drop of 50 µL 80% buffered glycerine, 0.1 M Tris, pH 9.0 was placed over each sample on the slide.

Coverslips were gently placed on the top of the slide and gentle pressure was applied to remove air bubbles. Excess 80% buffered glycerine was wiped away with a tissue, with care not to allow the cover slip to slide around. Slides were sealed with nail polish, and stored at 4°C in the dark.

4.1.6.2. Slide analysis using confocal microscopy.

All fluorescent images were taken with a Leica confocal microscope (Leica Microsystems) using a 63x objective oil lens.

An experienced experimenter with knowledge of neuropathology and who was blind to each treatment group captured digital photos of four fields of view (250 μm x 250 μm) from three representative samples per mouse brain. The average number of β-amyloid deposits from these images was used as representative of the total β-amyloid depositions in each brain section. All representative samples were taken from 5 μm sagittal sections that were cut parallel to the midbrain and represent similar brain regions.

Slide images were coded using the Histology Core Laboratory (Melbourne Brain Centre) coding system, so that the experimenter completing analysis was blinded to each treatment group.

Consistent with the first aim of this project, β-amyloid deposits were characterised using immunofluorescent techniques. Beta-amyloid deposits can be intracellular or extracellular, it was therefore appropriate to classify β-amyloid deposits based on their association and co-localization with DAPI staining using confocal microscopy. These classification types are described in detail in the results section (pp. 213-214, Figure 8). Briefly, three types of deposits were observed.

1. Bright β-amyloid deposits co-localized with diffuse DAPI cores that lacked the integrity and structure of surrounding intact cells (Figure 8A, Figure 9C). These diffuse DAPI cores spread over an area that was 3-4 times larger than that of intact nuclei, which is characteristic of necrosis.

Therefore, this type of staining was interpreted as representing necrotic cells.

2. Extracellular β-amyloid deposits that were large and diffuse. These deposits contained, but did not co-localize with, intact nuclei (Figure 8B).

3. Extracellular β-amyloid deposits that were associated with GFAP staining along blood vessels, and were therefore classified as deposits that were associated with the blood brain barrier (Figure 8C, Figure 10D).

The β- amyloid deposits (as classified above) were counted and measured using LAS AF Lite software (Leica Application Suite, Advanced Fluorescence, version 2.6.0., Leica Microsystems, Mannheim). The total number of β-amyloid deposits was counted for each 250 μm^2 field of view. Deposits were then scored according to their classification type. The percentage of each deposit type was calculated for three fields of view per mouse brain. The averages of these percentages were used to represent the population of deposits in each mouse brain.

It was also noted that some intact neurons contained high levels of amyloid. These neurons were not included in the counts of β-amyloid deposits. These neurons, and the role that they may play in β-amyloid pathology, are discussed in the results and discussion sections of this chapter (pp. 213-214, and pp. 247-252 respectively).

Data was stored in an Excel spreadsheet (Microsoft, 2007). All statistical analysis was carried out using GraphPad Prism® (Prism 5 for windows, version 5.4, GraphPad Prism Software). Comparison of β-amyloid deposit types within groups was made with one-way ANOVA (repeated measures) and Bonferroni post tests. Differences between groups were compared with Students *t*- tests. All data is reported as mean ±SEM. All differences where $p<0.05$ were considered significant.

4.2. Results.

4.2.1. Brain weights of 15 and 18 month old normal and Amy mice.

Brain shrinkage and neuro-atrophy are common features of AD. Table 4 provides details of the weight of brains collected from 15 and 18 month old Amy mice. There were no Amy mice that were fed the Oz-AIN Supp diet for 18 months as they were not required to achieve the aims of that study (see section 4.1.1.).

Table 4. Brain weight (g) of 15 and 18 month old normal and Amy mice.

	Normal mice Oz-AIN diet	Amy mice AIN93-M diet	Amy mice Oz-AIN diet	Amy mice Oz-AIN Supp diet
15 mo	0.47 ±0.02	0.47 ±0.02 $^{\Phi}$	0.48 ±0.02	0.50 ±0.01
18 mo	0.45 ±0.01 $^{\#}$	0.55 ±0.02 $^{\Phi}$	0.47 ±0.02 $^{\Xi}$	----------

Brain weights of 15 and 18 month old mice. All values are mean ±SEM. A one-way ANOVA detected diet-type effects on brain weights of 18 month old mice (p=0.006). Bonferroni post tests revealed significant differences relative to 18 month old Amy mice that were fed the AIN93-M diet. (Ξ) p=0.025. (#) p=0.002.
A two-way ANOVA indicated an age-diet-type interaction (p=0.06). Bonferroni post tests revealed significant differences relative to 18 month old Amy mice that were fed the AIN93-M diet. (Φ) p=0.05.

A one-way ANOVA revealed that there were no differences in brain weight amongst 15 month old mice (p=0.58, Table 4). However, a one-way ANOVA of the brain weights of 18 month old mice revealed significant differences (p=0.0006, Table 4). Bonferroni post tests revealed that 18 month old Amy mice that were fed the AIN93-M diet had significantly heavier brains than either Amy mice that were fed the Oz-AIN diet (p=0.025, Table 4) or normal mice that were fed the Oz-AIN diet (p=0.002, Table 4).

A two-way ANOVA was carried out on the brain weights of 15 and 18 month old Amy mice that had been fed the AIN93-M diet or the Oz-AIN diet. An age-diet-type interaction accounted for 10.11% of the total variance of brain weights (p=0.06, Table

4). Although this is not significant at $p<0.05$, it is suggestive of trends for an age-diet-type interaction on brain weight of Amy mice that are fed either the AIN93-M diet or the Oz-AIN diet. Bonferroni post tests revealed that this interaction effect could be attributed to the brain weights of Amy mice that were fed the AIN93-M diet. The brains of 18 month old Amy mice that were fed the AIN93-M diet were significantly heavier than those of 15 month old Amy mice that had been fed the AIN93-M diet ($p=0.05$, Table 4). Neither diet-type nor age had a significant effect on brain weights of mice, and accounted for 6.24% and 5.58% of overall variance respectively ($p=0.14$ and $p=0.16$ respectively, Table 4).

4.2.2. A description of β-amyloid staining in Amy mouse brains using bright field microscopy at low magnification.

The β-amyloid neuropathology of Amy mice was examined and classified using immunohistochemical and immunofluorescent techniques. Mice were killed at 15 or 18 months of age, their brains were collected and cut at 5μm sections and mounted onto slides.

Figure 2 shows representative samples of brain sections from 15 months old Amy mice that were fed either the AIN93-M diet (Figure 2C), the Oz-AIN diet (Figure 2E), or the Oz-AIN Supp diet (Figure 2G), and from 18 month old Amy mice that were fed the AIN93-M diet (Figure 2D) or the Oz-AIN diet (Figure 2F). Representative brain sections from normal mice that were fed the Oz-AIN diet demonstrate an absence of β-amyloid in the 15 and 18 month old normal mouse brain (Figure 2A and 2B respectively).

Figure 2. Representative samples of sagittal brain sections from 15 and 18 month old normal and Amy mice that have been stained for β-amyloid.

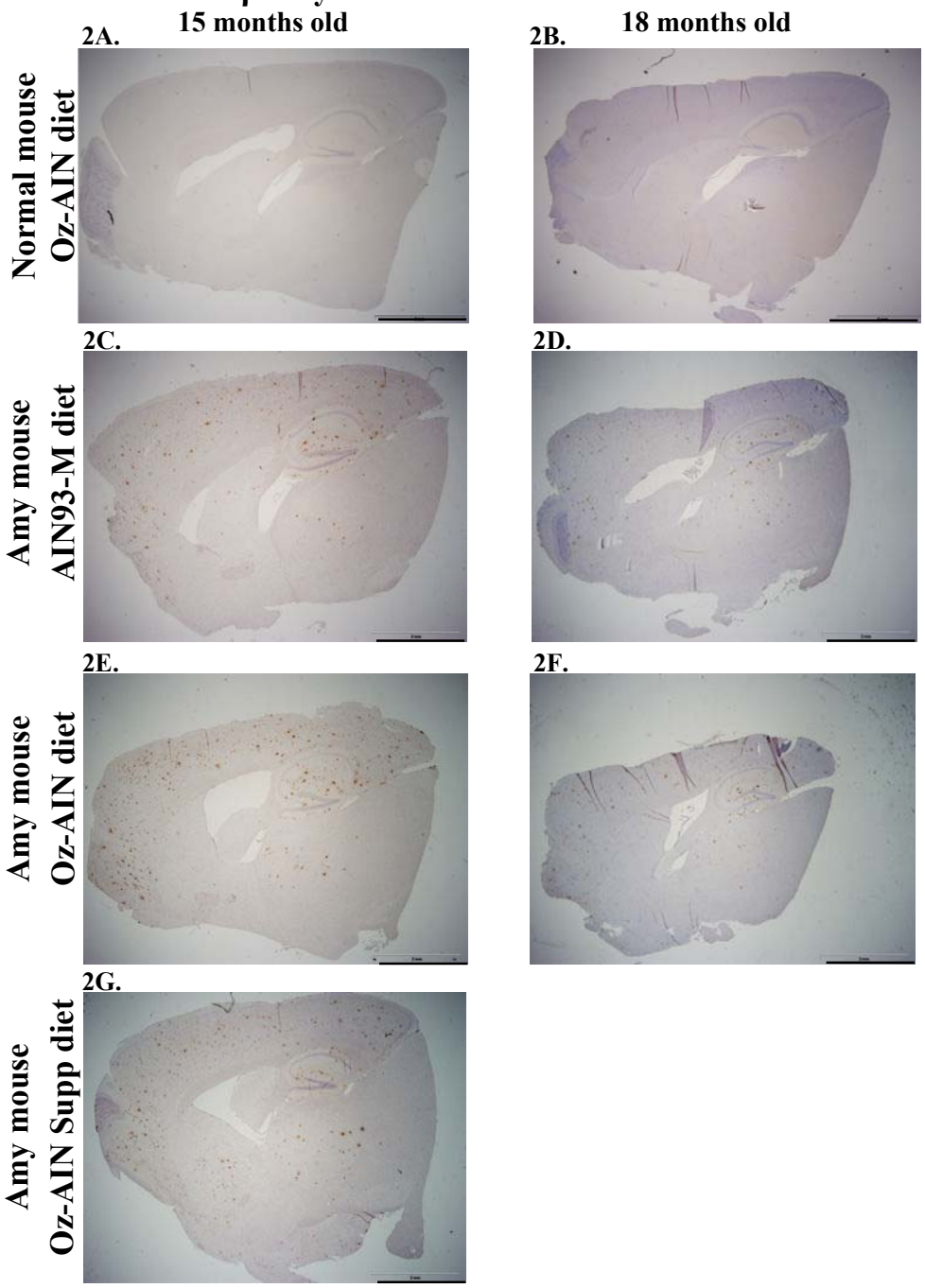


Figure 2. Brightfield images of normal and Amy mouse brain at 15 and 18 months old at low power. (**A**) Sagittal section of a 15 month old normal mouse brain that does not contain any staining for β-amyloid. (**B)** Sagittal section of an 18 month old normal mouse brain that does not contain any staining for β-amyloid. (**C**), (**E)** and (**G)** are representative sagittal brain sections of 15 month old Amy mice fed the AIN93-M diet (**C**), the Oz-AIN diet (**E**) and the Oz-AIN Supp diet (**G**). (**D)** and (**F)** are representative sagittal brain sections of 18 month old Amy mice fed the AIN93-M diet (**D**) and the Oz-AIN diet (**F**). Beta-amyloid was detected using the 6E10 antibody. Scale bars in all images are 2 mm.

4.2.2.1. A description of β-amyloid staining in the brains of 15 month old mice at low magnification.

There was no β-amyloid staining detected in the representative samples of the brains from 15 month old normal mice fed the Oz-AIN diet (Figure 2A). Therefore, the brains of the 15 month old normal mice that had been fed the Oz-AIN diet have been excluded from all further analysis of neuropathology.

The representative samples of the brains from the 15 month old Amy mice that were fed either the AIN93-M diet, Oz-AIN diet, or the Oz-AIN Supp diet stained positive for β-amyloid (Figures 2C, 2E, and 2G respectively). The β-amyloid positive staining was similar to that observed in other mouse models of AD [132, 136, 137]. This confirmed that, irrespective of diet, the 15 month old mice that had been used in the present study developed β-amyloid neuropathology that, when viewed under low power, is similar to the β-amyloid neuropathology that seen in AD.

The Amy mice that were fed the Oz-AIN diet appeared to have more β-amyloid deposits than Amy mice that were fed either the AIN93-M diet or the Oz-AIN Supp diet (Figure 2E compared to Figures 2C and 2G respectively). This was investigated further with quantitative analysis of amyloid load and the number of β-amyloid deposits (sections 4.2.3. and 4.2.4.).

4.2.2.2. A description of β-amyloid staining in the brains of 18 month old mice at low magnification.

There was no β-amyloid staining detected in the representative samples of the brains from 18 month old normal mice that were fed the Oz-AIN diet (Figure 2B). This confirmed that even in old age, normal mice that are fed a sub-optimal diet do not develop the β-amyloid neuropathology that is observed in AD. The brains from 18

month old normal mice that were fed the Oz-AIN diet were excluded from all further analysis of neuropathology.

The representative samples of brains from 18 month old Amy mice that were fed either the AIN93-M diet or the Oz-AIN diet stained positive for amyloid. Beta-amyloid positive staining appeared similar to that observed in other mouse models of AD [132, 136, 137]. This confirms that the Amy mouse model used in the present study develops β-amyloid deposits that appear similar to the β-amyloid deposits in AD, under low power.

The representative samples of the brains from 18 month old Amy mice that had been fed the Oz-AIN diet appeared to have less β-amyloid positive staining than the brains from age matched Amy mice fed the AIN93-M diet (Figures 2D and 2F). This was explored further with quantitative analysis of amyloid load and the number of β-amyloid deposits (sections 4.2.3. and 4.2.4.).

4.2.3. The effect of diet on amyloid load in the brains on 15 and 18 month old Amy mice.

Amyloid load is a measurement of the percentage area of each representative brain sample that has stained positive for a β-amyloid marker (6E10). Diet did not have a significant effect on amyloid load in 15 or 18 month old Amy mice ($p=0.45$ and $p=0.12$ respectively, Table 5, Figures 3A and 3B). While the brains of 15 month old Amy mice that were fed the AIN93-M diet had higher amyloid loads than brains from Amy mice that were fed the Oz-AIN diet or the Oz-AIN Supp diet, this was not significant with Bonferroni post tests ($p=0.44$ and $p=0.55$ respectively, Table 5). The amyloid loads in the brains of 18 month old Amy mice that were fed the AIN93-M were lower than those of 18 month old Amy mice that were fed the Oz-AIN diet, however this was also not significant ($p=0.11$, Table 5, Figure 3).

Table 5. Amyloid load in the brains of 15 and 18 month old Amy mice.

	Amy mice AIN93-M diet	Amy mice Oz-AIN diet	Amy mice Oz-AIN Supp diet
15 months	3.50 ±0.16%	2.97 ±0.32% $^{\Phi}$	3.05 ±0.26%
18 months	4.16 ±0.32%	5.19 ±0.21% $^{\Phi}$	----N/A------

All values are mean ±SEM. A two-way ANOVA revealed a significant effect of age (p=0.008). Numbers with matching symbols are significantly different with Bonferroni post tests. (Φ) p=0.02.

To determine whether or not age had an effect on amyloid load in Amy mice brains, a two-way ANOVA was carried out on the amyloid load in the brains of 15 and 18 month old Amy mice that were fed either the AIN93-M or the Oz-AIN diet (Figure 4). The brains of mice that were fed the Oz-AIN Supp diet were not included in these calculations, as there were no 18 month old mice that had been fed the Oz-AIN Supp diet. The two-way ANOVA revealed that age accounted for 27.62% of the variance of amyloid load (p=0.0008, Table 5). Diet-type and an age-diet-type interaction only accounted for 0.82% and 8.14% of the variance respectively (p=0.62 and p=0.12, Table 5). Bonferroni post tests indicated that the age effect could be attributed to significant differences between amyloid load in the brains of 15 and 18 month old Amy mice that were fed the Oz-AIN diet (p=0.02, Table 5, Figure 4).

The finding that there was a greater percentage of positive staining for β-amyloid in the brains of 18 month old mice compared to those of 15 month old mice contrasts with qualitative observations under the low power (section 4.2.2., Figure 2). To elucidate whether or not the amyloid load that was measured digitally truly reflected the β-amyloid positive staining, individual deposit counts were made in each representative section.

Figure 3A. The effect of diet on amyloid load in the brains of 15 month old Amy mice.

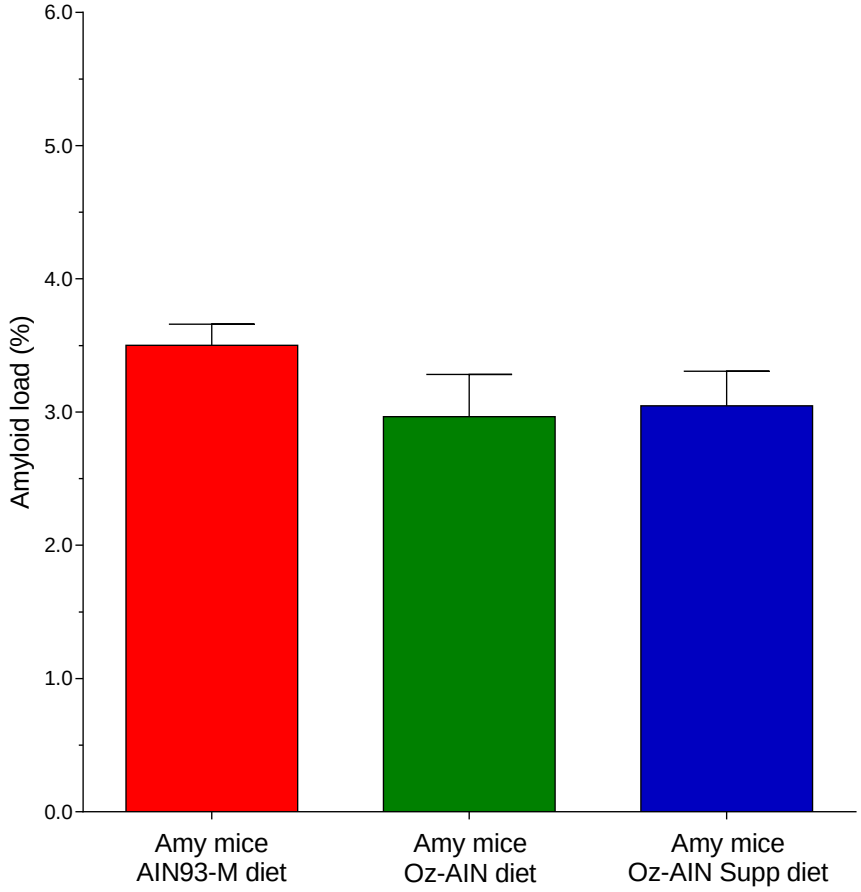


Figure 3A. Amyloid load was measured in the brains of 15 month old mice fed the AIN93-M diet (red bars, n=7), Oz-AIN diet (green bars, n=12), or the Oz-AIN Supp diet (blue bars, n=12). Amyloid load is expressed as percentage area that stained positive for β-amyloid in a 5 μm section of brain tissue. Bars represent mean ±SEM.

Figure 3B. The effect of diet on amyloid load in the brains of 18 month old Amy mice.

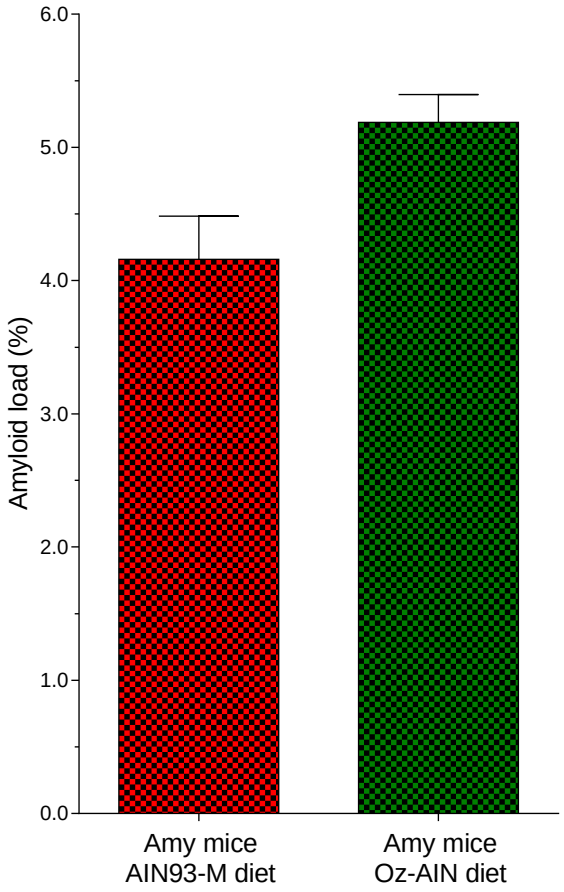


Figure 3B. Amyloid load was measured in the brains of 18 month old mice fed the AIN93-M diet (red checked bar, n=6), or the Oz-AIN diet (green checked bar, n=6). Amyloid load is expressed as percentage area that stained positive for β-amyloid in a 5 μm section of brain tissue. Bars represent mean ±SEM.

Figure 4. The effect of age on amyloid load in the brains of 15 and 18 month old Amy mice.

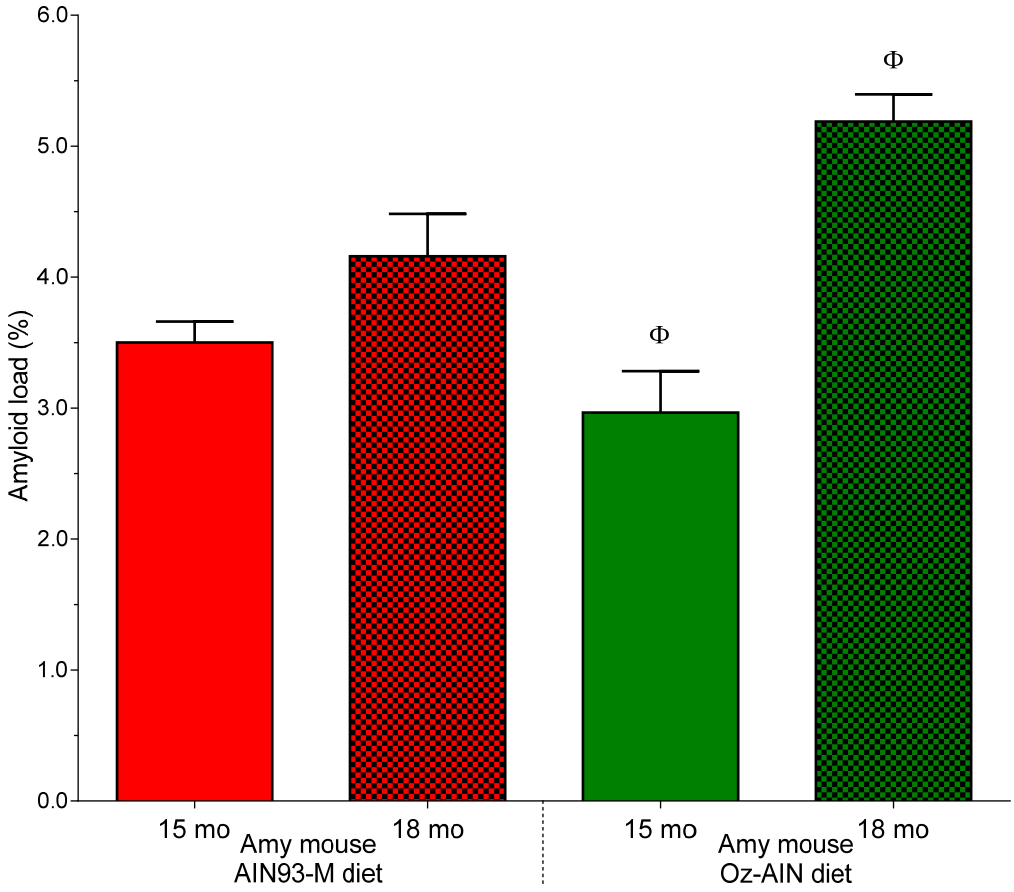


Figure 4. Amyloid load was compared between 15 month old Amy mice that were fed the AIN93-M diet (red bar, n=7) and 18 month old Amy mice that were fed the AIN93-M diet (red checked bar, n=6), and between 15 month old Amy mice that were fed the Oz-AIN diet (green bar, n=12) and 18 month old Amy mice that were fed the Oz-AIN diet (green checked bar, n=6). Amyloid load is expressed as percentage area that stained positive for β-amyloid in a 5 μm section of brain tissue. Bars represent mean ±SEM. A two-way ANOVA detected a significant effect of age (p=0.008). Bars with matching symbols are significantly different with Bonferroni post tests. (#) p=0.02.

4.2.4. Characterising the existence and total number of β-amyloid deposits in the brains of 15 and 18 month old Amy mice.

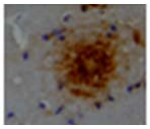
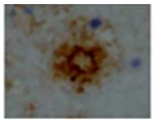
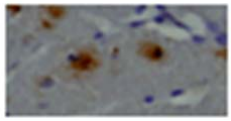
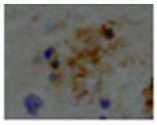
Beta-amyloid deposits in the brains of 15 and 18 month old Amy mice were counted and scored based on size. Large deposits were >35 μm wide, medium deposits were 15 – 35 μm wide, and smaller deposits were <15 μm (Figure 5). Deposits often consisted of a dense core, surrounded by lighter staining for amyloid, which was considered to be debris. However, not all deposits had debris, and not all debris contained a core. To maintain consistency throughout counts, only deposits that contained a core were counted and were measured across the widest point of the core. Debris without a core was not scored in the counts as it was too diffuse to measure size accurately (Figure 5).

4.2.4.1. Beta-amyloid deposit counts in the brains of 15 month old Amy mice.

There were no significant differences in the total number of β-amyloid deposits that were counted in the brains of 15 month old Amy mice that were fed the AIN93-M diet, the Oz-AIN diet or the Oz-AIN Supp diet ($p=0.36$, Figure 6A). This confirms that the numbers of each deposit size (small, medium, and large) would be comparable between each of the dietary groups. Although differences were not significant, there appeared to be fewer β-amyloid deposits in the brains of 15 month old Amy mice fed the AIN93-M diet than in the brains of Amy mice fed either the Oz-AIN diet ($p=0.33$, Table 6, Figure 6A) or the Oz-AIN Supp diet ($p=0.69$, Table 6, Figure 6A). This is most likely attributed to the finding that Amy mice that were fed the AIN93-M diet had fewer large deposits than mice that were fed the Oz-AIN diet or the Oz-AIN Supp diet.

Figure 5. Small, medium and large deposits in the Amy mouse brain and measured under low power microscopy.

5A.

Large deposits (>35um)	
Medium deposits (15um – 35um)	
Small deposits (<15um)	
Debris	

5B.

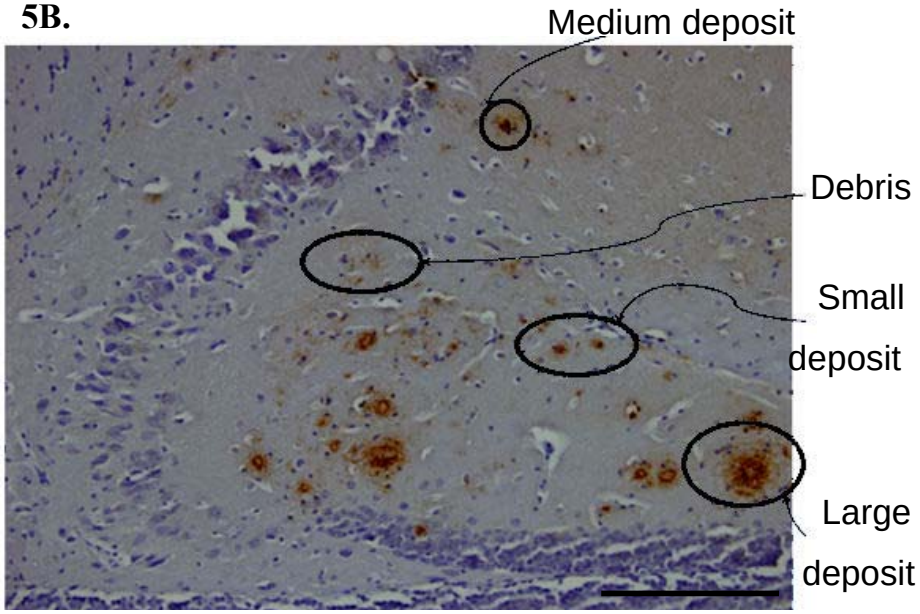


Figure 5A. Representative images of large (>35 μm), medium (15 – 35 μm) and small (<15 μm) deposits and debris that are seen in low power images of the Amy mouse brain.

Figure 5B. Representative image of an Amy mouse brain stained for β-amyloid (6E10) demonstrating the distribution and variety of large, medium and small deposits and debris in the hippocampus. Scale bar in image B is 200 μm.

Figure 6. Total, small, medium and large deposits in the 15 old Amy mouse brain and counted at 45x magnification.

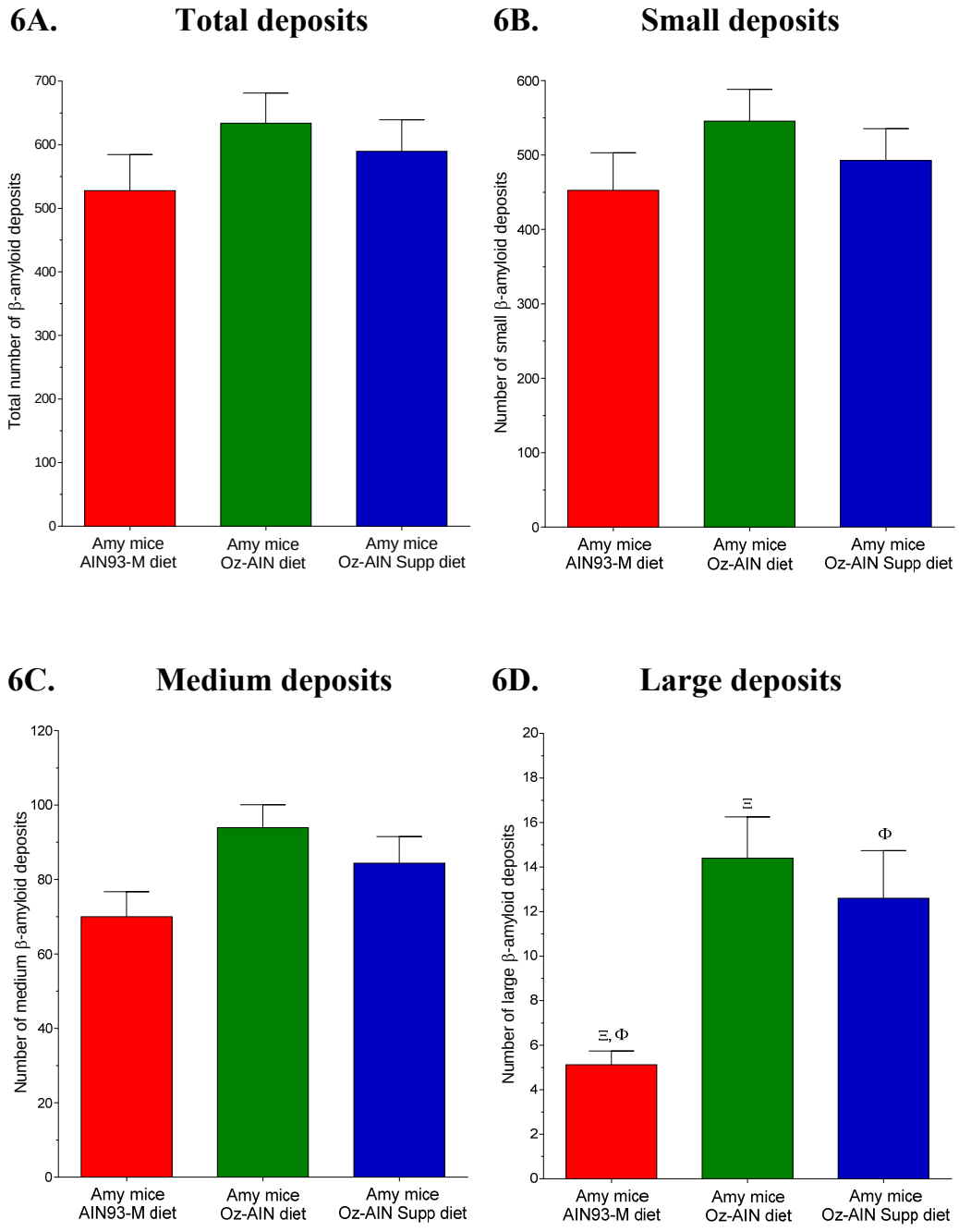


Figure 6A. The total number of β-amyloid deposits in representative sections in the brains of 15 month old mice fed the AIN93-M diet (red bars, n=7), Oz-AIN diet (green bars, n=12), or the Oz-AIN Supp diet (blue bars, n=12). Deposits were counted at 45x magnification. Bars represent mean ±SEM.

Figure 6B. The number of small (<15um) β-amyloid deposits in representative sections in the brains of 15 month old mice fed the AIN93-M diet (red bars, n=7), Oz-

AIN diet (green bars, n=12), or the Oz-AIN Supp diet (blue bars, n=12). Deposits were counted at 45x magnification. Bars represent mean ±SEM.

Figure 6C. The number of medium (15um - 35um) β-amyloid deposits representative sections in the brains of 15 month old mice fed the AIN93-M diet (red bars, n=7), Oz-AIN diet (green bars, n=12), or the Oz-AIN Supp diet (blue bars, n=12). Deposits were counted at 45x magnification. Bars represent mean ±SEM.

Figure 6D. The number of large (>35um) β-amyloid deposits representative sections in the brains of 15 month old mice fed the AIN93-M diet (red bars, n=7), Oz-AIN diet (green bars, n=12), or the Oz-AIN Supp diet (blue bars, n=12). Deposits were counted at 45x magnification. Bars represent mean ±SEM. A one-way ANOVA detected significant differences (p=0.004). Bars with the same letter are significantly different with Bonferroni post tests. (Ξ) p=0.004. (Φ) p=0.02.

Table 6. Beta-amyloid counts and integrated densities from brain sections of 15 month old mice.

	Amy mice AIN93-M diet	**Amy mice Oz-AIN diet**	**Amy mice Oz-AIN Supp diet**
Total β-amyloid deposits	527.80 ±56.75	633.90 ±47.16	589.70 ±49.31
Large deposits (> 35 µm)	5.13 ±0.61	14.40 ±1.86 $^{\Xi}$	12.60 ±2.14 $^{\Phi}$
Medium deposits (15-35 µm)	70.00 ±6.75	93.90 ±6.18	84.4 ±7.14
Small deposits (< 15 µm)	452.60 ±50.50	545.60 ±42.83	492.70 ±42.66
Integrated density	6.36 x 10^8 ±1.19 x 10^8	11.10 x 10^8 ±1.69 x 10^8	6.93 x 10^8 ±1.03 x 10^8

Beta-amyloid deposits in 5 µm brain sections of 15 month old Amy mice that were fed the AIN93-M diet, Oz-AIN diet or Oz-AIN Supp diet. All values are mean ±SEM. A one-way ANOVA detected significant diet-type effects on the number of large deposits (p=0.004). Bonferroni post tests revealed (Ξ) p=0.004. (Φ) p=0.02 relative to large deposits of Amy mice fed the AIN93-M diet.

The brains from 15 month old Amy mice that were fed the AIN93-M diet had fewer large deposits, medium deposits and small deposits than the brains of mice fed either the Oz-AIN diet or the Oz-AIN Supp diet (Table 6, Figure 6B, 6C and 6D, respectively). This was not significant for small deposits (Figure 6B) or medium deposits (Figure 6C). However, there was a significant diet-type effect on the number of large deposits ($p=0.004$, Figure 6D). The representative samples of brains from 15 month old Amy mice that had been fed the AIN93-M diet had significantly fewer large deposits than Amy mice that had been fed either the Oz-AIN diet ($p=0.004$, Table 6, Figure 6D) or the Oz-AIN Supp diet ($p=0.02$, Table 6, Figure 6D).

4.2.4.2. Beta-amyloid deposit counts in the brains of 18 month old Amy mice.

There were more β-amyloid deposits in the brains of 18 month old Amy mice that were fed the AIN93-M diet than in the brains of age matched mice fed the Oz-AIN diet (635 ±206 and 451 ±70 respectively, Table 7, Figure 7A). Unlike the brains of 15 month old mice, the 18 month old Amy mice that had been fed the AIN93-M diet had more small deposits than Amy mice that were fed the Oz-AIN diet. However, owing to the large variation of the number of small deposits in the brains of 18 month old Amy mice that were fed the AIN93-M diet, this failed to achieve significance ($p=0.50$, Figure 7B). Similar trends were seen for medium deposits ($p=0.51$, Figure 7C) and large deposits ($p=0.15$, Figure 7D).

Table 7. Beta-amyloid counts and integrated densities from brain sections of 18 month old mice.

	Amy mice AIN93-M diet	**Amy mice Oz-AIN diet**
Total β-amyloid deposits	635.0 ±206.0	451.0 ±70.0
Large deposits (> 35 µm)	10.0 ±3.0	3.0 ±0.5
Medium deposits (15-35 µm)	105.5 ±28.5	76.0 ±23.0
Small deposits (< 15 µm)	519.0 ±171.5	372.0 ±47.0
Integrated density	$2.90 \times 10^8 \pm 5.53 \times 10^7$	$2.67 \times 10^8 \pm 9.53 \times 10^6$

Beta-amyloid deposits in 5 µm brain sections of 18 month old Amy mice that were fed the AIN93-M diet or Oz-AIN diet. All values are mean ±SEM.

Figure 7. Total, small, medium and large deposits in the 18 month old Amy mouse brain and counted at 45x magnification.

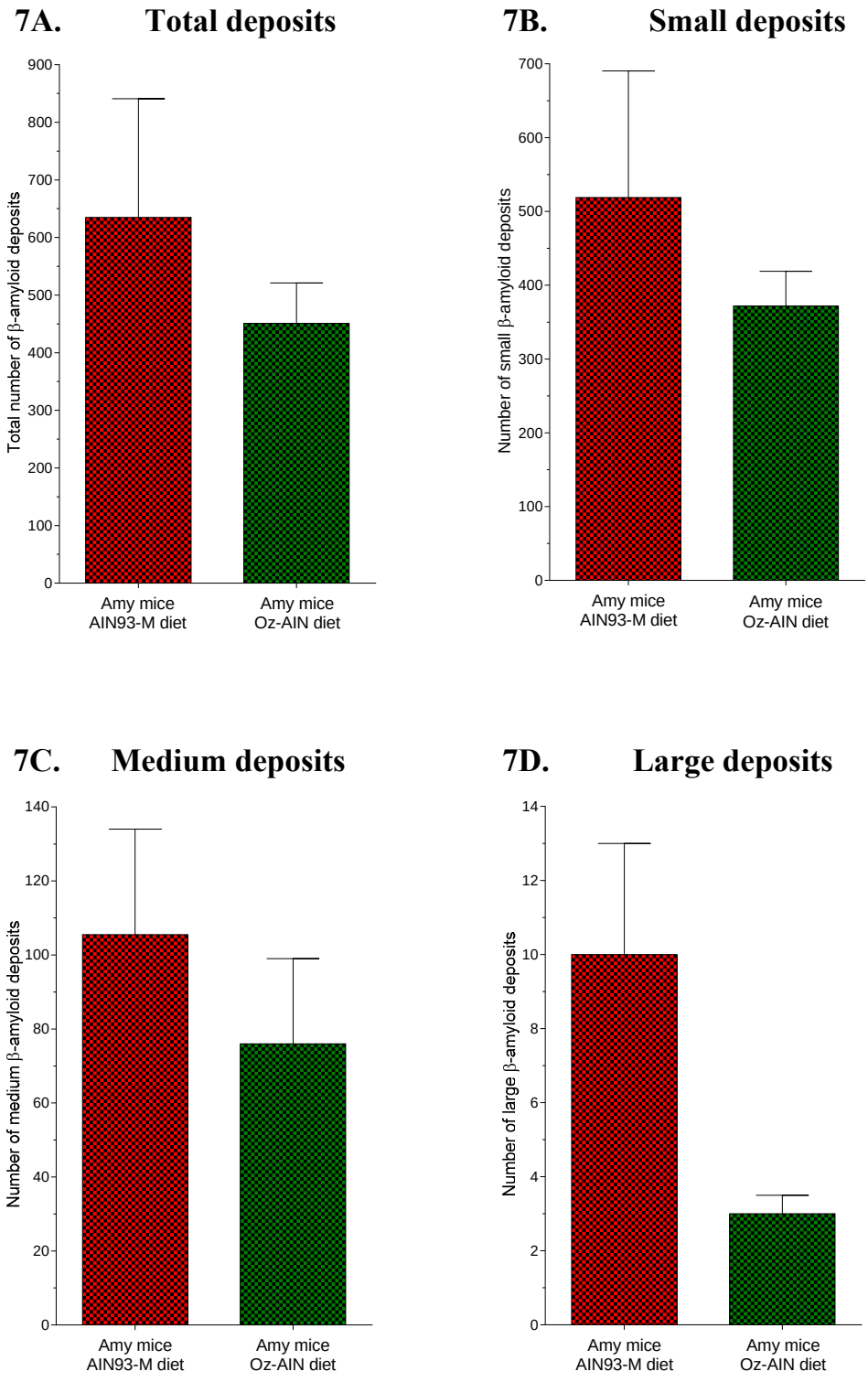


Figure 7A. The total number of β-amyloid deposits in representative sections in the brains of 18 month old mice fed the AIN93-M diet (red checked bars, n=6) or the Oz-AIN diet (green checked bars, n=6). Deposits were counted at 45x magnification. Bars represent mean ±SEM.

Figure 7B. The number of small (<15um) β-amyloid deposits in representative sections in the brains of 18 month old mice fed the AIN93-M diet (red checked bars, n=6) or the Oz-AIN diet (green checked bars, n=6). Deposits were counted at 45x magnification. Bars represent mean ±SEM.

Figure 7C. The number of medium (15um - 35um) β-amyloid deposits in representative sections in the brains of 18 month old mice fed the AIN93-M diet (red checked bars, n=6) or the Oz-AIN diet (green checked bars, n=6). Deposits were counted at 45x magnification. Bars represent mean ±SEM.

Figure 7D. The number of large (>35um) β-amyloid deposits in representative sections in the brains of 18 month old mice fed the AIN93-M diet (red checked bars, n=6) or the Oz-AIN diet (green checked bars, n=6). Deposits were counted at 45x magnification. Bars represent mean ±SEM.

4.2.5. Integrated densities of the brains of 15 and 18 month old Amy mice.

Integrated densities were also measured using Image J software to give further indication of the total amount of β-amyloid in mouse brain sections. Integrated densities are a measure of the mean gray value of a digital image. A higher integrated density indicates higher levels of positive staining across the representative image.

Brain sections of 15 month old Amy mice that were fed the Oz-AIN diet had higher integrated densities than those from Amy mice that were fed the Oz-AIN Supp diet or the AIN93-M diet (Table 6). While this is similar to the qualitative assessment of the effect of diet on β-amyloid deposition (section 4.2.2.) and the total numbers of β-amyloid deposits (section 4.2.4.), the differences between integrated densities were not significant ($p=0.15$, Table 6).

Brain sections from 18 month old Amy mice that were fed the Oz-AIN diet had lower integrated densities than age matched litter mates fed the AIN93-M diet. However these differences did not achieve significance (Table 7).

Figure 5B shows a representative sample that contains small, medium and large deposits and debris. The integrated densities may differ from deposit counts because they have also measured the amount of debris. It is acknowledged that the β-amyloid that was deposited as debris may have had a bearing of β-amyloid neuropathology. However, direct measurement of the amount of debris was not carried out in these low power images, as it was hard to separate debris from deposits.

4.2.6. Confocal analysis of β-amyloid deposits in Amy mouse brains.

4.2.6.1. Different deposit formations that were observed using confocal microscopy.

Beta-amyloid deposits were also characterised under confocal microscopy. First, it was determined whether deposits of β-amyloid were intracellular or extracellular. This was determined by co-localisation with DAPI nuclear staining. Interactions between β-amyloid and different cell-types were also investigated by looking for β-amyloid co-localisation with markers for neurons (MAP2, Figure 9), astrocytes (GFAP, Figure 10), and oligodendrocytes (P25, Figure 11). An attempt to co-localize β-amyloid with microglia was made, but staining was unsuccessful.

Confocal images revealed that, irrespective of diet, the 15 and 18 month old Amy mouse brains contained three different types of β-amyloid deposits: (i) intracellular β-amyloid (Figure 8A, Figure 9A, 9B and 9C), or extracellular β-amyloid in either (ii) large, diffuse deposits (Figure 8B), or (iii) smaller diffuse deposits that were associated with the blood-brain barrier (Figure 8C, Figure 10C).

(i) Intracellular β-Amyloid.

Neurons co-localised with light staining for intracellular β-amyloid (Figure 9A). DAPI staining in these cells was bright and had a well defined structure indicative of an intact nucleus in amyloid-containing neurons. There were also neurons that contained brighter staining for β-amyloid (Figure 8A, Figure 9B). A minority of these cells were intact with a preserved nucleus and appeared to secrete β-amyloid into the extracellular space (Figure 9B). Other neurons appeared to have ruptured with a disintegrating nucleus, as indicated by diffuse DAPI staining that spanned an area 3-4 times greater than that of surrounding intact nuclei (Figure 8A, Figure 9C). This is

suggestive of necrotic morphology. The β-amyloid deposits that co-localised with necrotic cells had a bright core that was 14.93 ±0.70 μm across the centre of the widest point, and were surrounded by diffuse β-amyloid staining that extended 32.69 ±1.84 μm from the centre of the core (Figure 8A, Figure 9C).

(ii) Extracellular β-Amyloid – Large diffuse deposits.

Extracellular β-amyloid deposits were either large diffuse deposits (92.85 ±8.94 μm across the centre of the widest point) that often covered the entire field of view (250 μm). There were occasional small extracellular deposits (28.9 ±4.43 μm across the centre of the widest point) (Figures 8B and 8C). The larger deposits contained DAPI staining of intact nuclei. Smaller extracellular deposits were not associated with DAPI staining of either intact nuclei or necrotic cells.

(iii) Extracellular β-Amyloid – Blood Brain Barrier.

Astrocytes are glial cells that connect to and protect neurons via foot processes. Astrocyte foot processes can also insheathe endothelial cells and form a part of the blood brain barrier. Beta-amyloid deposits that were 57.65 ±4.89 μm across the widest point of a bright region, co-localised with astrocyte foot processes that appeared to be the astrocytes processes that line the blood brain barrier (Figure 10D).

There appeared to be gaps between astrocytes processes that were associated with β-amyloid (Figure 10D). While these gaps may have been due foot processes moving out of the plane at which images were taken, they could equally have represented breaks in the blood brain barrier. This is addressed further in the discussion section of this chapter (pp. 253-256). The β-amyloid deposit-blood brain barrier association was accompanied by large areas of diffuse β-amyloid staining extending 133.30 ±59.24 μm the blood vessel (Figure 8C).

Figure 8A. Intracellular β-amyloid associated with necrotic cells.

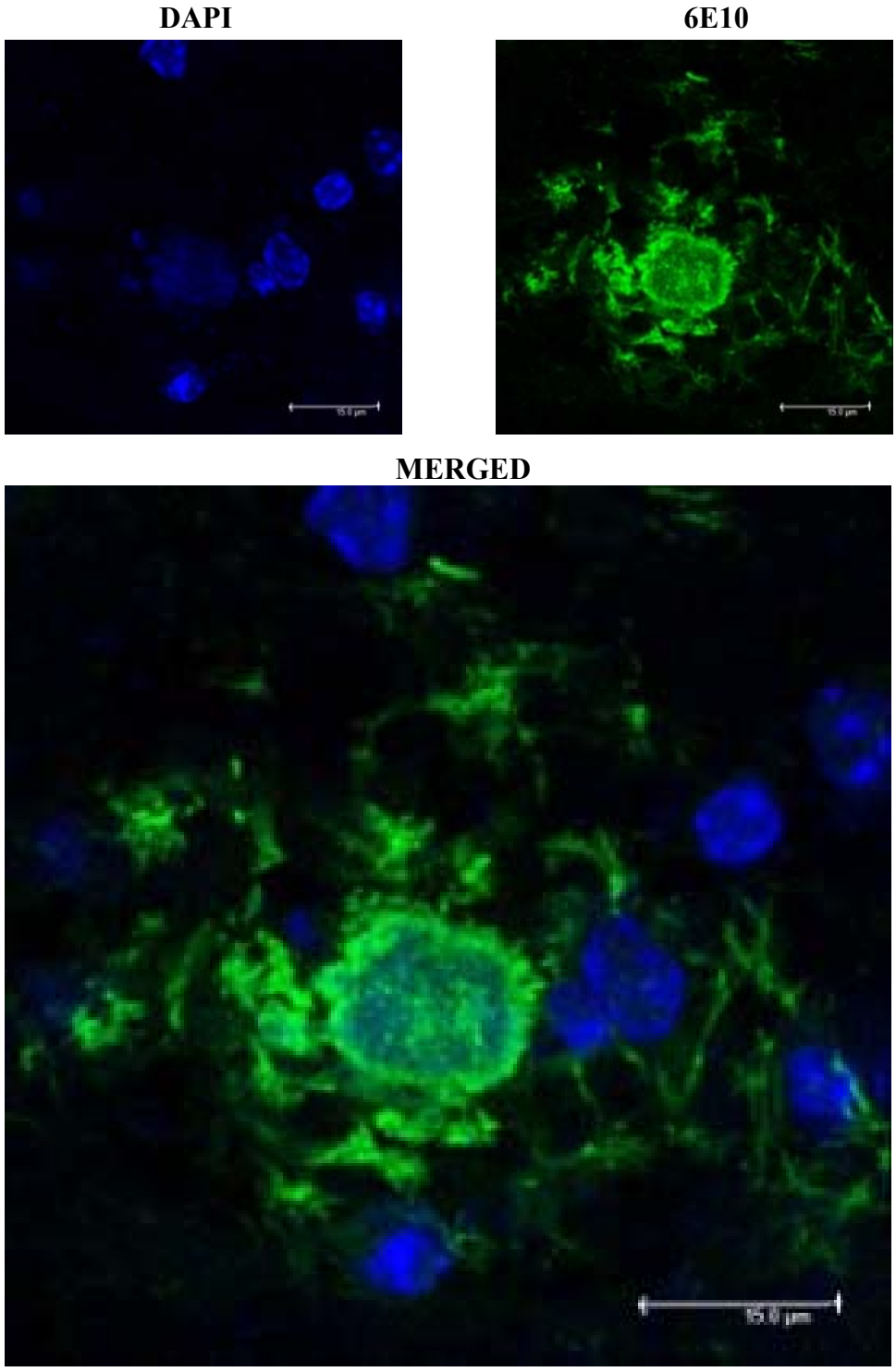


Figure 8A. Necrotic cells had diffuse DAPI staining, which spread 4-5 times the area of surrounding cells and co-localised with bright β-amyloid staining. Beta-amyloid deposits were co-localised with DAPI nuclear staining. Beta-amyloid was stained with 6E10. Nuclear tissue was stained with DAPI. Scale bars are 15 µm.

Figure 8B. Extracellular β-amyloid in large diffuse deposits of β-amyloid.

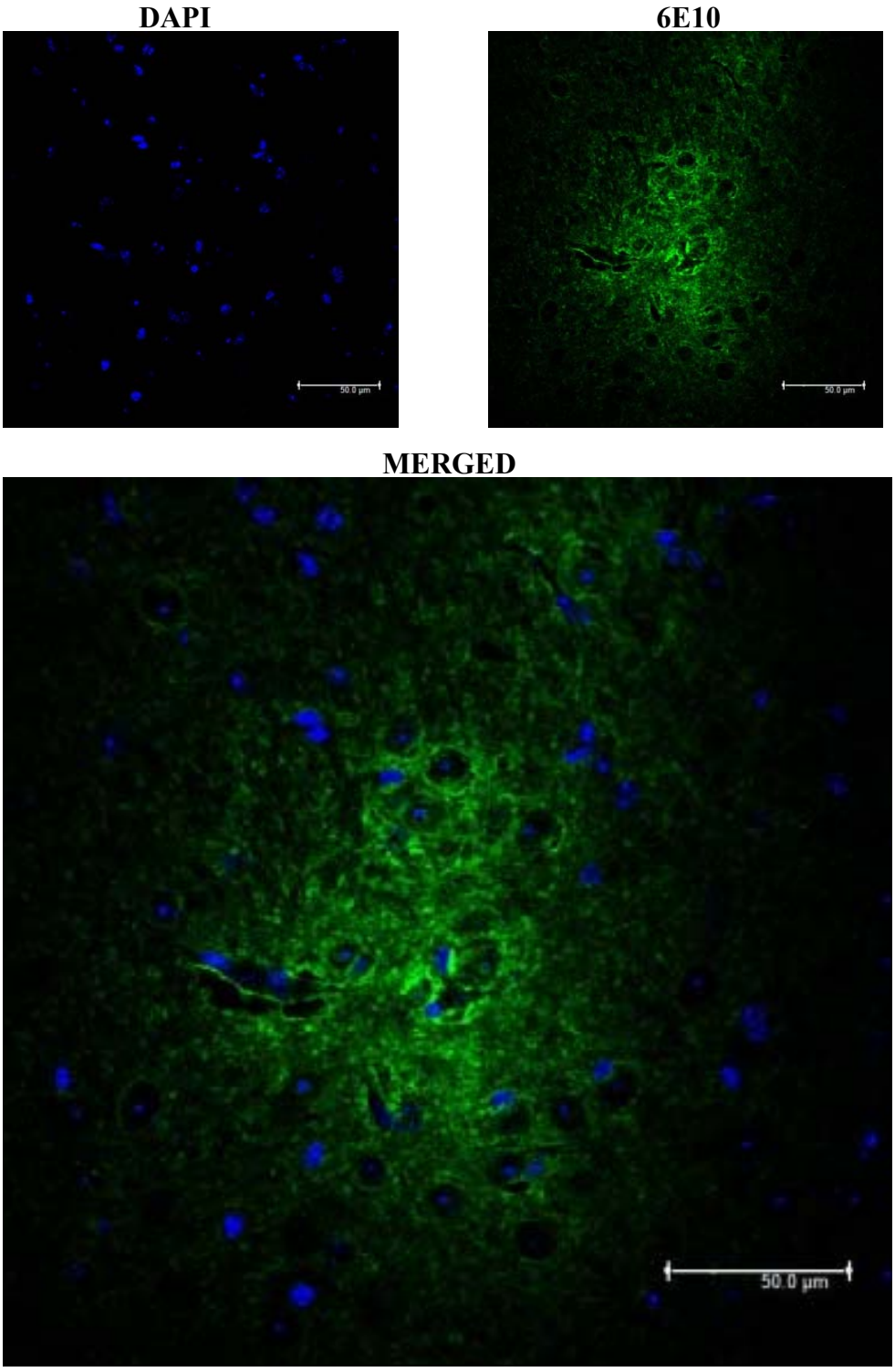


Figure 8B. Diffuse deposits of β-amyloid were large and surrounded intact nuclei. Beta-amyloid was stained with 6E10. Nuclear tissue was stained with DAPI. Scale bars are 50 µm.

Figure 8C. Extracellular β-amyloid at the Blood Brain Barrier.

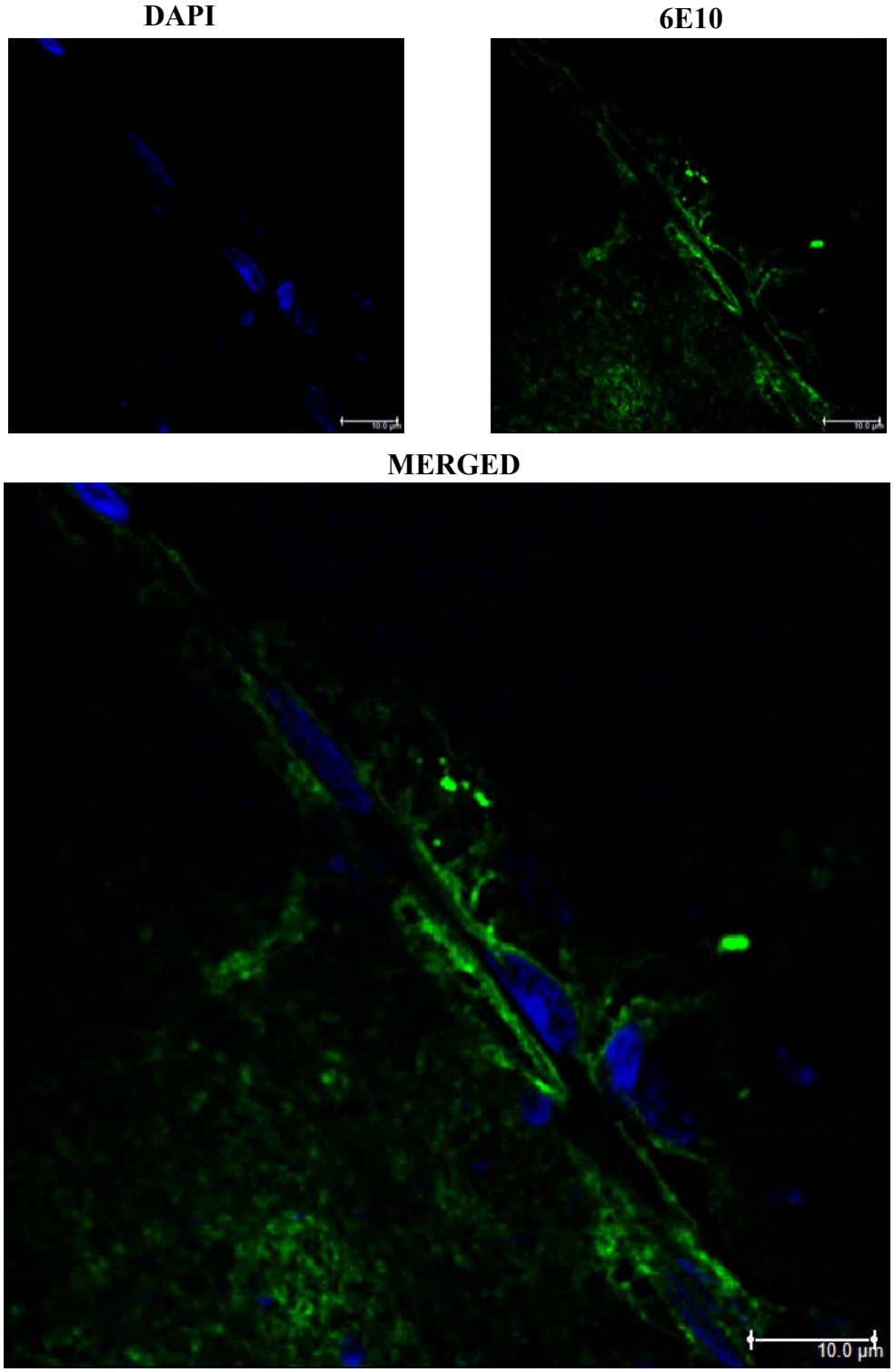


Figure 8C. Diffuse deposits that associated with blood vessels had a bright core and did not co-localise with any of the surrounding cells. Beta-amyloid was stained with 6E10. Nuclear tissue was stained with DAPI. Scale bars are 10 µm.

Figure 9A. Beta-amyloid co-localised with neurons.

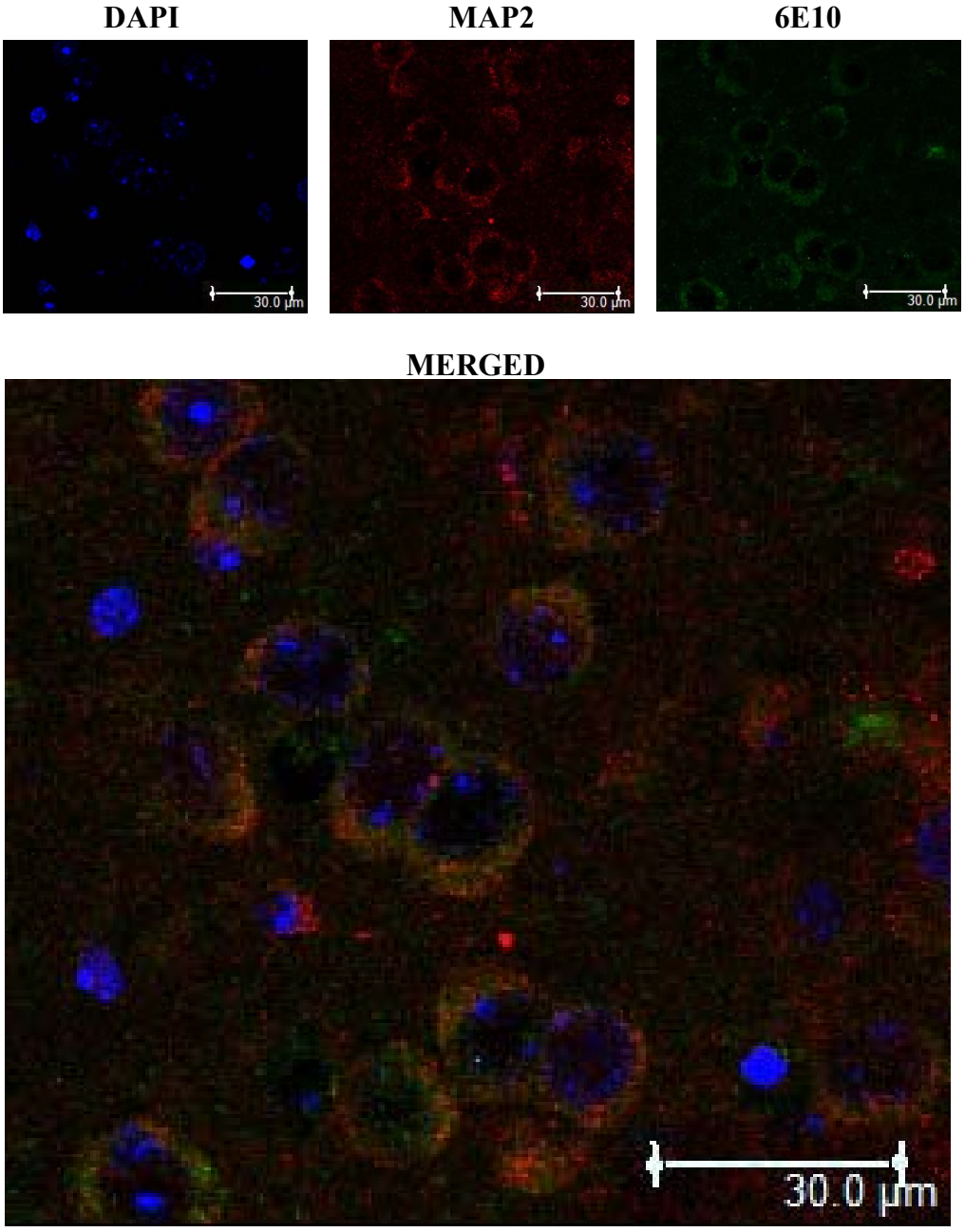


Figure 9A. Most neurons co-localised with light staining for amyloid. Neurons were stained with MAP2. Beta-amyloid was stained with 6E10. Nuclear tissue was stained with DAPI. Scale bars are 30 µm

Figure 9B. Neurons with intact nuclei appeared to secrete β-amyloid.

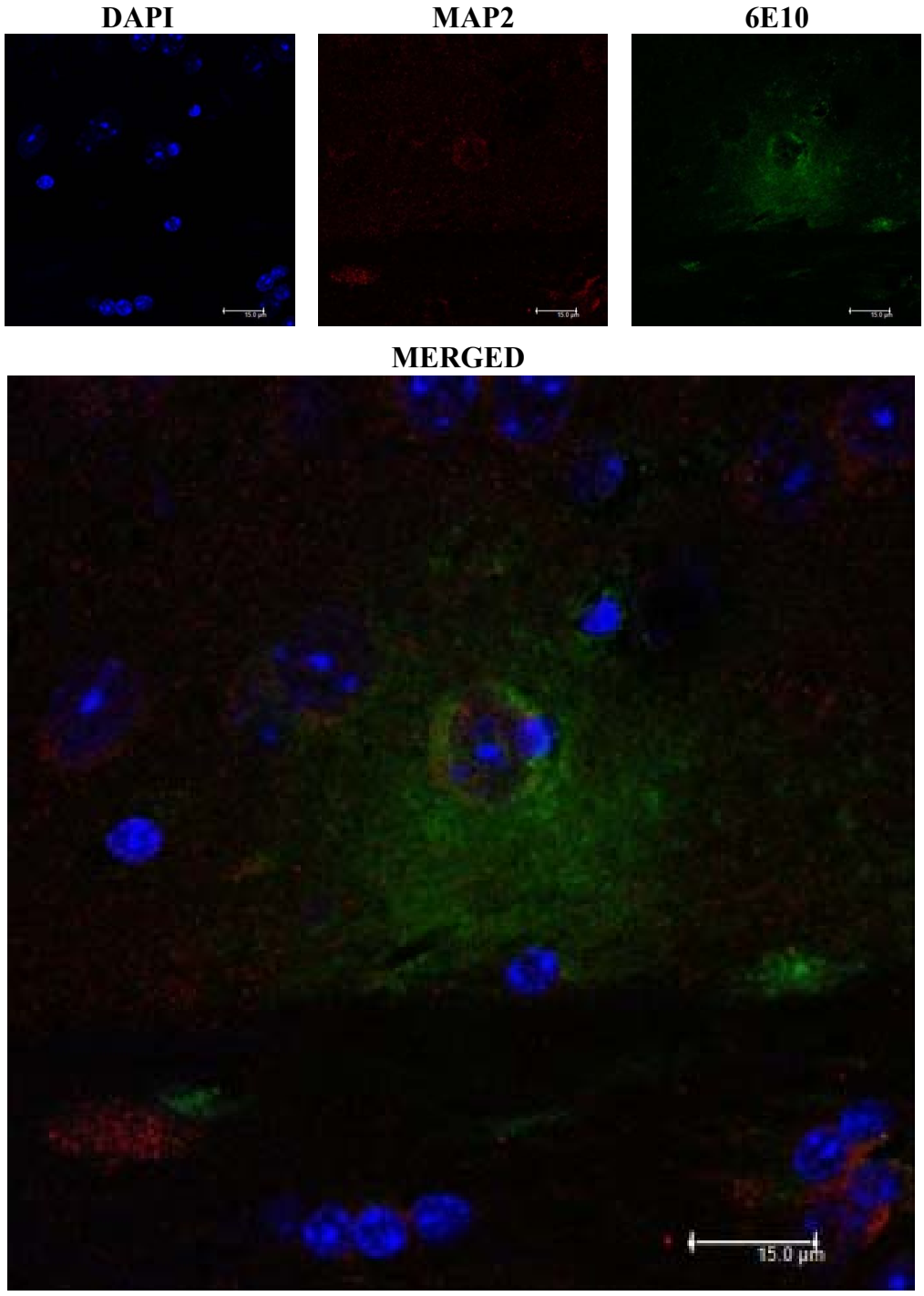


Figure 9B. Extra-cellular β-amyloid that surrounded neurons appeared to be secreted by neurons that contained higher amounts of β-amyloid than surrounding cells and had intact nuclei. Neurons were stained with MAP2. Beta-amyloid was stained with 6E10. Nuclear tissue was stained with DAPI. Scale bars are 15 µm.

Figure 9C. Cells that did not secrete β-amyloid underwent necrosis.

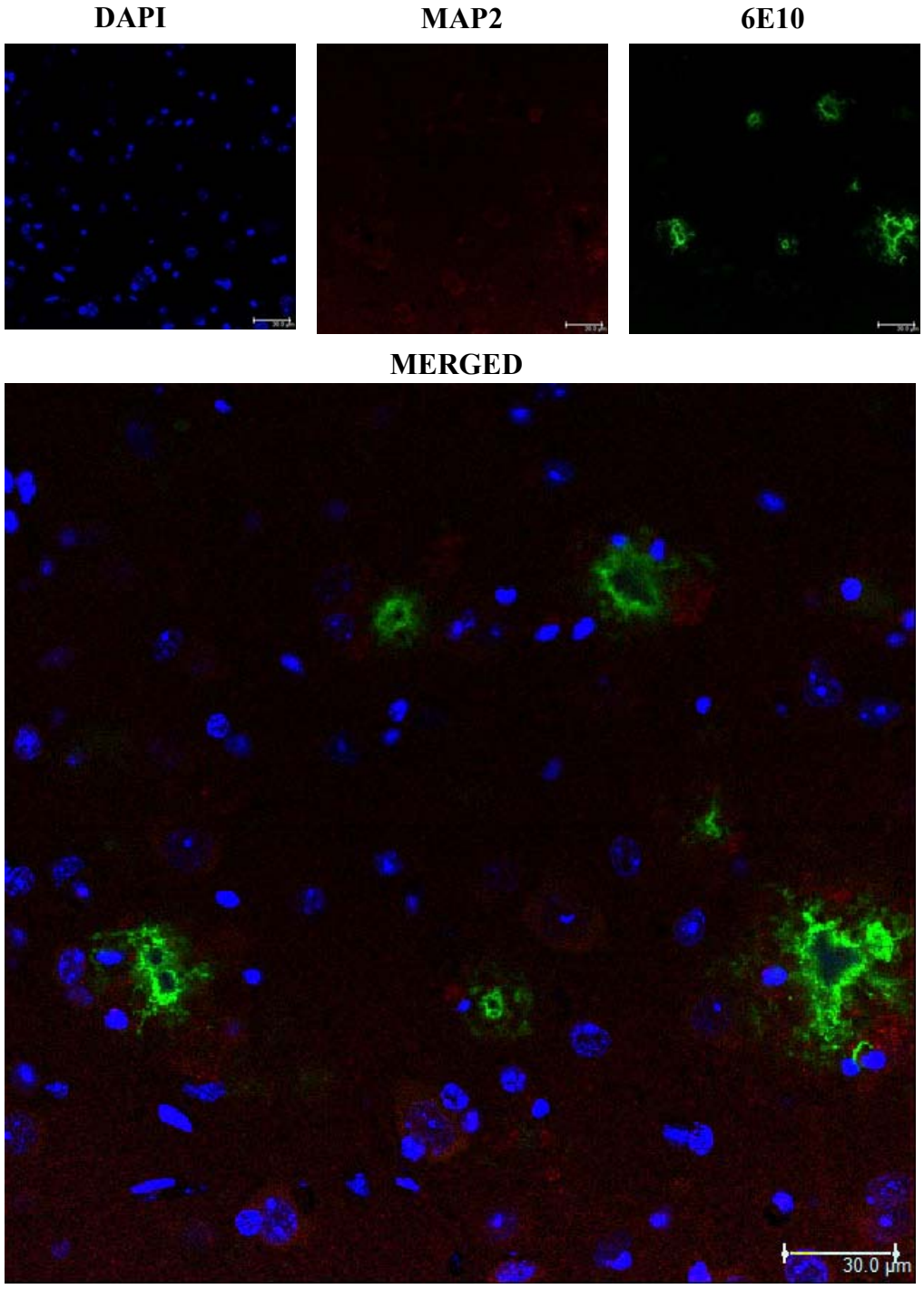


Figure 9C. Neurons that contained higher amounts of β-amyloid than surrounding cells appeared to undergo necrosis. Neurons were stained with MAP2. Beta-amyloid was stained with 6E10. Nuclear tissue was stained with DAPI. Necrotic cells had collapsed nuclei that spread 4-5 times the area of surrounding cells. Scale bars are 30 µm.

Figure 10A. Astrocytes did not co-localise with necrosis-associated or diffuse deposits of β-amyloid.

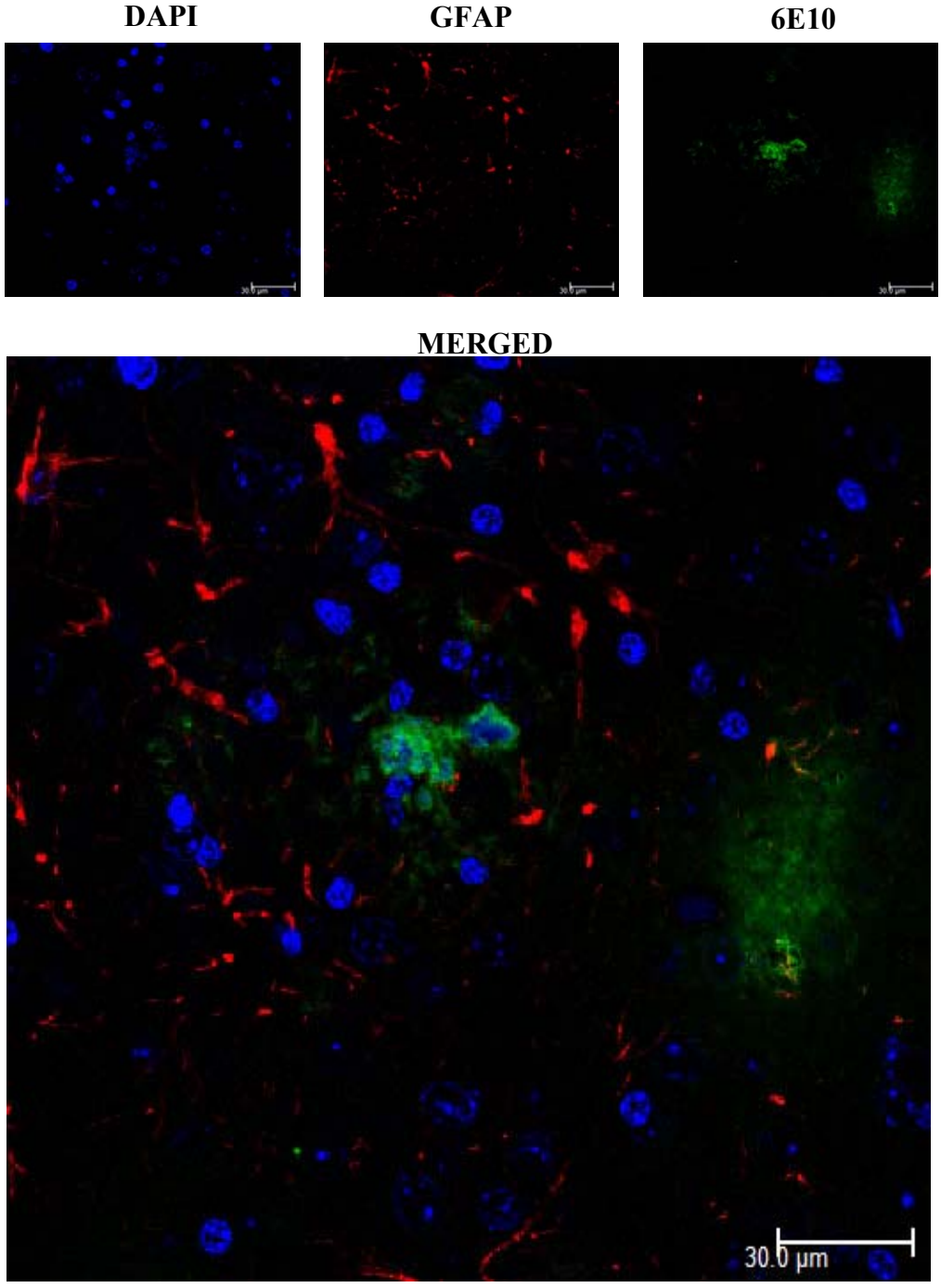


Figure 10A. Astrocytes had no preference for necrosis-associated or diffuse deposits of β-amyloid. Astrocytes were stained with GFAP. Beta-amyloid was stained with 6E10. Nuclear tissue stained with DAPI. Scale bars are 30 µm.

Figure 10B. Endfoot processes of astrocytes line the Blood Brain Barrier.

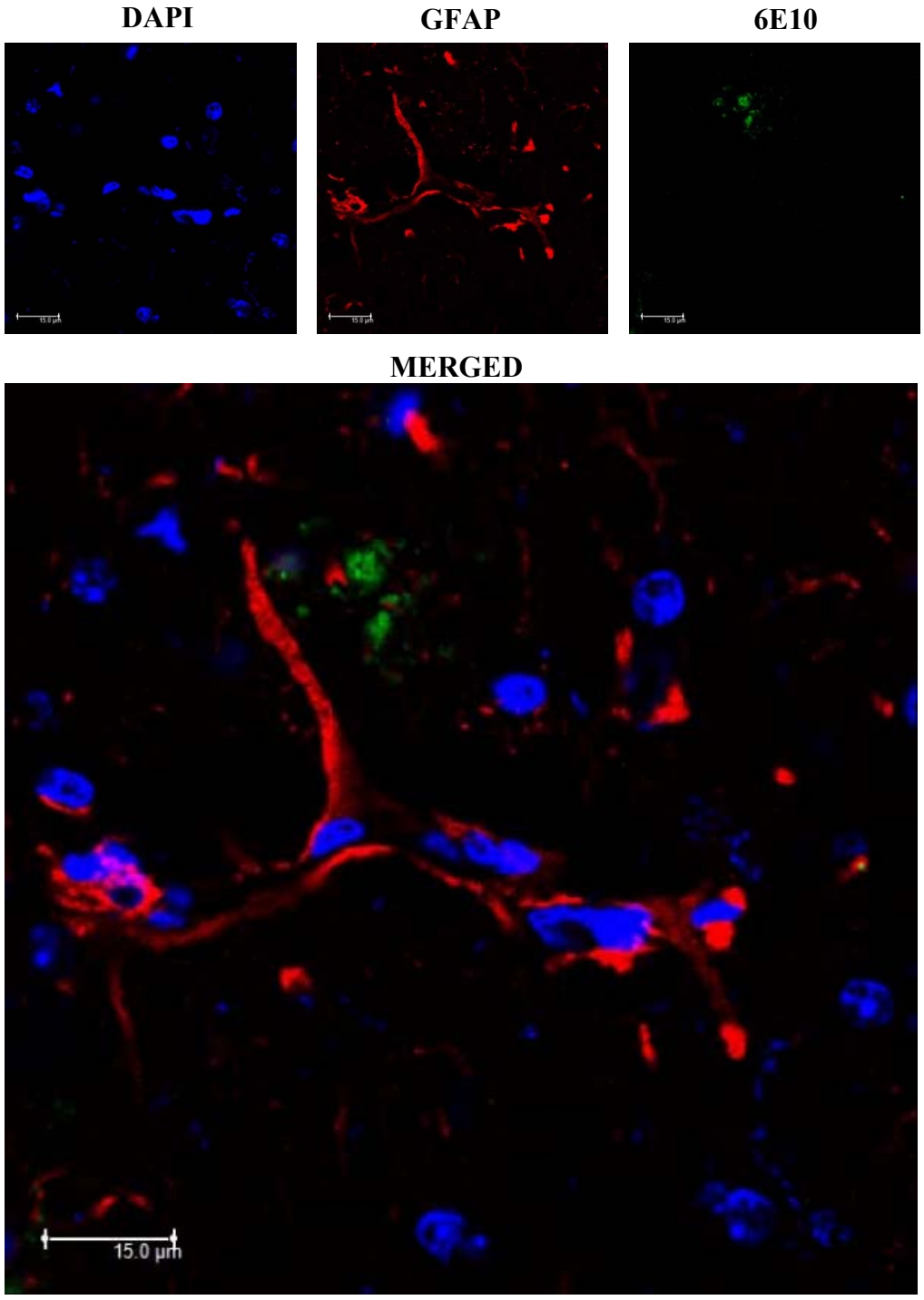


Figure 10B. Endfoot processes of astrocytes line the blood brain barrier. Astrocytes were stained with GFAP. Beta-amyloid was stained with 6E10. Nuclear tissue stained with DAPI. Scale bars are 15 µm.

Figure 10C. Amyloid was associated with possible breaks in endfoot processes that were associated with the Blood Brain Barrier.

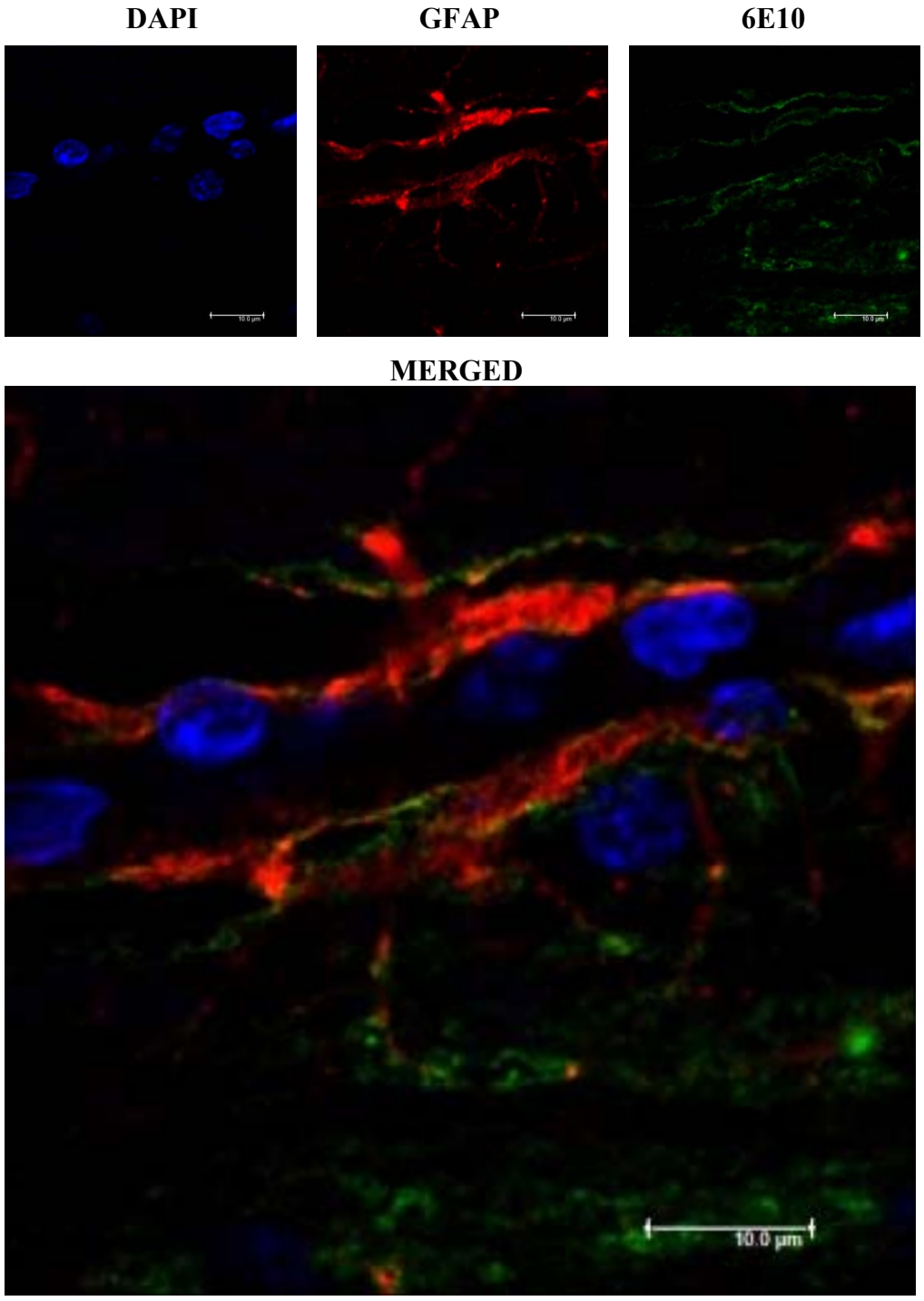


Figure 10C. Amyloid was associated with possible breaks in the blood brain barrier. Astrocytes were stained with GFAP. Beta-amyloid was stained with 6E10. Nuclear tissue stained with DAPI. Scale bars are 10 µm.

Figure 11A. Oligodendrocytes did not co-localize with necrotic cells and only occasionally co-localised with extracellular amyloid.

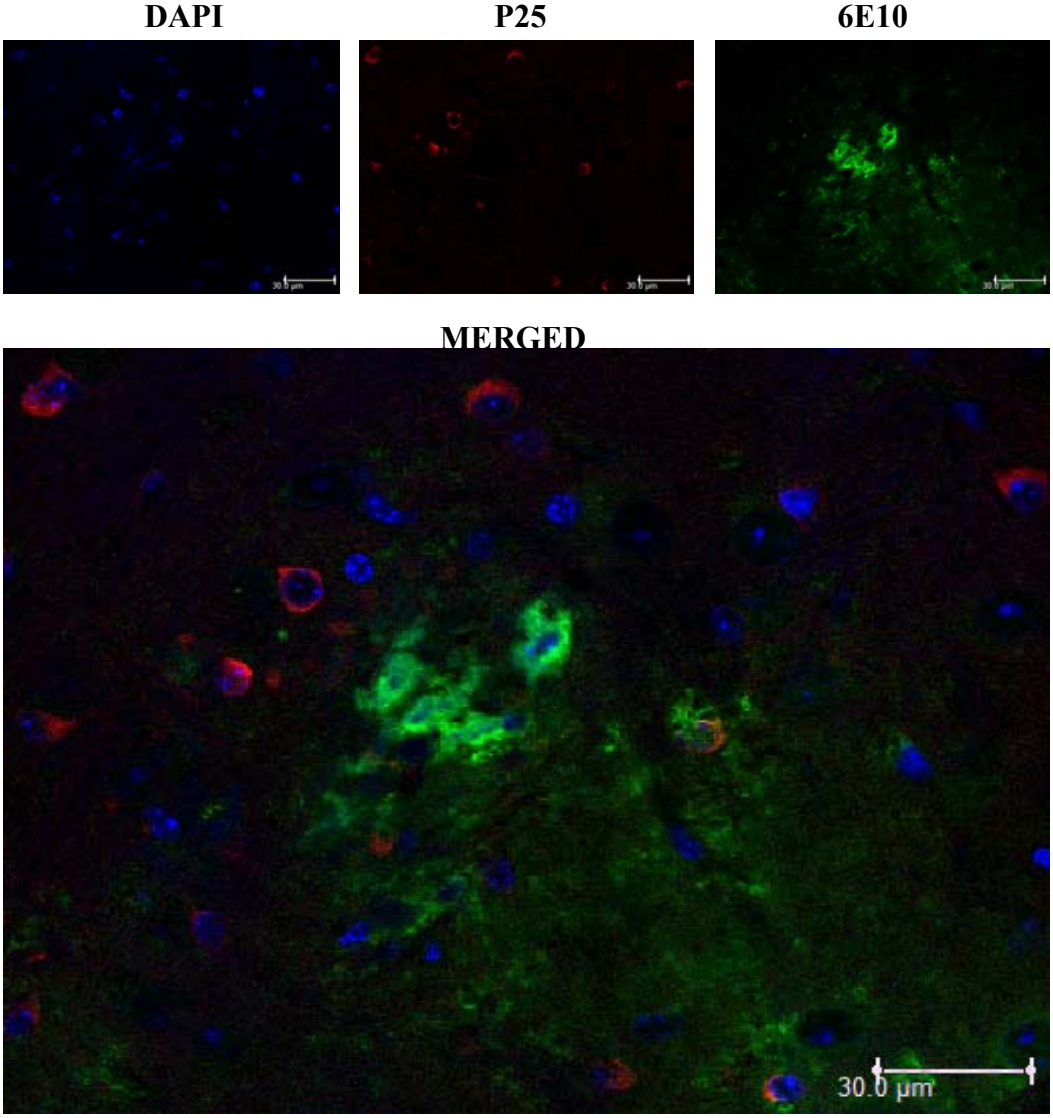


Figure 11A. Oligodendrocytes had intact nuclei and were not associated with necrotic cells or co-localise with amyloid. Oligodendrocytes were stained with P25. Beta-amyloid was stained with 6E10. Nuclear tissue was stained with DAPI. Scale bars are 30 µm.

Figure 11B. Oligodendrocytes did not co-localize with cells that secreted β-amyloid.

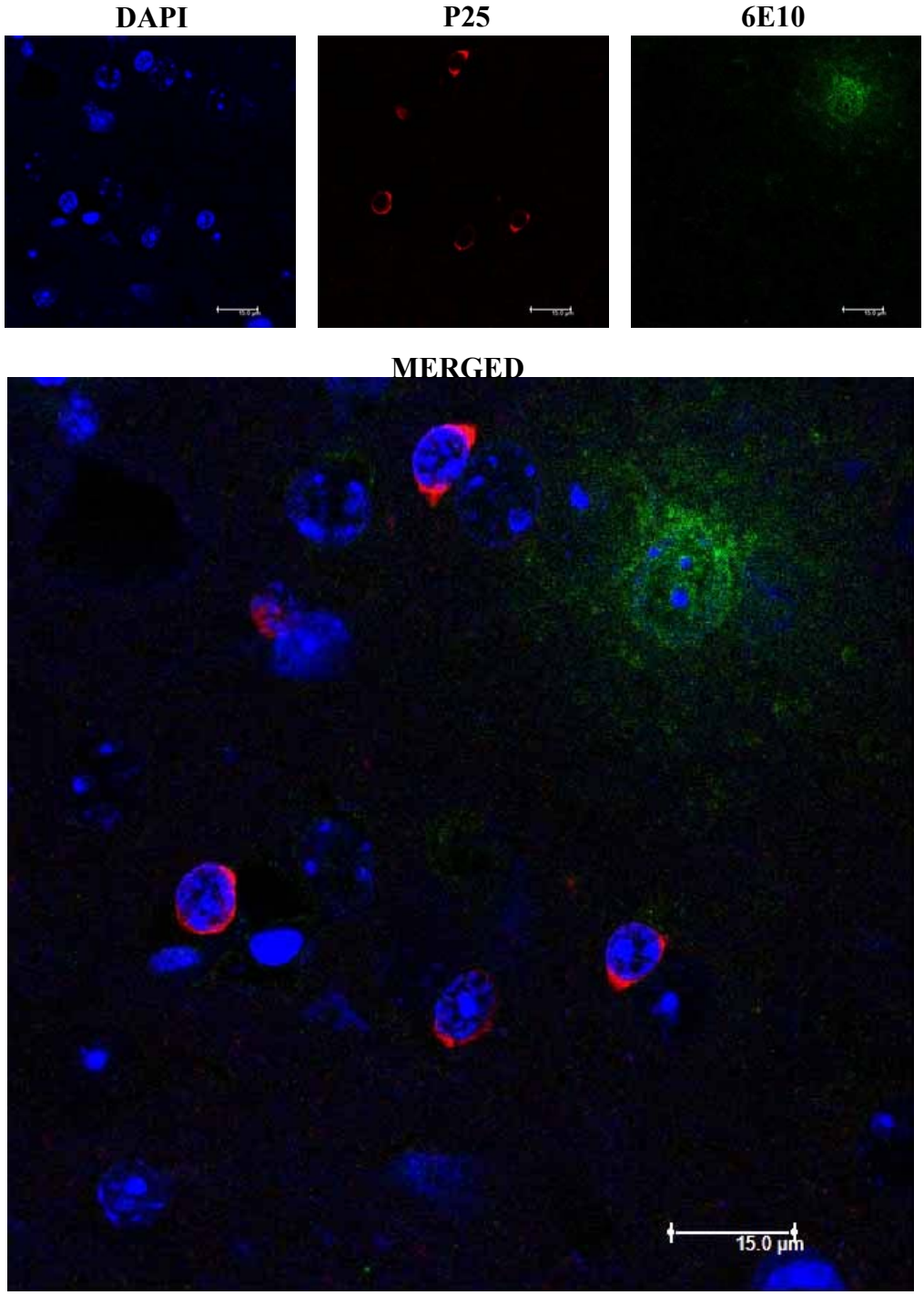


Figure 11B. Oligodendrocytes were not associated with cells that secreted β-amyloid. Oligodendrocytes were stained with P25. Beta-amyloid deposit types were stained with 6E10. Nuclear tissue was stained with DAPI. Scale bars are 15µm.

4.2.6.2. Beta-amyloid co-localisation with astrocytes, oligodendrocytes, microglia and neurons.

Cell populations that were associated with the different deposits of β-amyloid were viewed with confocal microscopy (Figure 9, Figure 10, Figure 11). Whilst microglial staining was unsuccessful, staining for neurons, astrocytes and oligodendrocytes revealed that each cell type co-localised differently with amyloid.

(i) Neurons.

As described in section 4.2.6.1. (p. 213), most neurons contained amyloid in low levels (Figure 9A). However, there were some neurons that either: had intact nuclei and secreted amyloid (Figure 9B), or appeared necrotic and contained high levels of intracellular amyloid (Figure 9C).

(ii) Astrocytes.

While some astrocytes co-localised with external deposits of amyloid, this was not consistent. There were many external deposits that did not contain astrocytes, despite there being multiple groups of astrocytes within 50 μm of the deposit (Figure 10A). Astrocytes did not co-localise with the necrotic cells, indicating that the astrocytes were not involved with the detoxification of necrotic cells that contained β-amyloid (Figures 10A).

As highlighted in section 4.2.6.1. astrocyte processes line the blood brain barrier (Figure 10B). These processes co-localised with β-amyloid deposits that were 57.65 ±4.89 μm across the centre, and extended 133.30 ±59.24 μm away from the blood vessel (Figure 10C). There appeared to be breaks in the astrocyte processes that co-localised with amyloid, which may be suggestive of a dysfunctional blood brain barrier. However, this study has not investigated blood brain barrier integrity, so this

was not determined. Furthermore, it was not determined whether this is clearance or invasion of β-amyloid from vascular sources across the blood brain barrier.

(iii) Oligodendrocytes.

Oligodendrocytes occasionally co-localised with the β-amyloid in the large diffuse deposits, but this did not happen frequently enough to confirm a relationship between the two (Figure 11A). While the oligodendrocytes associated with other cells that had intact nuclei, they never associated with cells that were undergoing necrosis (Figures 11A and 11B). Similarly, oligodendrocytes did not associate with intact cells that secreted β-amyloid (Figure 11B).

The effect of diet on cell population within the brains of 15 and 18 month old Amy mice was not characterised due to (i) the failure to produce successful staining for microglia; and (ii) the lack of interaction between astrocytes and oligodendrocytes with amyloid.

4.2.7. The effect of diet on deposit type in the brains of Amy mice.

As outlined in 4.2.6.1. (pp. 213-214), β-amyloid was present in low levels in all neurons (Figure 9A), and there were three other distinct β-amyloid deposit-types in the Amy mouse brains:

(i) Intracellular β-amyloid that was associated with necrotic cells.

These were small, bright β-amyloid deposits with a diffuse DAPI core. Whilst DAPI staining of intact nuclei is bright and structured, the DAPI cores that were associated with β-amyloid were diffuse, with little structure and spread over an area 3-4 times that of surrounding cells, which is suggestive of necrotic morphology (Figure 8A, Figure 9C).

These deposits will be referred to as "necrosis-associated deposits" for the remainder of this thesis.

(ii) Extracellular deposits of β-amyloid that were large and diffuse.

These were large, diffuse, extracellular deposits of β-amyloid that spread 92 ±8.94 μm and contained small intact cells (Figure 8B). These deposits will be referred to as "diffuse deposits" for the remainder of this thesis.

(iii) Extracellular deposits of β-amyloid that were associated with end foot processes that lined the blood brain barrier.

These were extracellular deposits that were smaller deposits than the diffuse deposits (57.65 ±4.89 μm), which associated closely with breaks in foot processes of astrocytes that appeared to line the blood brain barrier (Figure 8C, Figure 10D). These deposits will be referred to as "BBB-associated deposits" for the remainder of this thesis.

To characterise the effect of diet on deposit type in the brains of Amy mice, each deposit type was counted and expressed as a percentage of total deposits in 10 representative sections spanning 250 μm^2 from the brains of 15 month old (Table 8, Figure 11) and 18 month old Amy mice (Table 9, Figure 12).

There were no differences in the total number of deposits counted confirming that percentages of deposit types made were a true reflection of deposit populations in the brains of 15 month old Amy mice that were fed the AIN93-M diet, Oz-AIN diet or the Oz-AIN Supp diet (Table 8, Table 9).

4.2.7.1. The effect of diet on deposit type in the brains of 15 month old Amy mice.

There was no overall effect of diet on the percentage of necrosis-associated deposits or diffuse deposits in the brains of 15 month old Amy mice (Table 8, Figure 11). However, the percentage of BBB-associated deposits was affected by diet ($p<0.001$, Table 8, Figure 12). The percentage of BBB-associated deposits in the brains of 15 month old Amy mice that had been fed the Oz-AIN Supp diet was significantly higher than those in the brains of Amy mice that had been fed either the AIN93-M diet ($p<0.01$) or the Oz-AIN diet ($p<0.001$) (Table 8, Figure 12).

Further investigation lead to a comparison of the deposit-type population profile within each dietary group (Figure 13). Amy mice that were fed the AIN93-M diet had a similar profile of distribution as Amy mice that had been fed the Oz-AIN diet (Table 8, Figures 13A and 13B). Both dietary groups had significantly more necrosis-associated deposits than diffuse deposits ($p<0.0001$, Table 8, Figures 13A and 13B). Similarly, both diet groups had significantly more defuse deposits than BBB-associated deposits ($p<0.0001$, Table 8, Figures 13A and 13B), with negligible percentages of BBB-associated deposits. This indicates that diet-type does not affect deposit profile in the brains of Amy mice.

However, in the brains of Amy mice that were fed the Oz-AIN Supp diet, there was an equal distribution of each of the deposit types (Table 8, Figure 13C). It is likely that this was achieved through the increased amount of β-amyloid that was associated with the blood brain barrier.

Collectively, these results suggest that although overall diet-type did not affect deposit profile in the brains of 15 month old Amy mice, the nutrient supplements

were able to alter deposit distribution so that there were equal numbers of necrosis-associated, large, and BBB-associated deposits.

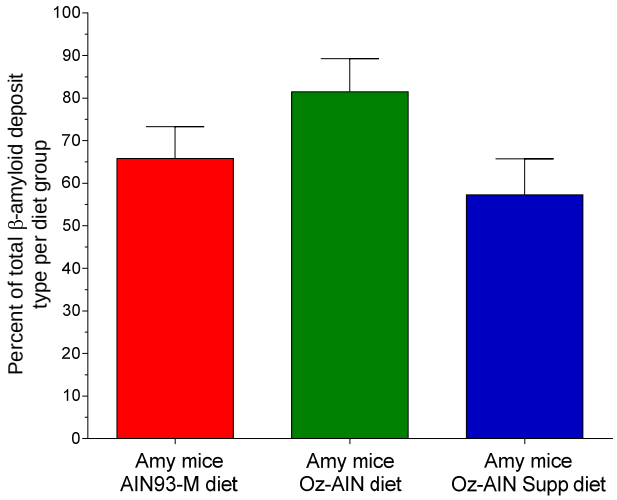
Table 8. Confocal counts and percentage of β-amyloid deposit types in the brains of 15 month old Amy mice.

	Amy mice AIN93-M diet	Amy mice Oz-AIN diet	Amy mice Oz-AIN Supp diet
Total β-amyloid deposits in 10 brain sections (mean ±SEM)	20.25 ±5.12	18.25 ±3.73	20.00 ±7.66
Necrosis associated β-amyloid deposit (% of total)	66.36 ±5.89	79.74 ±5.67	57.28 ±8.44
Large diffuse β-amyloid deposit (% of total)	28.64 ±6.00 $^{\Xi,\Theta}$	24.25 ±4.56 $^{\Xi,\Phi}$	38.83 ±8.83
BBB-associated β-amyloid deposit (% of total)	6.63 ±2.32 $^{\Xi,\chi}$	0.68 ±0.68 $^{\Xi,\chi}$	37.50 ±15.71

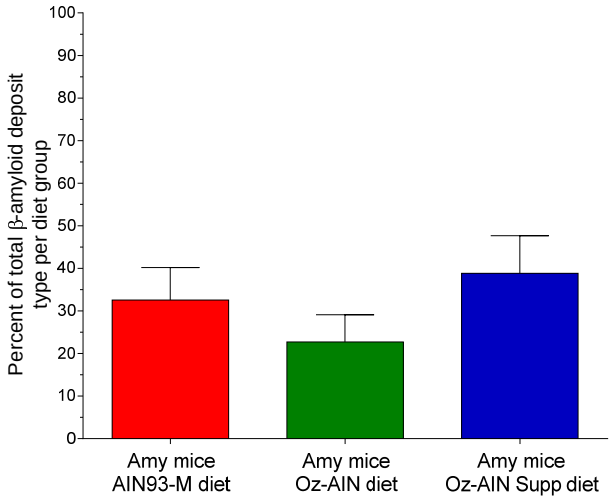
Percentage of necrosis-associated deposits, diffuse deposits and BBB-associated deposits in the brains of 15 month old Amy mice. All values are mean ±SEM. The percent of each deposit type differed significantly within dietary groups, and was detected with Bonferroni post tests. (Ξ) $p<0.0001$ relative to percentage of necrosis associated β-amyloid deposits within the same dietary group. (Θ) $p=0.008$, (Φ) $p=0.0004$ relative to percentage of BBB-associated within the same dietary group. The percentage of BBB-associated β-amyloid deposits differed significantly across dietary groups, and was detected with Bonferroni post tests. (χ) $p=0.02$ relative to BBB-associated deposits in the brains of Oz-AIN Supp diet mice.

Figure 12. The effect of diet on the percentage of necrosis-associated deposits, diffuse deposits, or BBB-associated deposits in the brains of 15 month old Amy mice.

12A. Necrosis-associated deposits



12B. Diffuse deposits



12C. BBB-associated deposits

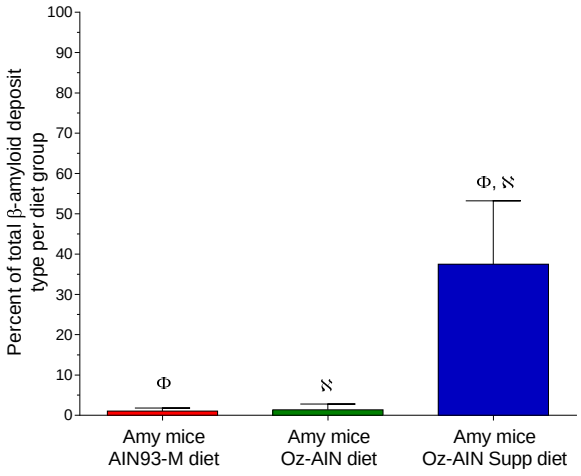


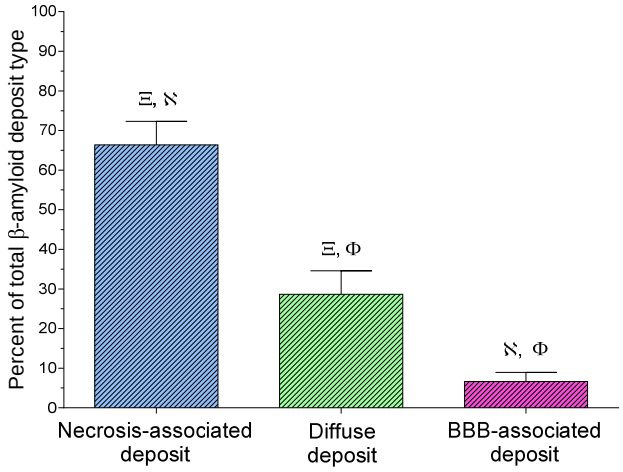
Figure 12A. The percentage of necrosis-associated deposits within the brains of 15 month old Amy mice that were fed either the AIN93-M diet (red bars, n=7), the Oz-AIN diet (green bars, n=12), or the Oz-AIN Supp diet (blue bars, n=12) were compared. Bars represent mean ±SEM.

Figure 12B. The percentage of diffuse deposits within the brains of 15 month old Amy mice that were fed either the AIN93-M diet (red bars, n=7), the Oz-AIN diet (green bars, n=12), or the Oz-AIN Supp diet (blue bars, n=12) were compared. Bars represent mean ±SEM.

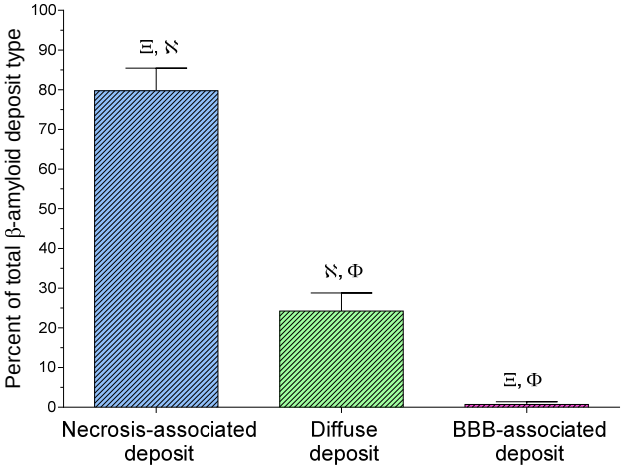
Figure 12C. The percentage of BBB-associated deposits within the brains of 15 month old Amy mice that were fed either the AIN93-M diet (red bars, n=7), the Oz-AIN diet (green bars, n=12), or the Oz-AIN Supp diet (blue bars, n=12) were compared. Bars represent mean ±SEM. Bars with matching symbols are significantly different with Bonferroni post tests. (ϰ) p=0.02. (Φ) p=0.02.

Figure 13. Beta-amyloid profiles in the brains of 15 month old Amy mice that had been fed the AIN93-M diet, the Oz-AIN diet or the Oz-AIN Supp diet.

13A. AIN93-M diet



13B. Oz-AIN diet



13C. Oz-AIN Supp diet

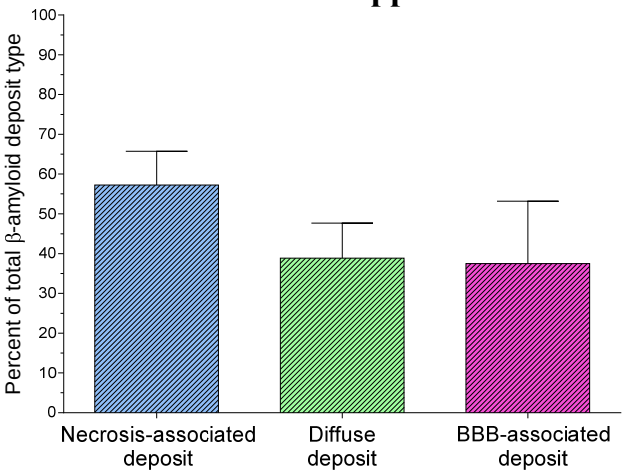


Figure 13A. The percentage of each deposit type in the brains of 15 month old Amy mice that had been fed the AIN93-M diet (n=7). Deposits were viewed under confocal microscopy and scored as either: (i) being associated with necrotic cells (necrosis-associated deposits, green bars), (ii) in large external deposits (diffuse deposits, red bars), or (iii) associated with blood brain barrier (BBB-associated deposits, yellow bars). Bars represent mean ±SEM. Bars with matching symbols are significantly different with Bonferroni post tests. (Ξ) $p<0.0001$. (ϰ) $p<0.0001$. (Φ) $p=0.008$.

Figure 13B. The percentage of each deposit type in the brains of 15 month old Amy mice that had been fed the Oz-AIN diet (n=6). Deposits were viewed under confocal microscopy and scored as either: (i) being associated with necrotic cells (necrosis-associated deposits, green bars), (ii) in large external deposits (diffuse deposits, red bars), or (iii) associated with blood brain barrier (BBB-associated deposits, yellow bars). Bars represent mean ±SEM. Bars with matching symbols are significantly different with Bonferroni post tests. (Ξ) $p<0.0001$.(ϰ) $p<0.0001$. (Φ) $p=0.0004$.

Figure 13C. The percentage of each deposit type in the brains of 15 month old Amy mice that had been fed the Oz-AIN Supp diet (n=4). Deposits were viewed under confocal microscopy and scored as either: (i) being associated with necrotic cells (necrosis-associated deposits, green bars), (ii) in large external deposits (diffuse deposits, red bars), or (iii) associated with blood brain barrier (BBB-associated deposits, yellow bars). Bars represent mean ±SEM.

4.2.7.2. The effect of diet on deposit type in the brains of 18 month old Amy mice

Diet did not affect the distribution of deposit type in the brains of 18 month old Amy mice (Table 9, Figure 14). The percentage of necrosis-associated deposits and the percentage of diffuse deposits did not differ between the brains of Amy mice that were fed either the AIN93-M diet or the Oz-AIN diet (Table 9, Figure 14). However, the 18 month old Amy mice that were fed the Oz-AIN Supp diet had no β-amyloid that was associated with the blood brain barrier (Table 9, Figures 14 and 15B).

Table 9. Confocal counts and percentages of β-amyloid deposits types in the brains of 18 month old Amy mice.

	Total deposits in 10 brain sections (mean ±SEM)	**Necrosis-associated deposit (% of total)**	**Diffuse deposit (% of total)**	**BBB-associated deposit (% of total)**
Amy mouse AIN93-M diet	61 ±6.70	75.69 ±4.57%	27.08 ±6.67% $^{\Phi}$	3.33 ±2.22% $^{\Phi,\Xi}$
Amy mouse Oz-AIN diet	58 ±8.47	68.51 ±12.41%	25.00 ±12.91% $^{\Delta}$	0.00 ±0.00%

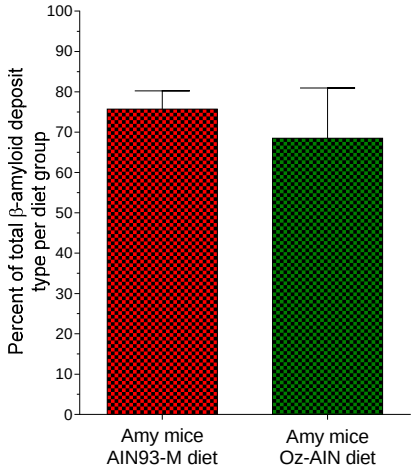
Percent of β-amyloid deposits that were associated with necrosis-associated deposits, diffuse deposits, and BBB-associated deposits. All values are mean ±SEM. The percent deposit type differed significantly with dietary groups, and was detected with Bonferonni's post tests. (Δ) $p=0.02$, (Φ) $p<0.0001$ relative to percentage of necrosis-associated deposits from the same diet group; (Ξ) $p=0.02$ relative to percentage of diffuse deposits from the same diet group.

A comparison of the β-amyloid deposit population profile within each group revealed similar profiles for deposit types in the brains of 18 month old Amy mice that had been fed either the AIN93-M diet or the Oz-AIN diet. There were significant differences between the percentage of necrosis associated deposits, diffuse deposits and BBB-associated deposits in the brains of 18 month old Amy mice that had been fed the AIN93-M diet ($p<0.0001$, Table 9, Figure 15A). The necrosis associated deposits were the most common (75.69 ±4.57), followed by diffuse deposits (27.08 ±6.67%), with relatively negligible levels of BBB-associated deposits (3.33 ±2.22%, Table 9, Figure 15A).

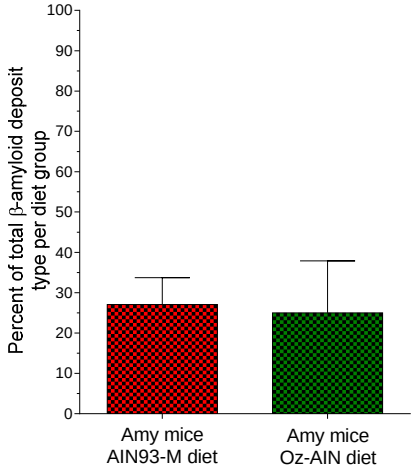
There was no β-amyloid associated with the blood brain barrier in the brains of 18 month old Amy mice that were fed the Oz-AIN diet. Necrosis associated deposits were significantly more abundant than diffuse deposits ($p=0.006$, Table 9, Figure 15B), which is similar to the profile observed in the brains of 18 month old Amy mice that had been fed the AIN93-M diet (Figure 15A).

Figure 14. The effect of diet on the percentage of necrosis-associated deposits, diffuse deposits, or BBB-associated β-amyloid deposits in the brains of 18 month old Amy mice.

14A. Necrosis-associated deposits



14B. Diffuse deposits



14C. BBB-associated deposits

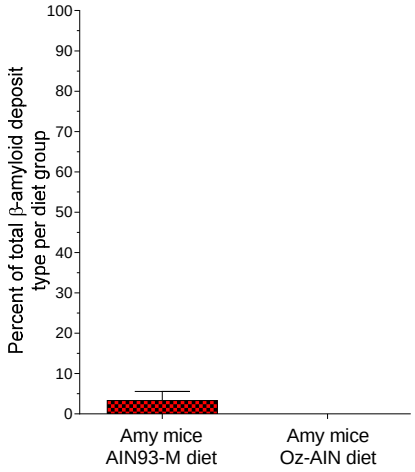


Figure 14A. The percentage of necrosis-associated deposits within the brains of 18 month old Amy mice that were fed either the AIN93-M diet (red checked bars, n=6) or the Oz-AIN diet (green checked bars, n=6) were compared. Bars represent mean ±SEM.

Figure 14B. The percentage of necrosis-associated deposits within the brains of 18 month old Amy mice that were fed either the AIN93-M diet (red checked bars, n=6) or the Oz-AIN diet (green checked bars, n=6) were compared. Bars represent mean ±SEM.

Figure 14C. The percentage of necrosis-associated deposits within the brains of 18 month old Amy mice that were fed either the AIN93-M diet (red checked bars, n=6) or the Oz-AIN diet (green checked bars, n=6) were compared. Bars represent mean ±SEM.

Figure 15A. Distribution of each of the β-amyloid deposit types through the brains of 18 month old Amy mice that were fed either the AIN93-M diet.

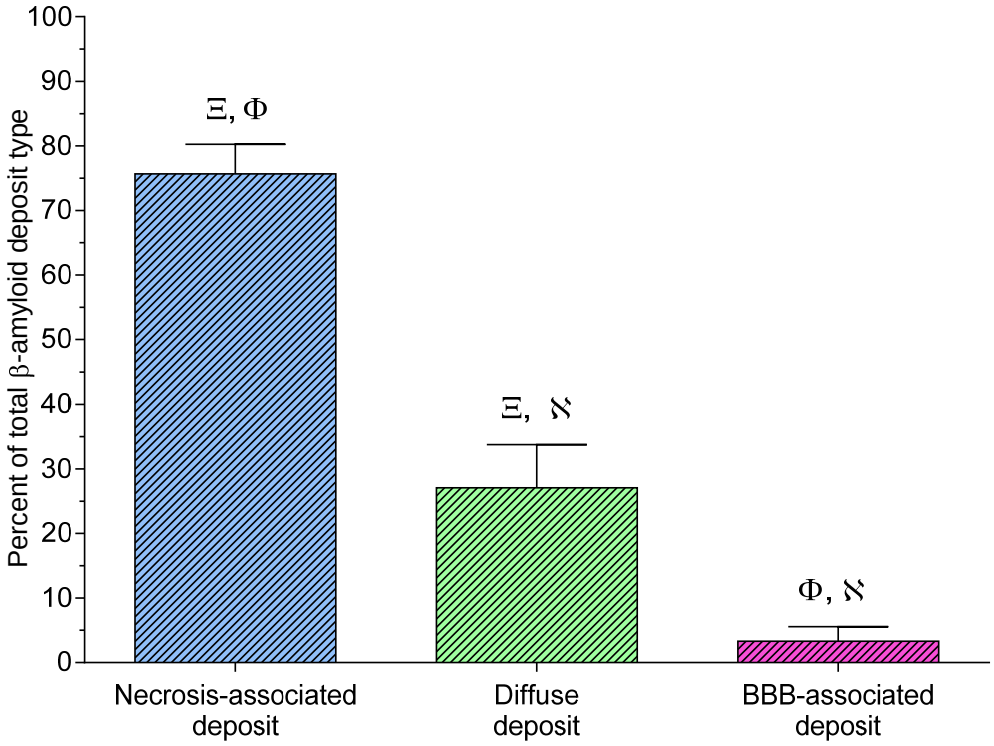


Figure 15A. The percentage of each deposit type in the brains of 18 month old Amy mice that had been fed the AIN93-M diet (n=6). Deposits were viewed with confocal microscopy and scored as either: (i) being associated with necrotic cells (necrosis-associated deposits, green bars), (ii) in large external deposits (diffuse deposits, red bars), or (iii) associated with blood brain barrier (BBB-associated deposits, yellow bars). Bars represent mean ±SEM. Bars with matching symbols are significantly different with Bonferroni post tests. (Ξ) $p<0.0001$. (Φ) $p<0.0001$. (ϰ) $p=0.02$.

Figure 15B. Distribution of each of the β-amyloid deposit types through the brains of 18 month old Amy mice that were fed either the Oz-AIN diet.

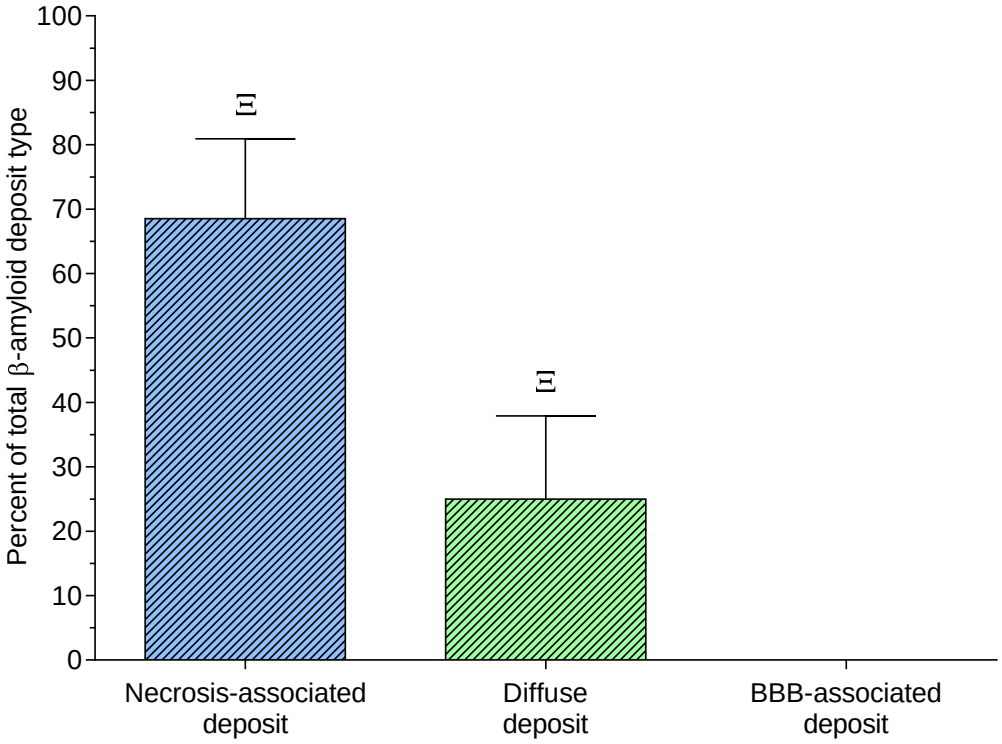


Figure 15B. The percentage of each deposit type in the brains of 18 month old Amy mice that had been fed the Oz-AIN diet (n=6). Deposits were viewed with confocal microscopy and scored as either: (i) being associated with necrotic cells (necrosis-associated deposits, green bars), (ii) in large external deposits (diffuse deposits, red bars), or (iii) associated with blood brain barrier (BBB-associated deposits, yellow bars). Bars represent mean ±SEM. Bars with matching symbols are significantly different with Bonferroni post tests. (Ξ) p=0.006

4.3. Discussion.

4.3.1. Bright field analysis

All Amy mouse brains had amyloid.

Long term high-fat diets increase amyloid precursor protein levels in normal mice [167]. It was therefore important to establish whether or not the Oz-AIN diet, which is also high in fat, could induce increased levels of β-amyloid in the brains of normal mice. However, no β-amyloid staining was observed in the representative samples of 15 or 18 month old normal mice, demonstrating that prolonged feeding with a sub-optimal diet did not increase β-amyloid deposition in the normal mice used in this study.

Low power brightfield microscopy confirmed that there was β-amyloid throughout the brains of 15 or 18 month old Amy mice. This is consistent with reports from others [374-376] and validates the use of this mouse model to demonstrate the effects of diet and age on β-amyloid deposition in the mouse brain.

Increased β-amyloid load, but decreased number of deposits in the brains of aging Amy mice that were fed the Oz-AIN diet may be explained by changes in the numbers of neurons.

The aging effect on amyloid deposition that has been reported here is consistent with reports from others [377]. However, despite having lower amyloid loads, the 15 month old Amy mice that were fed the Oz-AIN diet had more deposits than 18 month old Amy mice that were fed the same diet. This suggests that although β-amyloid load increases with age, the total number of β-amyloid deposits decreases with age, and this only happens in the brains of Amy mice that are fed the Oz-AIN diet.

Two possible causes for the decrease in β-amyloid deposit number are either: (i) decreased β-amyloid production; or (ii) increased β-amyloid clearance. In the event of either of these occurring, it would be expected that there would be some indication that β-amyloid processing or clearance mechanisms differed at 15 and 18 months. The number or small, medium and large β-amyloid deposits may provide some indication that these events are taking place. However, the percentage of small, medium and large deposits was the same in the brains of the 15 and 18 month old Amy mice that had been fed the Oz-AIN diet. This indicates that similar percentages of each type of deposit were being made at each age, and therefore β-amyloid processing and clearance mechanisms were probably similar for both age groups. Direct assessments of β-amyloid processing and degradation would be required to make this conclusion. This may be achieved by Western-blot analysis of β-secretase or amyloid precursor protein intracellular domain, both of which are involved in β-amyloid production [46]; or insulin-degrading enzyme and neprilysin, which are involved in β-amyloid clearance [378, 379].

Neurodegeneration and brain atrophy are two well established features of AD-type brains [380, 381]. The hippocampus, which is a region of the brain that plays a role in learning and memory, undergoes rapid neuronal loss in AD [382]. By the late stages of the disease, hippocampal neuronal populations can be almost 60% less than neuronal populations in normal aged brains [382, 383]. It is possible that the lower β-amyloid deposit counts in the brains of 18 month old Amy mice that were fed the Oz-AIN diet may be due to neuronal loss and less opportunity for β-amyloid production. Neuronal loss would mean that there was less amyloid precursor protein available for β-amyloid production as mice aged, and subsequently fewer deposits.

If the reduced total number of deposits was due to neuronal loss, the percentage of β-amyloid in each field of view would be greater relative to the overall amount of tissue in each section. This would explain the high β-amyloid burden in the brains of 18 month old mice that were fed the Oz-AIN diet, whose brains were lighter than those of mice fed the AIN93-M diet. It is possible that there was a higher degree of neurodegeneration in the brains of Amy mice that were fed the Oz-AIN diet, as these brains were significantly lighter than littermates fed the AIN93-M diet. Certainly, brain weight has been used by others to evaluate degree of neurodegeneration, and in human studies has correlated better with cognitive deficits than either amyloid plaques or neurofibrillary tangles [384]. Cellular counts of neurons and glial cells relative to the number of β-amyloid deposits could to be made to gain further support for this hypothesis.

Beta-amyloid burden does not indicate the type, size, toxicity or the effect of β-amyloid deposits; it reflects the total amount of β-amyloid in brain sections. Beta-amyloid burden can be the same between groups of mice irrespective of deposit size, irrespective of whether it is in an oligomeric or monomeric state [385, 386]. Hamaguchi *et al.* suggest that the dissociation between plaque burden and measurement of soluble β-amyloid may be due each measuring different forms of β-amyloid (ie. soluble or insoluble amyloid) and that the soluble β-amyloid measurements may also include small toxic intracellular β-amyloid deposits [385]. While there is a growing understanding that soluble β-amyloid is more toxic than the extracellular deposits of insoluble amyloid, the discrepancies between overall β-amyloid load and β-amyloid type indicate that there is still more to learn about β-amyloid deposition in the AD-type brain. Therefore, the β-amyloid deposits in the Amy mouse brains were characterised and compared.

Diet had an effect on β-amyloid deposit size at 15 and 18 months, due to total fat.

To quantify the differences in β-amyloid deposition in the Amy mice fed different diets, β-amyloid deposits were counted and scored as either small (<15 μm), medium (15-35 μm) or large (>35 μm) deposits (Figure 5).

The majority of deposits in the brains of all three dietary groups were small deposits, with large deposits being the least common deposit type. While there was no dietary effect on the number of small deposits or the number of medium deposits in the brains of 15 month old Amy mice, there was a clear dietary effect on the number of large deposits. The brains of 15 month old Amy mice that had been fed the AIN93-M diet had significantly fewer large β-amyloid deposits than those from Amy mice that had been fed either the Oz-AIN diet or the Oz-AIN Supp diet. This suggests that there is a dietary element that is similar between the Oz-AIN diet and the Oz-AIN Supp diet, and that is different to the AIN93-M diet, that enables accumulation of β-amyloid into larger deposits.

Table 10. Comparison of the energy content (kJ/g) and P:M:S ratio of the AIN93-M diet, Oz-AIN diet and Oz-AIN Supp diet.

	Total energy (kJ/g)	Carbohydrate (% energy)	Protein (% energy)	Fat (% energy)	P:M:S
AIN93-M diet	16.66	75.9%	14.1%	10.0%	3.7: 1.5: 1.0
Oz-AIN diet	20.11	46.7%	16.8%	33.0%	1.0: 2.4: 2.7
Oz-AIN Supp diet	20.98	47.4%	15.7%	36.9%	1.0: 1.7: 1.9

The AIN93-M diet contains optimal levels of macronutrients. However, the macronutrient content of the Oz-AIN diet and the Oz-AIN Supp diet had been

adjusted to reflect that of Australian women. As such, they were higher in total fat (at the expense of carbohydrate), with P:M:S ratios that favoured saturated and monounsaturated fat intake (Table 10). This suggests that the adjusted macronutrient content may have partially played a role in the higher numbers of large plaques in the brains of Amy mice that were fed the two high-fat diets.

Transgenic mouse models of AD have revealed strong relationships between dietary fat and β-amyloid deposition. Diets that are high in saturated fats or cholesterol increase β-amyloid neuropathology [191, 197, 198, 218, 387, 388]. However, the effect of polyunsaturated fats on β-amyloid deposition is not so clearly defined.

The ω-6 and ω-3 fatty acids have different roles in pathways that are associated with AD, such as inflammation. Arachidonic acid, which is an ω-6 fatty acid, promotes inflammation. On the other hand, the ω-3 fatty acid eicosapentaenoic acid, which competes with arachidonic acid for cycloxygenase active site, has anti-inflammatory properties [342, 389]. Owing to the relationship between inflammation and β-amyloid deposition, this suggests that ω-6 fatty acids increased β-amyloid deposition, whilst ω-3 fatty acids reduce β-amyloid deposition and arachidonic acid levels in mouse brain [342, 390].

However, different ω-3 polyunsaturated fats have different effects on β-amyloid expression. Docosahexaenoic acid decreases β-amyloid levels in AD-type mouse brains [86, 191, 391], whilst eicosapentaenoic acid has been demonstrated to increase β-amyloid deposition in AD mouse brains [342]. This suggests that when discussing the benefits of dietary polyunsaturated fatty acids on β-amyloid deposition, while the ω-3: ω-6 ratio needs to be considered, so to do the specific types of ω-3 that are present in diet [392-394].

Amtul *et al.* demonstrated that the protective effects of ω-3 fatty acid supplements are dependent on the diet to which they are added. They report that supplementing a low-fat diet with docosahexaenoic acid decreases amyloid in transgenic mice. However, supplementing a diet that is high in saturated fats with docosahexaenoic acid enhanced β-amyloid neuropathology to levels above that seen in mice that were fed high-saturated fat diets without supplements [395]. In the current study, the Oz-AIN Supp diet is a high-fat diet (39% kcal) and was associated with significantly greater numbers of large β-amyloid deposits than mice that were fed an ideal rodent diet, which is low in fat. This suggests that in the context of a high-fat diet, docosahexaenoic acid does not have a protective effect against β-amyloid deposition.

Alternately, these results could reflect a desensitisation to the beneficial effects of dietary docosahexaenoic acid after prolonged feeding. Optimal uptake of dietary docosahexaenoic acid into the brain occurs after 2-3 months feeding [220]. However, longer-term feeding with docosahexaenoic acid reduces brain uptake of docosahexaenoic acid across the blood brain barrier [396]. Therefore, it is possible that any beneficial effects of docosahexaenoic acid on amyloid deposition, blood brain barrier integrity or on cerebral function may have been lost by the time mice were 15 months old. To confirm this, it would be necessary to study amyloid deposition and diffusion of dietary docosahexaenoic acid across the blood brain barrier in young mice as well as 15 month old mice.

These results may also be interpreted as indicating that total fat content, not fat type, alters β-amyloid deposition in the Amy mouse brain. The Oz-AIN diet and Oz-AIN Supp diet both had a high total fat content (33.0% and 36.9% respectively). However, their P:M:S ratios and ω-3: ω6 ratios were different. The Oz-AIN diet has a P:M:S ratio that favoured saturated fats (1.0: 2.4:2.7) and had an ω-6:ω-3 ratio that was well

in favour of ω-6 PUFA's (10.0:1.0). This is similar to other diets that promote amyloid neuropathology [218]. The Oz-AIN Supp diet, on the other hand, had a more equal P:M:S ratio (1.0:1.7:1.9) and a well balanced ω-6:ω-3 ratio of 1.4:1.0. This is similar to diets that have been demonstrated to prevent or delay AD neuropathology [157, 296, 329]. Yet both the Oz-AIN diet and the Oz-AIN Supp diet were associated with greater percentages of large β-amyloid deposits than the AIN93-M diet. This suggests that total fat content, not fat type, increases β-amyloid neuropathology.

Julien *et al.*, demonstrated that high dietary saturated fat, and not imbalanced ω-3: ω-6 intake, increases β-amyloid deposition. Julien *et al.* fed mice a (i) low-fat diet (5%), (ii) low-fat diet (5%) with a low ω-3: ω-6 ratio (0.01), or (iii) a high-fat diet (60%) with a low ω-3: ω-6 ratio (0.01). They report that while imbalanced ω-3 fatty acids and high saturated fat intake were both associated with increased tau deposition, only mice that were fed the high-fat diet had increased soluble and insoluble β-amyloid levels [388].

It is possible that, in the current study, the high-saturated fat content of the Oz-AIN Supp diet lead to enhanced oxidation of docosahexaenoic acid after it had been ingested. This may have increased the inflammation and oxidative stress that is already associated with aging and AD . This hypothesis could be tested by assessing mouse brains for hexanonyl lysine and propanoyl lysine, which are indicative of ω-6 and -3 fatty acid oxidation, respectively [397].

4.3.2. Confocal analysis.

Confocal microscopy confirmed that most neurons contained amyloid. This is consistent with observations by others who report that neurons contain low levels of β-amyloid that does not appear to be toxic [398, 399]. At low levels β-amyloid may

have a role in neuroprotection [15, 400], neurogenesis [401, 402], synaptic plasticity and ion-channel function and expression [403-405]. However amyloid, in either a soluble, non-aggregated state or as insoluble, aggregated deposits is associated with oxidative stress and inflammation. The inflammatory response that is initiated by β-amyloid leads to microglial activation, cytokine and p38MAPK release, neuro-toxicity, apoptosis and programmed cell death [406-408].

Beta-amyloid deposits that were associated with necrotic cells were the most common deposit-type observed.

The finding that there were cells with necrotic morphology that co-localised with bright β-amyloid deposits, suggested that β-amyloid may play a role in necrosis in the Amy mouse brain.

While there have been some reports of necrosis in AD [409], most of the current literature attributes neuronal cell death in AD to apoptosis. It is consistently reported that β-amyloid increases intracellular levels of oxidative stress [407] and this leads to neuronal damage, apoptosis and programmed cell death [410]. However, in the current study, none of the diffuse DAPI deposits or the bright β-amyloid deposits co-localised with markers for apoptosis such as Caspase 3. This supports the conclusion that the diffuse DAPI staining that was associated with bright β-amyloid deposits represented cells undergoing necrosis, and not apoptosis.

Large diffuse β-amyloid deposits did not appear to be toxic.

Some cells contained higher levels of amyloid than others, and were surrounded by light, diffuse amyloid deposits. It is suggested here that this was the source of the larger, diffuse extracellular deposits. Potentially, neurons secrete β-amyloid once intracellular amyloid levels reach a certain thresh-hold. This may be protective

mechanism against build up of intracellular amyloid, as cells that appeared to be secreting amyloid had intact nuclei.

The neuroprotective role of secretion of β-amyloid and amyloid precursor protein has been suggested by other research groups [411, 412]. Perez-Gonzalez *et al.* report that exosomes in the brains of two different AD mouse models, but not normal mice, contain amyloid precursor protein and the β-carboxyl terminal fragment. They suggest that this exosomal secretion may be both neuroprotective and neurotoxic. Exosome secretion may be beneficial to the neuron as it allows release of neurotoxic β-amyloid and prevents intracellular accumulation. However, exosome secretion may also be neurotoxic, as it provides the opportunity for extracellular build up of toxic β-amyloid [411].

It is possible that the diffuse β-amyloid that surrounded cells containing higher levels of β-amyloid was actually being absorbed into cells, rather than being excreted. However, such an event is unlikely, owing to the fact that intracellular build up of β-amyloid is neurotoxic. Furthermore, it was never confirmed whether or not there was β-amyloid moving into or out of neurons, as the current study had not been designed to make such assessments. This would be beneficial, as it may provide some insight into the source of the diffuse extracellular deposits.

The small intact cells within the larger diffuse deposits that were observed in this study may have been involved in clearing β-amyloid. Oligodendrocytes are characteristically smaller than other glial cells, suggesting that they may have been the cells within the diffuse deposits [413-415]. However, under confocal microscopy, co-localisation between oligodendrocyte markers and the nuclei in the diffuse deposits did not occur frequently enough to demonstrate a clear relationship between oligodendrocytes and the external diffuse β-amyloid deposits. Alternately, the cells in

the diffuse deposits may have been microglial cells. This is more likely, as microglial activation plays a key role in the amyloid-mediated inflammatory response [406, 408]. Unfortunately, while with the primary antibody that was used for microglial cells works well in human tissue, it was not very effective on the mouse tissues used in the present study and results were hard to interpret. This does not necessarily mean that the cells in the diffuse deposits are not microglia. To confirm if these events have occurred, staining needs to be repeated with other antibodies against microglial markers that have been successfully demonstrated in for microglia in mice [416], such as the ionised calcium-binding molecule 1 (Iba1) (Ab15690, Abcam, Australia).

All nuclei within the diffuse deposits were well structured and intact. This suggests that they were not under any stress and that the extracellular β-amyloid in large diffuse deposits was not toxic to these cells.

It is proposed herein that most neurons in the Amy mouse brain produce amyloid and are able to maintain some level of homeostasis. However, some neurons are unable to clear β-amyloid as fast as they produce it. A small proportion of these neurons have the ability to secrete the excess β-amyloid. Most of the neurons that expressed high levels of β-amyloid retained the β-amyloid, which built up to toxic levels and led to necrosis (Figure 16).

This is not an entirely new concept. Early hypotheses of AD acknowledge that loss of β-amyloid homeostasis leads to increased β-amyloid levels [66]. It has recently been shown that β-amyloid is cleared via proteolysis in late endosomes. However, high levels of β-amyloid can overwhelm proteolytic machinery resulting in an accumulation of intracellular β-amyloid and compromised cellular function [19]. The current study adds to this by demonstrating that the accumulation of intracellular β-amyloid led to necrosis.

Figure 16. Formation of large diffuse and necrosis-associated β-amyloid deposits.

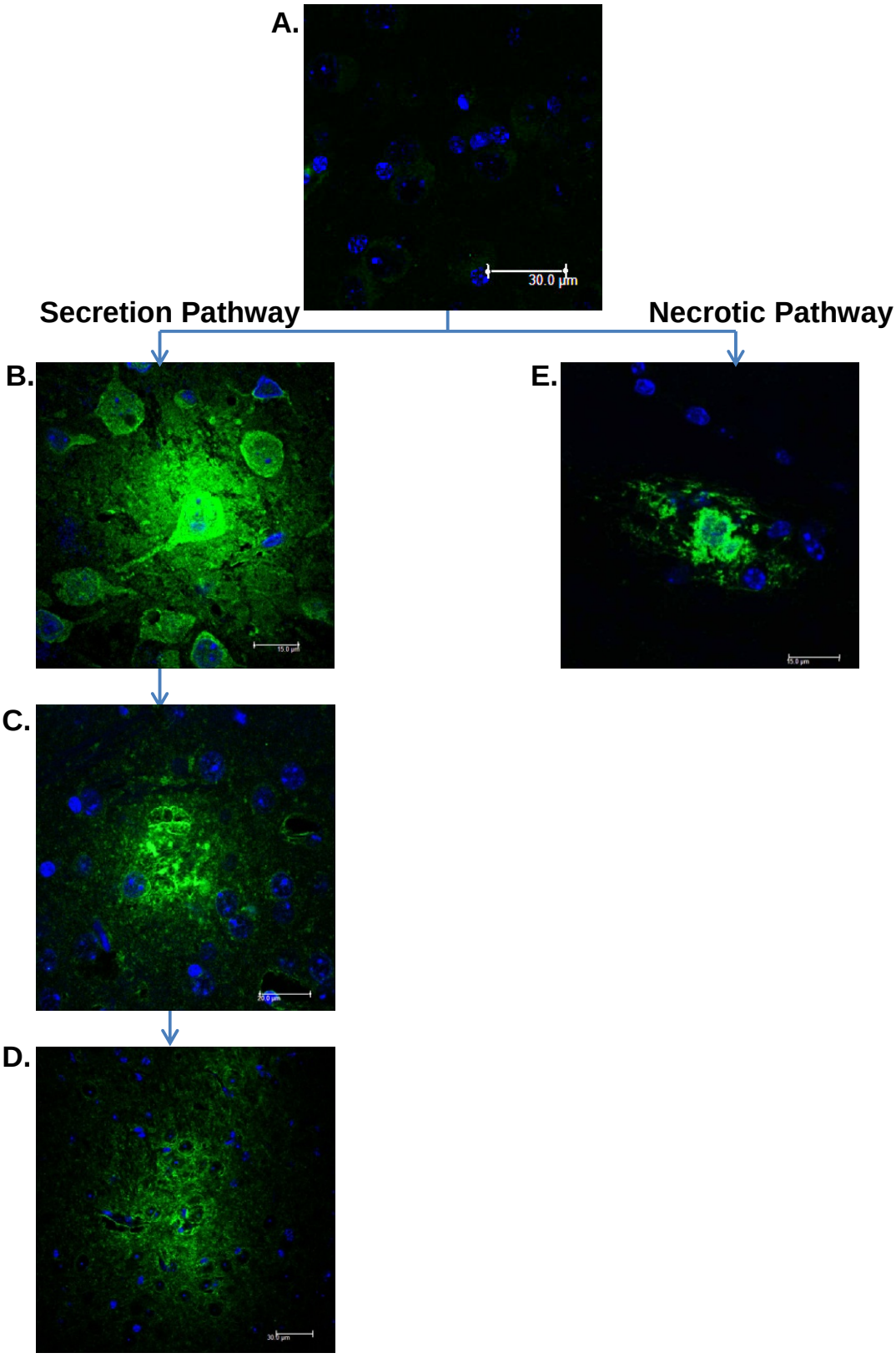


Figure 16. Formation of large diffuse and necrosis associated deposits.
(**A**) Neurons normally contain intracellular β-amyloid at low levels. (**B**) When β-amyloid levels become too high the neuron begins to secrete β-amyloid into the surrounding extracellular space. This does not appear to be toxic, as all cells maintain intact nuclei. (**C**) The extracellular β-amyloid then forms a small diffuse deposit.
(**D**) Beta-amyloid secreted from neurons build up into large, extracellular diffuse deposits of β-amyloid. (**E**) Alternately, accumulation of β-amyloid can cause increased stress on the cell, which can not for whatever reason, secrete the excessive amyloid. The cell then undergoes necrosis.

Beta-amyloid that was associated with the Blood Brain Barrier.

A third, and rarer form of β-amyloid deposit, was associated with the Blood Brain Barrier. These deposits had cores that were 57.65 ±4.89 μm across the widest point and were surrounded by more diffuse β-amyloid that extended 133.30 ±59.24 μm from the blood vessel (Figure 10C).

Beta-amyloid deposits along cerebrovascular walls are a common neuropathology associated with AD and occurs in 78%-98% of AD [417]. Beta-amyloid deposits between vascular smooth muscle cells lead to disrupted perivascular drainage and blood brain barrier dysfunction [418]. Amyloid-induced damage to the blood brain barrier is not confined to its effects on vascular smooth muscle cells or endothelial cells, but also effects support cells such as astrocytes. Winkler *et al*. reported that when β-amyloid is confined to the vessel wall, astrocyte endfeet remain intact and associated with the vessel wall. However, when β-amyloid accumulates in the parenchyma endfeet lose their association with vessel walls, leading to blood brain barrier dysfunction and rupture [419]. This is similar to more recent reports that in the presence of diffuse β-amyloid deposits, astrocyte endfeet lose contact with vessel walls [420].

In the current study, the blood brain barrier was identified using confocal microscopy by the astrocytes processes that encase the endothelial layer forming the blood brain barrier (Figure 10C). The astrocyte processes that formed the blood brain barrier co-localised with small, extracellular, diffuse deposits that spanned 57.65 ±4.89 μm along their longest point (Figure 10D). These BBB-associated deposits were the most infrequent form of deposit observed, but were distinct as they were always associated with what appeared to be breaks in the blood brain barrier (Figure 10D).

The association between β-amyloid deposits and breaks in the blood brain barrier indicates that β-amyloid may be moving across the blood brain barrier. Beta-amyloid movement across the blood brain barrier is bi-directional. Clearance of β-amyloid from the brain into the blood occurs via the low-density lipoprotein receptor 1 (LRP1), whilst uptake from the blood into the brain is mediated by the receptor for advanced glycation end-products (RAGE). Enhanced blood brain barrier permeability to β-amyloid has previously been demonstrated to play a role in AD pathogenesis [421]. The present study had not been designed to investigate the direction of movement of β-amyloid across the blood brain barrier. It is possible that the β-amyloid that was associated with the blood brain barrier was representative of invasion of β-amyloid (blood-to-brain movement), clearance of β-amyloid (brain-to-blood movement), or both.

Cerebral β-amyloid binds to LRP1 on the endothelial cell membrane, and undergoes rapid clearance from the brain into the plasma [422-425]. Soluble LRP1 acts as an β-amyloid "sink" and takes up 70%-90% of β-amyloid that has crossed the blood brain barrier, preventing free β-amyloid from crossing back into the brain [422]. Once bound to soluble LRP1, β-amyloid can be transported to the liver, where it is systemically cleared via LRP1 [426].

Just as the liver plays a role in clearance of amyloid, it also has the ability to produce β-amyloid [425, 427-429]. Amyloid precursor protein is expressed in high levels in the liver and the brain, and amyloid precursor protein processing in the liver is similar to that in the brain [430]. Amyloid precursor protein undergoes proteolysis and is cleaved by γ-secretase to produce β-amyloid [428]. Beta-amyloid is then transported through the blood stream by Apolipoprotein J or Apolipoprotein B, where it can cross the blood brain barrier by RAGE mediated mechanisms [427, 429, 431-433]. Free β-

amyloid in the plasma can also cross the blood brain barrier. These processes of β-amyloid movement between the brain and the liver are outlined in Figure 17.

Amyloid can also have a direct effect on blood brain barrier function. Circulating β-amyloid 40 increases reactive oxygen species in endothelial cells that line the blood brain barrier, which decreases electrical resistance of endothelial cells, causing disruption to tight junctions along the blood brain barrier [434].

The observation that β-amyloid was associated with the blood brain barrier gives rise to a barrage of questions. First, is this representative of invasion, clearance or both? Owing to the fact that the present study does not report any differences in total β-amyloid in the brains of Amy mice that were fed different diets, it is not likely that these deposits are *only* clearance or *only* invasion, but it is more likely to represent a combination of the two. This suggests that not all β-amyloid at the blood brain barrier would be toxic. Therefore, it needs to be determined whether or not the breaks in astrocyte endfeet reflect dysfunctional blood brain barrier. This could be achieved through immunohistochemical techniques to visualize astrocyte endfeet (with GFAP, as used here) and the endothelial proteins claudin-5 (which is found in all endothelial cells) and claudin-3 or claudin-11 (which are both associated with neurological disorders) [435]. This would enable co-localisation between astrocytes and markers for breaks in endothelial tight junctions, confirming that the breaks in the astrocyte endfeet observed here reflect breaks in the blood brain barrier. If these breaks are reflective of a dysfunctional blood brain barrier, then measurements of plasma levels of free β-amyloid and β-amyloid bound to lipoproteins such as ApoE, could provide some indication as to direction of β-amyloid across the blood brain barrier.

Beta-amyloid has been demonstrated to cause toxicity through increased oxidative damage and monocyte infiltration across the blood brain barrier [434, 436]. This

suggests that the β-amyloid that was associated with the blood brain barrier in the current study may also have had the potential to increase monocyte infiltration and increase inflammation in the brains of Amy mice. This, however did not appear to be the case. The brains that contained the most BBB-associated β-amyloid deposits were the brains from Amy mice that had been fed the nutrient supplements. There did not appear to be an up-regulation of activated astrocytes in the brains of Amy mice that had been fed the Oz-AIN Supp diet compared to other mice, as would be the case if there were higher levels of inflammation [437, 438]. There are two possible reasons for this. Firstly, the amount of inflammation brought about by monocyte infiltration may have been negligible compared to the degree of inflammation that was caused by neurons that were undergoing necrosis. Secondly, the Oz-AIN Supp diet contained ω-3 fatty acids, which are recognised for their powerful anti-inflammatory properties. Therefore, had there been a rise in cerebral inflammation, this may have been countered by the high levels of dietary ω-3 fatty acids. Further analysis of the brains from Amy mice that were fed the Nut Supp diets needs to be carried out before either of these conclusions can be made. First and foremost cerebral inflammation needs to be assessed in the brains of Amy mice that were fed the AIN93-M, Oz-AIN or the Oz-AIN Supp diet to determine whether or not diet alters inflammation in this mouse model. Correlations could then be made between the levels of inflammation and the frequency of necrosis-associated deposits or BBB-associated deposits. This would provide insight as to whether either of the β-amyloid pathologies was associated with increased inflammation.

Figure 17. Beta-amyloid invasion and clearance across the Blood Brain Barrier.

Figure 17. Beta-amyloid can be transported out of the brain via low-density lipoprotein receptor 1 (LRP1). The free-soluble β-amyloid is then taken up by soluble LRP1 (sLRP1) and transported through the blood system to the liver, where it is taken up by hepatic cells and broken down. Alternately, β-amyloid is synthesised in the liver, and can be transported on ApoJ or ApoB back to the brain, where it crosses the blood brain barrier via the receptor for advanced glycation end-products (RAGE). Alternately, free plasma β-amyloid can diffuse across the blood brain barrier.

The effect of diet and nutritional supplementation on β-amyloid deposit type as viewed with confocal microscopy.

The Amy mice that were fed the Oz-AIN Supp had a different ratio of necrosis associated deposits: large diffuse deposits: BBB-associated deposits compared to Amy mice that were fed the AIN93-M or the Oz-AIN diet. The change in deposit profile in the brains of mice that were fed the Oz-AIN Supp diet may have been due to either a decrease in necrosis associated deposits or an increase in BBB-associated deposits.

It was not determined whether the β-amyloid that was associated with the blood brain barrier was a reflection of invasion or clearance of amyloid. It is also unclear whether or not the BBB-associated β-amyloid deposits are toxic. The increased levels of folate in the Oz-AIN Supp diet may potentially reduce necrosis, on the other hand, the increase in total fats may have altered β-amyloid association with the blood brain barrier. Both possibilities are discussed further below.

Folate supplements may have reduced the number of necrosis associated deposits.

Dietary folate crosses the blood brain barrier where it can play a role in homosyteine metabolism, DNA damage repair and neuronal survival [439-441].

There is increasing evidence that elevated homocysteine plays a role in neurodegenerative disorders such as AD [287, 439, 442]. Build up of extra-cellular homocysteine in the central nervous system can lead to excito-toxicity through prolonged activation of NMDA receptors, and damages DNA, which triggers apoptosis [439]. Metabolism and clearance of homocysteine requires folate as a methyl group donor for the conversion of methionine to *S*-adenosylmethionine, which

is the major methyl donor for most methyltransferase reactions. [290]. In fact, *S*-adenosylmethionine is required for methylation of DNA, proteins, and neurotransmitters. Therefore, under low folate conditions, essential processes within the central nervous system cannot be maintained, leading to neurological impairments.

Furthermore, folate deficiency enhances lipid peroxidation, mitochondrial toxicity and cell death in the brains of normal and AD-type mice [159]. Folate supplementation is able to reduce these events in areas of the brain that are most effected by AD, such as the hippocampus [443], but this does not appear to be dose dependent [440]. It is possible that in the current study, folate supplements reduced the numbers of necrosis associated cells, through alleviating mitochondrial damage induced by β-amyloid or high-fat diet feeding. The role of folate in prevention of neural cell loss is primarily through prevention of apoptosis. Nonetheless, the ability of folate to promote cellular survival and repair oxidative damage to DNA imply that it may have played a role in neuronal survival in the brains of Amy mice that were fed the Oz-AIN Supp diet.

Nutrient supplements may have had an effect on the movement of β-amyloid across the blood brain barrier, increasing the amount of β-amyloid that was associated with the blood brain barrier.

Integrity of the blood brain barrier in normal mice brains is affected by aging and diet. Cerebrovascular integrity and fluidity decrease with aging [391, 444, 445] causing an increase in vascular permeability [446]. Feeding mice a high-fat diet accelerates the age related changes in blood brain barrier integrity, and can lead to breaks in the blood brain barrier allowing infiltration of pro-inflammatory mediators in normal mice [192, 271, 429, 446, 447]. The changes in the cerebrovascular

pathology that are induced by a high-fat diet are different to atherosclerotic pathology, and are more like the vasculature that is observed in AD-type mice that are fed normal chow [190]. This suggests that a high-fat diet and AD neuropathology may have similar effects on cerebral architecture and the blood brain barrier.

The relationship between a high-fat diet, blood brain barrier dysfunction and β-amyloid deposition has been demonstrated in several different mouse models. Takechi *et al*. demonstrated that high-fat diet feeding increases β-amyloid deposition in Amy mice [429]. Beta-amyloid is associated with triglyceride rich lipoproteins that are secreted by the liver and the intestine such as Apolipoprotein B. Takechi *et al*. demonstrate that normal mice that are fed a high-fat diet have increased Apolipoprotein B movement across the blood brain barrier, and that Apolipoprotein B – β-amyloid co-localisation was the same as that observed in AD-type mice. This is similar to reports of others that high-fat diet feeding increases circulating Apolipoprotein E and Apolipoprotein B, and this correlates with cerebral β-amyloid levels [198]. Takechi *et al*. suggest that the interference of saturated fats with blood brain barrier function, and subsequent transport of β-amyloid-laden Apolipoprotein B across the blood brain barrier may explain how dietary fats increase AD risk [429].

However, in the present study the high-fat content of the Oz-AIN diet did not appear to have an effect on the amount of β-amyloid in the Amy mouse brain, nor did it have an effect on cerebrovascular architecture. The blood brain barrier appeared intact in the brains of mice that were fed the Oz-AIN diet compared to those that were fed the AIN93-M diet, which contained lower levels of saturated fats. Mice that were fed the Oz-AIN Supp diet did appear to have breaks in the blood brain barrier, suggesting that the nutrient supplements may have interfered with blood brain barrier integrity. The most likely candidates for this are oxidation of the ω-3 polyunsaturated

supplements docosahexaenoic acid and eicosapentaenoic acid, increasing oxidative stress levels at the blood brain barrier, causing blood brain barrier dysfunction.

Docosahexaenoic acid and eicosapentaenoic acid are prone to oxidation, and for this reason have been added to the Oz-AIN Supp diet in combination with anti-oxidant polyphenolic compounds and B vitamins. However, docosahexaenoic acid and eicosapentaenoic acid both undergo rapid oxidation upon uptake by the brain [224]. The blood brain barrier is vulnerable to oxidative stress [177, 448], which can be enhanced by diets high in SFA [449]. It is possible that high levels of oxidative stress, caused by high SFA levels, overwhelmed the oxidative capabilities of the polyphenolic compounds and B vitamins in the Oz-AIN Supp diet. The increased oxidative stress could then have led to breaks in the blood brain barrier allowing easier movement of β-amyloid across the blood brain barrier [177]. This however, does not indicate the direction in which the β-amyloid is moving.

Despite its potential oxidation, dietary docosahexaenoic acid has been demonstrated to have beneficial effects on blood brain barrier function through enhanced cerebrovascular volume and reduced plasma β-amyloid levels in AD mice and AD patients [86, 450]. In a cross sectional study of aged, cognitively healthy adults, Gu *et al*. report that increased ω-3 intake (from fish, poultry, margarine and nuts) was associated with decreased plasma β-amyloid 40 and β-amyloid 42, suggesting that ω-3 fatty acids such as docosahexaenoic acid may facilitate amyloid clearance [450]. The polyphenolic compounds in the Oz-AIN Supp diet may have enhanced this function through maintenance of cerebral blood flow increasing the removal of waste being cleared across the blood brain barrier [451, 452].

It is possible that the diffuse deposits were not amyloid. The marker used in the current study, 6E10, may have detected amyloid precursor protein as well as amyloid.

The 6E10 monoclonal antibody is directed to the N terminus of β-amyloid [453]. Therefore, it also has the potential to detect amyloid precursor protein and it's cleavage products soluble APP-α and soluble APP-β [454].

This may explain the increase in blood brain barrier associated β-amyloid and the decrease in necrotic cells that was observed in the brains of mice that were fed the Oz-AIN Supp diet. It is possible that docosahexaenoic acid displaced amyloid precursor protein from the membrane through interactions with lipid-rafts.

Perez-Gonalez *et al.* report that exocytosis of amyloid precursor protein from the neuronal membrane is higher in the brains of transgenic AD-type mice compared to controls [411]. They suggest that this has a pleiotropic role. The release of amyloid precursor protein may lead to an increase in extracellular amyloid deposits. However, it may also be beneficial to the individual neuron as well, as it reduces the likelihood of a build-up of toxic intracellular β-amyloid [411]. It is possible that the docosahexaenoic acid in the Oz-AIN Supp diet may be responsible for increasing amyloid precursor protein from the cellular membrane, because docosahexaenoic acid has been demonstrated to alter membrane composition a manner that enables exocytosis of neurotransmitters and lipidated proteins [455, 456]. It is possible, that docosahexaenoic acid facilitated an up-regulation of amyloid precursor protein exocytosis, and that this was detected by the 6E10 antibody.

To confirm whether the 6E10 had stained for β-amyloid or for amyloid precursor protein and its cleavage products, brain sections would need to be re-stained with markers specifically for amyloid precursor protein or other downstream products such as soluble APP-α and soluble APP-β [454, 457].

However, 6E10 is well a established antibody specifically for β-amyloid, as it has been demonstrated to recognise the same short amino acids sequences as antibodies

generated from active immunisation with β-amyloid 42 [453]. Therefore, 6E10 positive staining is interpreted as representative of the β-amyloid deposits that are similar to those observed in human AD, and is commonly used in AD research [106, 131, 453, 458, 459].

4.4. Conclusion.

The aims of the study described in this chapter are to (i) classify the types of β-amyloid neuropathology in the Amy mouse model; (ii) investigate the effects of an Australian type diet with β-amyloid deposition in the brains of 15 and 18 month old Amy mice; and (iii) to demonstrate the effect of supplementing the Australian-type diet with nutritional supplements that delay or prevent AD pathology, on the β-amyloid deposits in the Amy mouse brain.

In accordance with the first aim of this study, it was demonstrated that the 15 and 18 month old Amy mice developed β-amyloid deposits. Confocal microscopy revealed that there are three separate and distinct pathologies of β-amyloid deposition: (i) dense, intracellular deposits of β-amyloid that were associated with necrosis; (ii) large diffuse extracellular deposits of β-amyloid that contained small, intact nuclei; and (iii) small diffuse extracellular deposits that were associated with the blood brain barrier.

While most neurons contained low levels of amyloid, there were neurons that expressed much greater amounts of amyloid. These neurons appear to either continue to accumulate β-amyloid and eventually undergo necrosis; or secrete β-amyloid at high levels, leading to the formation of the diffuse extracellular β-amyloid deposits. Three possibilities regarding the origins of the β-amyloid that was associated with the blood brain barrier have been put forward. (i) these deposits are representative of β-amyloid clearance, whereby β-amyloid is transported across the blood brain barrier to

the liver where it is broken down; or (ii) these deposits are representative of invasion of β-amyloid across the blood brain barrier into the CNS, as a consequence of a dysfunctional blood brain barrier; or (iii) these deposits are actually amyloid precursor protein, and reflect amyloid precursor protein that has been dislodged from neuronal membrane and transported to astrocytes for degradation.

In accordance with the second and third aims of this study, it was demonstrated that diet type does not have an effect on β-amyloid load or total deposit number in the brains of Amy mice. However, total fat content has an effect on the size of β-amyloid deposits. This was not prevented by nutritional supplementation demonstrating that, in the context of a high-fat diet, nutritional supplements do not prevent or alter β-amyloid deposition. However, nutrient supplements may have had an indirect effect on β-amyloid processing or transport in the CNS. Amy mice that were fed the Oz-AIN Supp diet had equal percentages of necrosis associated, large diffuse, and BBB-associated deposits. However, mice that were fed the AIN93-M diet or the Oz-AIN diet had a significantly higher percentage of β-amyloid that was associated with necrosis, and negligible deposits that were associated with the blood brain barrier. The increased β-amyloid associated with the blood brain barrier may be a result of increased transport across the blood brain barrier. However, whether this reflects invasion or clearance is undetermined and warrants further investigation.

Although reports of others indicate that β-amyloid levels change exponentially with age [372, 373], aging does not alter the parameters that have been measured in the present study. There was an association between increase in β-amyloid load and aging in the brains of Amy mice, however no other parameters changed. The distribution of large: medium: small deposits was the same at 15 months as compared to 18 months, as was the percentage of necrosis associated, large diffuse and BBB-associated deposits. It is possible that the time difference between 15 and 18 months was not

enough to see significant changes between mice. However, it was not possible to extend the study beyond 18 months due to difficulties with mice over grooming.

In conclusion, these studies have demonstrated that whilst diet type does not alter levels of β-amyloid in the Amy mouse brain, dietary components such as saturated fats or essential micronutrients may alter β-amyloid processing and transport within the brain. In particular, high total-fat content of a diet, rather than P:M:S ratio, may enhance β-amyloid aggregation into large deposits; whilst nutrient supplements may enhance transport of β-amyloid across the blood brain barrier, however whether this results in invasion or clearance of β-amyloid is undetermined.

Chapter 5: The effects of genotype and the Oz-AIN diet on spatial learning and spatial memory.

5. Background.

Alzheimer's disease (AD) is characterised by memory loss and cognitive decline [110, 122, 159, 460, 461]. Rodent models of AD can be used to explore cognitive deficits including decline in spatial reference and working memory [139, 462, 463]. Spatial learning and memory are hippocampal dependent processes. Their decline has been associated with neuro-inflammation, neurodegeneration, accumulation of intracellular β-amyloid and hyperphosphorylation of tau in AD transgenic mouse models [95, 460, 464].

Lifestyle factors such as diet can affect spatial learning and memory. Deficiency in the essential nutrients vitamin B12 and folate is associated with poor cognitive function of mice [330, 465]. Western-type high-fat diets impair spatial learning and memory in mice, while caloric restriction conserves these processes [86, 186, 466, 467].

A typical Australian-type diet can be characterised by increased saturated fat intake, with imbalanced levels of essential micronutrients. The purpose of the current study was to determine whether or not a rodent diet that reflects an Australian-type diet alters spatial learning and spatial memory in Amy mice.

The aims of the study described in this chapter are to:

1. **Demonstrate the effect of genotype on spatial learning and spatial memory in normal and Amy mice.**

   This was achieved by making two sets of comparisons:

   (i) Spatial learning abilities of diet-matched normal and Amy mice were assessed over a five day training period and compared.

   (ii) Spatial memory abilities of diet-matched normal and Amy mice were assessed in a Test Trial and compared.

2. **Demonstrate the effect of diet-type on spatial learning and spatial memory in normal and Amy mice that have been fed the AIN93-M diet or the Oz-AIN diet.**

   This was achieved by making two sets of comparisons:

   (i) Spatial learning abilities of genotype matched mice that were fed either the AIN93-M diet or the Oz-AIN diet were assessed over a five day learning period and compared.

   (ii) Spatial memory abilities of genotype matched mice that were fed either the AIN93-M diet or the Oz-AIN diet were assessed in a Test Trial and compared.

There are two hypotheses for the studies in this chapter. First, it was hypothesised that genotype will have an effect on spatial learning and spatial memory, and Amy mice will have poorer spatial learning and memory abilities than normal mice. Second, it was hypothesised that diet-type would have an effect on spatial learning and spatial memory, and mice that received the Oz-AIN diet will have poorer spatial learning and spatial memory abilities than mice that were fed the AIN93-M diet.

Decline of rodent spatial learning and memory ability can be monitored using behavioural tests such as the Morris Water Maze (MWM) [87, 89, 96, 468]. While initially developed for use with rats, the MWM has been successfully used to demonstrate changes in spatial learning and spatial memory in mice [96, 202, 469]. Spatial learning is demonstrated by improved latency (s) and distance travelled (m) to a submerged platform over a five day acquisition phase [87, 89, 96]. Spatial memory is demonstrated in a Test Trial on the following day when the platform is removed from the water maze [87, 89, 93, 96, 468]. The current study has utilized the MWM to assess spatial learning and memory in normal and Amy mice. The protocols that are used to do this are described in the first half of this chapter.

The principal findings of the study described in this chapter are that the Oz-AIN diet may have a beneficial effect on spatial learning and memory abilities of Amy mice. At 12 months of age, Amy mice were the only mice to make significant improvements in the MWM. Furthermore, at 18 months, the normal and Amy mice that were fed the Oz-AIN diet were the only mice to demonstrate intact spatial memory.

This study also reports that genotype does have a detrimental effect on spatial learning and memory in Amy mice. However, genotype effects on spatial memory may precede those on spatial learning, as no genotype effects on spatial learning were apparent until mice were 18 months old.

5.1. Methods.

5.1.1. Animals.

All experiments were approved by the Commonwealth Scientific and Industrial Research Organisation (CSIRO) Animal Welfare Committee, Australia in accordance with National Health and Medical Research Council guidelines.

Female Amy (APPswe/PSEN1dE9) mice and their female normal (C57bl/6) littermates were bred at and provided by Flinders University Animal Facility, Bedford Park, South Australia. Genotype was confirmed by PCR and agarose gel electrophoresis, as described in Appendix I. Amy mice and normal mice were each fed either the Oz-AIN diet or the AIN93-M diet from weaning until 18 months of age. Mice were housed (n<6) in cages lined with sawdust, and had free access to food and water.

Table 1. The numbers of mice in each treatment group that were tested in the MWM at 12, 15 and 18 months of age.

	Normal mice AIN93-M diet	**Normal mice Oz-AIN diet**	**Amy mice AIN93-M diet**	**Amy mice Oz-AIN diet**
12 months	n = 12	n = 12	n = 8	n = 9
15 months	n = 12	n = 11	n = 8	n = 9
18 months	n = 10	n = 6	n = 8	n = 6

Mice underwent testing in the MWM at 12, 15 and 18 months of age (Figure 1). Between 15 and 18 months of age, some mice had to be removed from the study due to difficulties managing over-grooming. Therefore, group sizes at 18 months of age are smaller than those at the start of the study. Treatment group sizes at 12, 15 and 18 months of age are reported in Table 1. Group sizes between five and twelve have

been reliably used to demonstrate cognitive changes in mice using the MWM, and should not affect the power of this study [86, 470, 471].

5.1.2. Study design.

This study made use of a two-factorial design. One factor was genotype (normal versus Amy mice) and the other was diet-type (AIN93-M diet versus Oz-AIN diet).

Aim 1: To determine GENOTYPE EFFECTS on spatial learning and spatial memory in normal and Amy mice.

The effect of genotype on spatial learning and spatial memory abilities was established by comparisons between Amy and normal mice that were fed the AIN93-M diet. Comparisons were also made between normal and Amy mice that were fed the Oz-AIN diet to demonstrate whether or not genotype effects are enhanced by a sub-optimal diet (Figure 1A).

Aim 2: To determine DIET-TYPE EFFECTS on spatial learning and spatial memory in normal and Amy mice.

The effect of the Oz-AIN diet on spatial learning and spatial memory was established by comparisons between normal mice that were fed either the AIN93-M diet or the Oz-AIN diet. Comparisons were also made between Amy mice that were fed either the AIN93-M diet or the Oz-AIN diet to establish whether or not dietary effects on spatial learning or spatial memory are enhanced in the Amy mouse (Figure 1B).

Figure 1A. The study design used to investigate the effects of genotype on spatial learning and memory in normal and Amy mice.

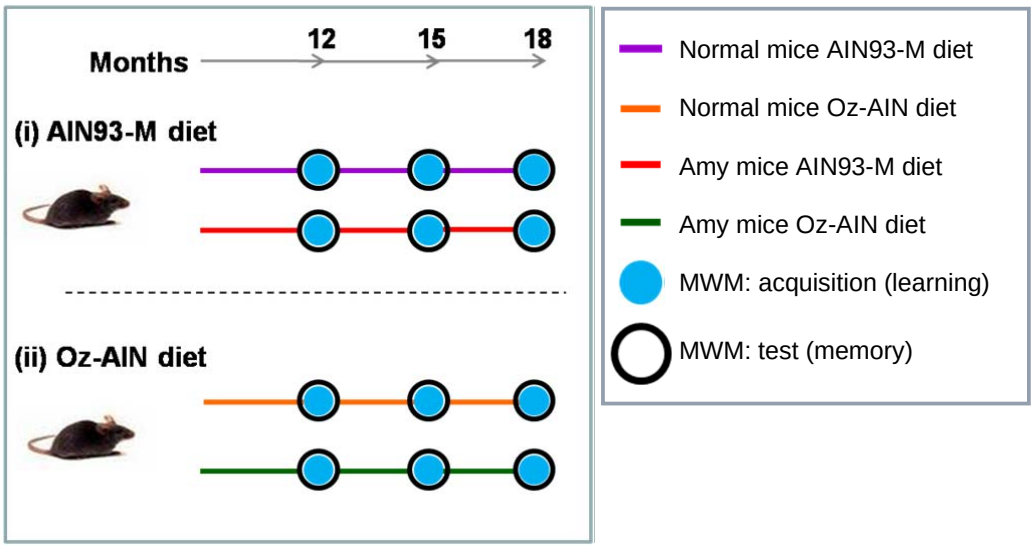


Figure 1A. The Morris Water Maze (MWM) was used to assess spatial learning (blue dots) and spatial memory (black circles). Mice underwent assessments at 12, 15, and 18 months.

Genotype effects on spatial learning and spatial memory were assessed by comparing diet-matched normal mice and Amy mice.

(i) Comparisons were made between normal mice fed the AIN93-M diet (purple line) and Amy mice fed the AIN93-M diet (red line) to demonstrate genotype effects on spatial learning and spatial memory.

(ii) Comparisons were made between normal mice fed the Oz-AIN diet (orange line) and Amy mice fed the Oz-AIN diet (green line) to demonstrate genotype effects when mice are challenged with a sub-optimal diet.

Figure 1B. The study design used to investigate the effects of diet-type on spatial learning and memory in normal and Amy mice.

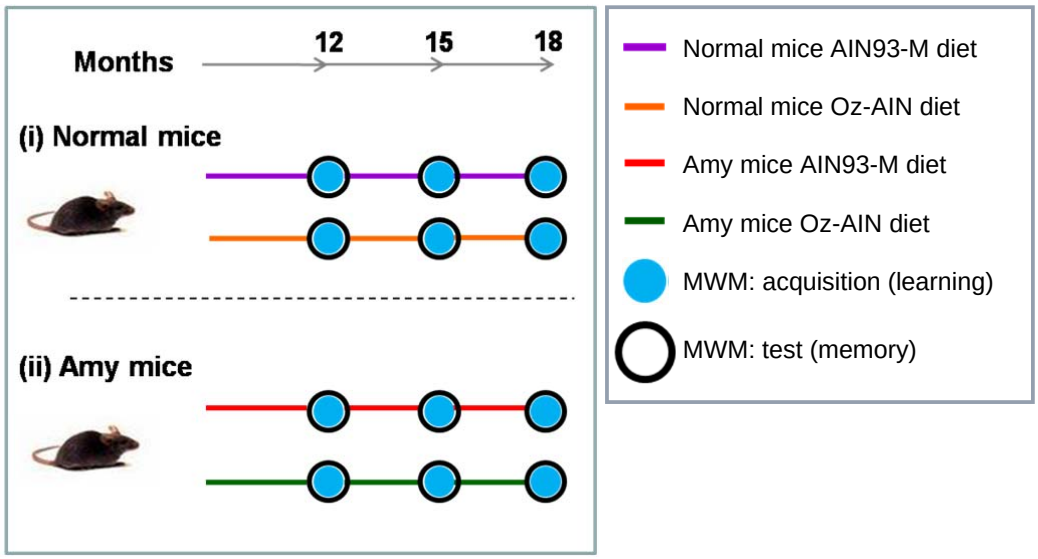


Figure 1B. The Morris Water Maze (MWM) was used to assess spatial learning (blue dots) and spatial memory (black circles). Mice underwent assessments at 12, 15, and 18 months.

Diet-type effects on spatial learning and spatial memory were assessed by comparing genotype-matched mice that were fed either the AIN93-M diet or the Oz-AIN diet.

(i) Comparisons were made between normal mice fed the AIN93-M diet (purple line) and normal mice fed the Oz-AIN diet (orange line) to demonstrate the effects of a sub-optimal diet on spatial learning and spatial memory.

(ii) Comparisons were made between Amy mice fed the AIN93-M diet (red line) and Amy mice fed the Oz-AIN diet (green line) to demonstrate the effects of a sub-optimal diet in mice that are genetically predisposed to develop AD.

5.1.3. Apparatus.

The MWM arena was a 1.2 m pool in the centre of a dark enclosure (3 m x 2 m x 2 m) under 4 fluorescent lights. The 1.2 m pool was filled with water (22°C – 24°C) made opaque with white paint (British Paints Ceiling Paint, flat acrylic) (Figure 2).

Coloured card-board shapes were placed around the arena as visual cues. A curtain separated the experimenter from the pool, so that they were out of the field of view of mice being tested.

A digital camera, connected to a laptop running Stoelting ANYmaze software (Stoelting Co., Wood Dale, USA), was positioned 3.2 m above the pool (see Appendix II for details on configuration of ANYmaze software for the MWM test). The arena was virtually divided into 4 equal quadrants. One of these quadrants was allocated to be the 'Test Quadrant' throughout the experiments.

A submerged platform (10 cm diameter) was placed in the centre of the Test Quadrant, 2 cm below the surface of the water.

Figure 2. Morris Water Maze Arena set up.

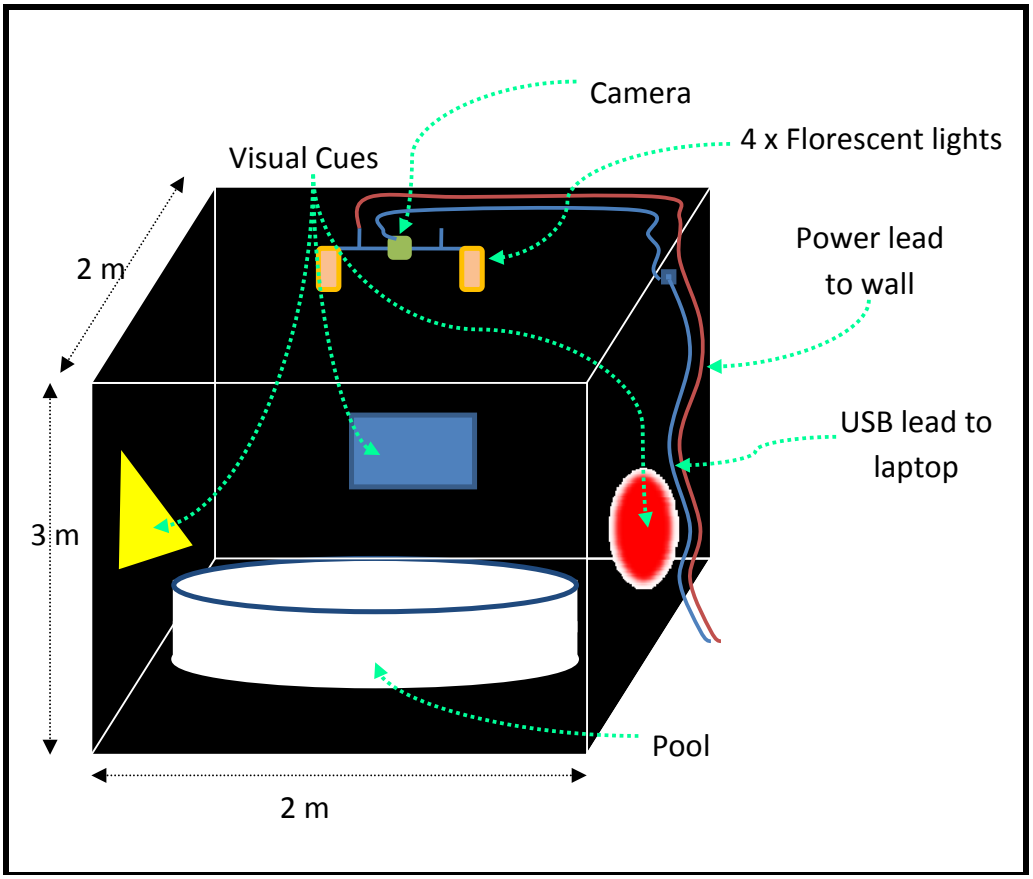


Figure 2. The Morris Water Maze is a 1.2 m diameter pool placed in the centre of a well-lit arena surrounded by controlled visual cues.

5.1.4. Protocol.

Cued trial.

A cued trial was used to validate that all mice could respond to visual cues to locate the platform. The platform was placed in the Opposite Quadrant (the quadrant opposite the Test Quadrant). A visible cue (a 15 cm coloured stand) was placed on top of the platform. All other visual cues were removed from the walls of the arena enclosure.

The mouse was placed in the Test Quadrant, facing the pool wall. The latency to reach the platform (s), distance travelled (m) and average speed (m/s) were recorded by Stoelting ANYmaze software (Stoelting Co.) (see Appendix II).

After locating the platform mice were collected and placed in a warm bath so that any paint residue could be removed. Mice were then returned to a warm cage with free access to food and water.

Acquisition phase.

Spatial learning was assessed during an acquisition phase. The acquisition phase lasted over five training days. Each of the five training days consisted of 4 x 120 s trials (two AM trials, and two PM trials).

Before the first trial of the first day, mice were placed on the platform for 30 s. This enabled mice to orientate themselves relative to the visual cues surrounding the pool.

In all trials mice were placed in either: a north, south, east or westerly position in the pool and given 120 s to locate the submerged platform. The latency to reach the platform (s), distance travelled (m) and average speed (m/s) were recorded by Stoelting ANYmaze software (Stoelting Co.) (see Appendix II). After each trial,

whether mice found or failed to locate the platform, they were placed on the platform for a further 10 s to re-enforce the location of the platform relative to visual cues. Mice were removed from the arena and rinsed in a warm bath before being returned to a warmed cage with free access to food and water.

Test Trial.

Spatial memory was assessed in a Test Trial. The Test Trial occurred the day following the acquisition phase. The platform was removed from the pool and mice were placed in Opposite Quadrant, facing the pool wall, and allowed a 120 s free swim. After 120 s, mice were removed from the arena and rinsed in a warm bath before being returned to a warmed cage with free access to food and water.

The number of passes made over the removed platform's position, the distance travelled (m) and average speed (m/s) were recorded by Stoelting ANYmaze software (Stoelting Co.) (see Appendix II).

5.1.5. Data collection.

Latency to platform (s), distance travelled (m) and average speed (m/s) during the cued trial and acquisition phase were recorded using ANYmaze software (Stoelting Co.) (see Appendix II). When mice were 15 months old, malfunctions with the ANYmaze software resulted in failure to record distance and average speed travelled by mice in the MWM. Therefore, the data for these parameters are lacking for 15 month old mice. Time taken to reach the platform was measured manually with a stopwatch, and these data were used to assess spatial learning at 15 months of age.

In the Test Trial, the number of times the mouse crossed the platform position, the amount of time (s) and distance travelled (m) by the mouse in the Test and Opposite Quadrants were recorded by ANYmaze software (Stoelting Co.) (see Appendix II).

To confirm that latencies, distances and average speed swam by mice were accurately detected by ANYmaze software, latencies to platform were also recorded manually from video footage by an experimenter that was blinded to the treatment condition of each mouse. Manual latencies were compared with latencies that were recorded by ANYmaze software. Any swims where manual latencies and the latencies recorded by ANYmaze differed by >2 s were deemed to not have detected mice accurately. These data were excluded from analysis.

5.1.6. Data analysis.

Spatial learning.

Improved latency (s) and distance travelled (m) before reaching the submerged platform throughout the acquisition phase were the primary indicators of spatial learning in the MWM.

Spatial learning within groups was established through comparison of performances on each day of training using one-way ANOVA. Bonferroni post tests were used to compare improvement relative to the first day of training.

Diet-type effects and genotype effects on overall improvement in latency (s) and distance travelled (m) after the five training days of the acquisition phase were compared using two-way ANOVA and Bonferroni post tests. Diet-type effects on overall improvement in latency and distance were determined by comparisons between normal mice that were fed either the AIN93-M diet or the Oz-AIN diet, and

between Amy mice that were fed the AIN93-M or the Oz-AIN diet. Genotype effects on overall improvement in latency and distance were determined by comparisons between normal and Amy mice that were fed the same diet.

Spatial memory.

The amount of time (s) and total distance travelled (m) within the Test Quadrant compared to the Opposite Quadrant was used as the primary indicator of degree to which mice recalled the location of the platform, and were compared within groups using Student's *t*-tests.

Two-way ANOVA and Bonferroni post tests comparing the percent of time and percent of distance travelled in the Test and Opposite Quadrants were used to determine genotype and diet-type effects on spatial memory. Diet-type effects on percent time and distance in each quadrant were determined by comparisons between normal mice that were fed either the AIN93-M diet or the Oz-AIN diet, and between Amy mice that were fed the AIN93-M or the Oz-AIN diet. Genotype effects on percent time and distance were determined by comparisons between normal and Amy mice that were fed the same diet.

A comparison of the number of passes over the position that the submerged platform had previously been located was used also as a measure of variance of spatial memory between treatment groups. Passes over platform location were compared with two-way ANOVA and Bonferroni post tests.

All data was stored in excel files and analysed using GraphPad Prism^{R} Software (Prism 5 for Windows, version 5.04, GraphPad Software inc., CA, USA). All data is reported as mean ±SEM. For all comparisons, statistical significance was set at $p<0.05$.

5.2. Results.

5.2.1. Validation that all mice could respond to visual cues.

At 12 months of age.

At 12 months of age, all mice were able to locate the platform in a cued trial (Table 2, Table 3). Differences in latency (s) or distance travelled (m) to the visible platform were not significantly different between treatment groups of mice. However, the 12 month old normal mice fed the AIN93-M reached the platform in less than two thirds the time taken by normal mice that were fed the Oz-AIN diet ($p>0.99$, Table 2), and less than half the time taken by Amy mice that were fed either the AIN93-M diet ($p=0.64$, Table 2) or the Oz-AIN diet ($p=0.37$, Table 2). Normal mice that were fed the AIN93-M diet also travelled shorter distances to the platform than littermates fed the Oz-AIN diet or Amy mice fed the AIN93-M diet or the Oz-AIN diet (Table 3).

As all mice were able to locate the platform with latencies (s) and distances (m) that were not significantly different, these results indicate that neither diet-type nor genotype impede a 12 month old mouse from responding to visual cues to locate a submerged platform (Table 2, Table 3).

At 15 months of age.

At 15 months of age, all mice were able to find the submerged platform in a cued trial (Table 2, Table 3). A two-way ANOVA indicated that genotype accounted for 4.28% of the overall variance of latency to the platform ($p=0.20$, Table 2), and diet-type and the genotype-diet-type interaction accounted for 0.02% and 4.70% of variance respectively ($p=0.93$ and $p=0.18$ respectively, Table 2). Whilst normal mice that were fed the Oz-AIN diet took the least time to reach the platform, this was not

significantly different to the latencies to platform by normal mice that were fed the AIN93-M diet ($p>0.99$, Table 2), Amy mice that were fed the AIN93-M diet ($p>0.99$, Table 2) or Amy mice that were fed the Oz-AIN diet ($p=0.40$, Table 2). Similarly, distance travelled before reaching the platform was not significantly affected by genotype (3.15% of variance, $p=0.26$, Table 3) or diet-type (0.67% of variance, $p=0.60$, Table 3).

These data confirm that at 15 months of age, normal and Amy mice that were fed either the AIN93-M or the Oz-AIN diet were motivated to escape the MWM arena, and were able to use visual cues to locate the platform.

At 18 months of age.

At 18 months of age, all mice were able to locate the submerged platform in a cued trial (Table 2, Table 3). A two way ANOVA indicated that genotype accounted for 5.25% of the overall variance of latency to the platform ($p=0.23$, Table 2), whilst diet-type and the genotype-diet-type interaction accounted for 0.45% and 3.57% respectively ($p=0.72$ and $p=0.32$ respectively, Table 2). The 18 month old Amy mice that were fed the Oz-AIN diet took the longest to reach the platform, but this was not significantly different to Amy mice that were fed the AIN93-M diet ($p>0.99$, Table 2), normal mice that were fed the Oz-AIN diet ($p=0.96$, Table 2) or normal mice that were fed the AIN93-M diet ($p>0.99$, Table 2). Distance travelled before reaching the platform was not different between mice ($p>0.99$ for all groups, Table 3), supporting the conclusion that there were no differences in motivation or visual ability between 18 month old mice.

These results suggest that at 18 months of age, all mice were able to use visual cues to locate the platform in the MWM and Amy mice that are fed the Oz-AIN diet may swim slower than other mice.

Table 2. Latency (s) for normal and Amy mice to reach the visible platform during the Cued Trial in the Morris water Maze at 12, 15 and 18 months old.

	Normal mice AIN93-M diet	Normal mice Oz-AIN diet	Amy mice AIN93-M diet	Amy mice Oz-AIN diet
12 months	8.28 ±2.13	12.21 ±3.71	15.52 ±3.32	16.74 ±2.32
15 months	10.13 ±2.19	7.37 ±1.05	10.01 ±1.77	12.43 ±2.16
18 months	12.41 ±5.49	9.57 ±1.97	13.35 ±2.34	19.33 ±3.68

All values are reported as mean ±SEM.

Table 3. Distance travelled (m) by normal and Amy mice before reaching the visible platform during the Cued Trial in the Morris water Maze at 12, 15 and 18 months old.

	Normal mice AIN93-M diet	Normal mice Oz-AIN diet	Amy mice AIN93-M diet	Amy mice Oz-AIN diet
12 months	1.31 ±0.45	2.30 ±0.70	2.66 ±0.63	2.72 ±0.67
15 months	1.91 ±0.31	1.07 ±0.21	1.61 ±0.33	2.11 ±0.42
18 months	2.49 ±1.11	1.70 ±0.38	2.12 ±0.30	2.64 ±0.23

All values are reported as mean ±SEM.

5.2.2. Spatial learning in the Morris Water Maze.

Spatial learning was assessed as per page 275.

5.2.2.1. Performance of 12 month old mice throughout the acquisition phase in the Morris Water Maze.

Normal mice fed the AIN93-M diet, normal mice fed the Oz-AIN diet, and Amy mice fed the AIN93-M diet.

One-way ANOVA did not detect significant improvement of latency to platform on any of the five training days by normal mice that were fed the AIN93-M diet (p=0.43,

Figure 3A), normal mice that were fed the Oz-AIN diet (p=0.14, Figure 3A) or Amy mice that were fed the AIN93-M diet (p=0.47, Figure 3A). Mice did not make day-to-day improvements (Figure 3A), and Bonferroni post tests did not detect significant improvement on Day 5 relative to Day 1 for normal mice that were fed the AIN93-M diet (p=0.55, Figure 3A), normal mice that were fed the Oz-AIN diet (p=0.34, Figure 3A) or Amy mice that were fed the AIN93-M diet (p>0.99, Figure 3A). Average speed travelled varied throughout the training period for all groups (Figure 3C). However, variances were not significant indicating that the latency data reflects impaired learning abilities (Figure 3C).

One-way ANOVA and Bonferroni post tests also did not detect significant changes in distance travelled on each of the training days for normal mice that were fed the AIN93-M diet (p=0.31, Figure 3B), normal mice that were fed the Oz-AIN diet (p=0.38, Figure 3B) or Amy mice that were fed the Oz-AIN diet (p=0.45, Figure 3B).

This indicates that irrespective of diet, 12 month old normal mice have impaired spatial learning abilities, and 12 month old Amy mice fed the AIN93-M diet also have impaired spatial learning abilities.

Amy mice fed the Oz-AIN diet.

Unlike other treatment groups, the 12 month old Amy mice that were fed the Oz-AIN diet made significant improvements in latency to platform throughout the acquisition phase relative to latencies on Day 1 (p=0.02, Figure 3A). Bonferroni post tests revealed that the significant improvement made by Amy mice that were fed the Oz-AIN diet was attributed to improvements on Day 3 (p=0.02, Figure 3A), Day 4 (p=0.02, Figure 3A), and Day 5 (p=0.04, Figure 3A). While the average speed travelled by Amy mice that were fed the Oz-AIN diet varied throughout the training

period, this was not significant. This indicates that the improved latencies to the platform reflect spatial learning (Figure 3C).

The Amy mice that were fed the Oz-AIN diet also made significant improvements in distance travelled to the platform throughout the acquisition phase ($p=0.006$, Figure 3B). They improved by 3.85 ±0.84 m on Day 2 ($p=0.04$, Figure 3B), 5.68 ±0.85 m on Day 3 ($p=0.002$, Figure 3B) and 4.56 ±0.94 m on Day 4 ($p=0.01$, Figure 3B). While they travelled 3.37 ±0.83 m less on Day 5 than Day 1 this was not significant ($p=0.07$, Figure 3B). These data indicate that the 12 month old Amy mice that were fed the Oz-AIN diet had intact spatial learning ability.

All treatment groups reduced average speed travelled while searching for the platform throughout the acquisition phase (Figure 3C). However, there were no significant differences in average speed travelled between treatment groups on any training day therefore performance of mice is still comparable.

Collectively, these data suggest that 12 month old Amy mice that are fed the Oz-AIN diet were able to learn the location of the platform, and that the 12 month old Amy mice that are fed the AIN93-M diet, and the normal mice that are fed either the AIN93-M or Oz-AIN diet have impaired spatial learning abilities.

Figure 3A. Latency (s) for 12 month old normal and Amy mice to reach a submerged platform in the Morris Water Maze over five training days.

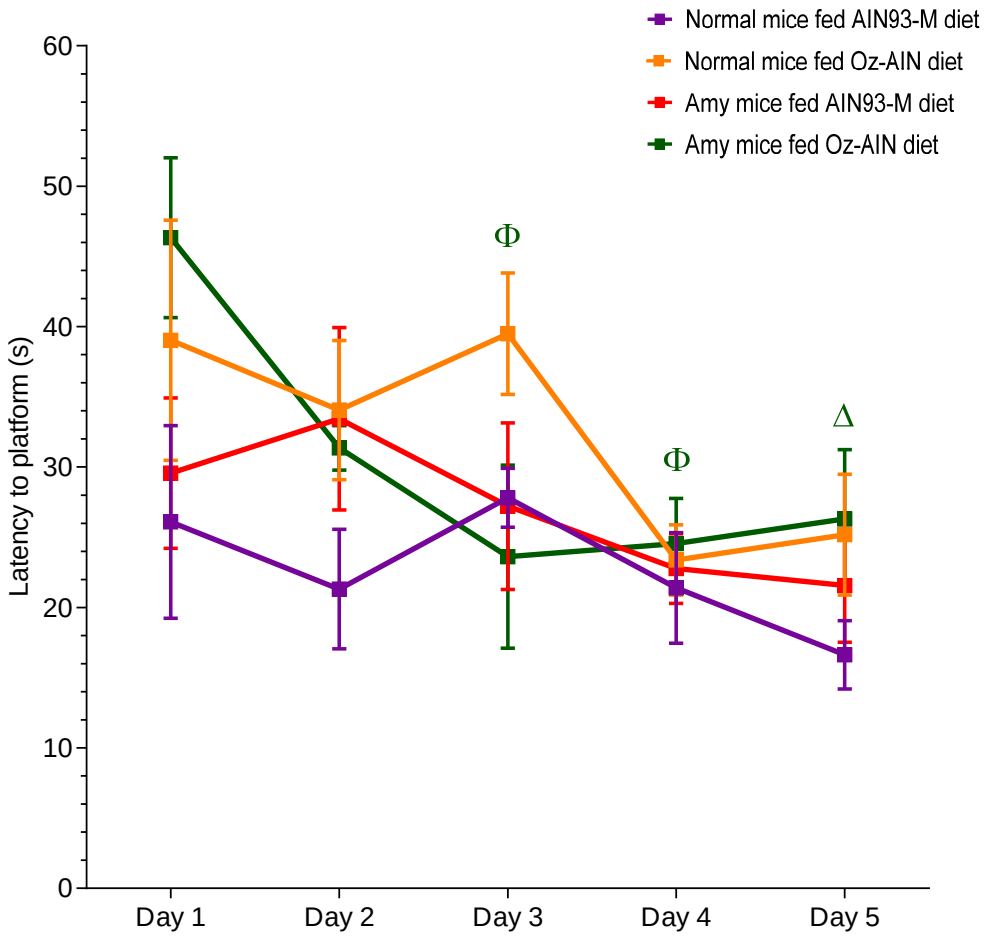


Figure 3A. The average latency (s) to reach the submerged platform on each of the five training days when mice were 12 months old. Normal mice fed the AIN93-M diet (purple line, n=12), normal mice fed the Oz-AIN diet (orange line, n=12), Amy mice fed the AIN93-M diet (red line, n=8), and Amy mice fed the Oz-AIN diet (green line, n=9). Error bars are mean ±SEM. Symbols indicate significant differences compared to latency on Day 1 within groups that are the same colour as the symbol, and were detected with Bonferroni post tests. (Φ) p=0.02 (green, Amy mice fed the Oz-AIN diet). (Δ) p=0.04 (green, Amy mice fed the Oz-AIN diet).

Figure 3B. Distance travelled (m) before 12 month old normal and Amy mice reached a submerged platform in the Morris Water Maze over five training days.

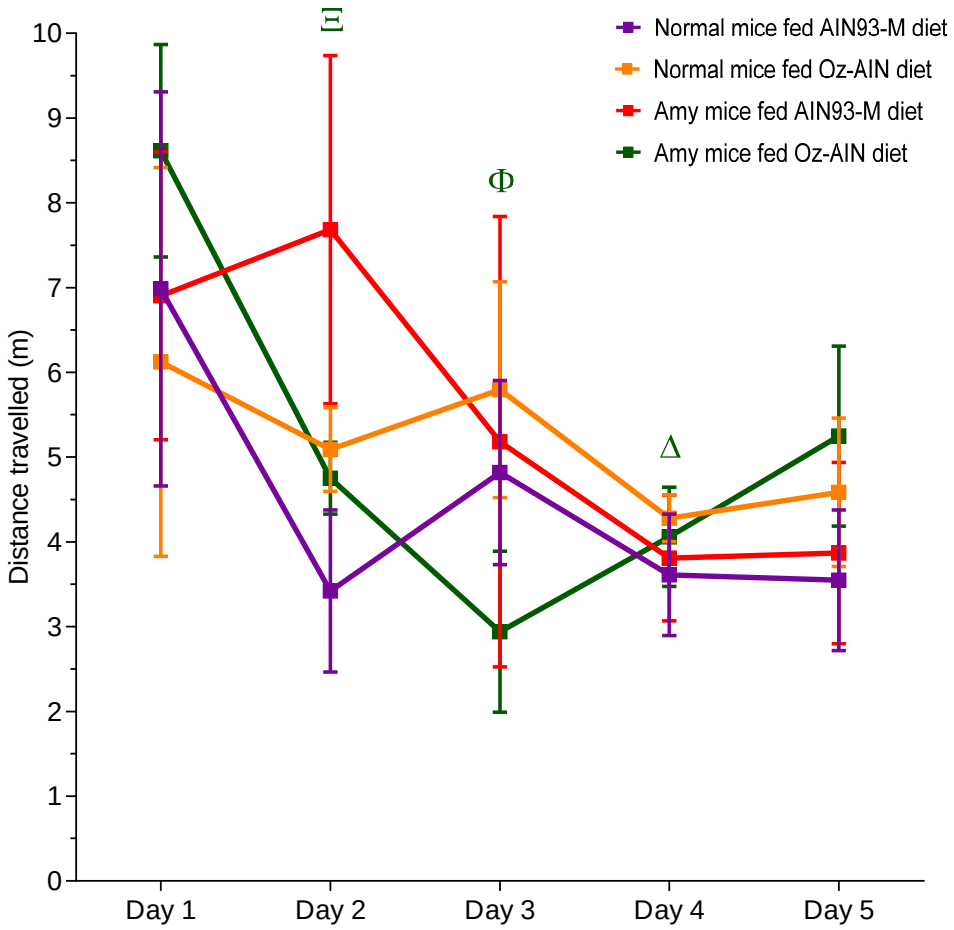


Figure 3B. The average distance travelled (m) during a swim to locate a hidden platform on each of the five training days when mice were 12 months old. Normal mice fed the AIN93-M diet (purple line, n=12), normal mice fed the Oz-AIN diet (orange line, n=12), Amy mice fed the AIN93-M diet (red line, n=8), and Amy mice fed the Oz-AIN diet (green line, n=9). Error bars are mean ±SEM. Symbols indicate significant differences compared to distance travelled on Day 1 within groups that are the same colour as the symbol, and were detected with Bonferroni post tests. (Φ) p=0.002 (green, Amy mice fed the Oz-AIN diet). (Δ) p=0.01 (green, Amy mice fed the Oz-AIN diet). (Ξ) p=0.04 (green, Amy mice fed the Oz-AIN diet).

Figure 3C. Average speed travelled (m/s) by 12 month old normal and Amy mice whilst looking for a submerged platform in the Morris Water Maze over five training days.

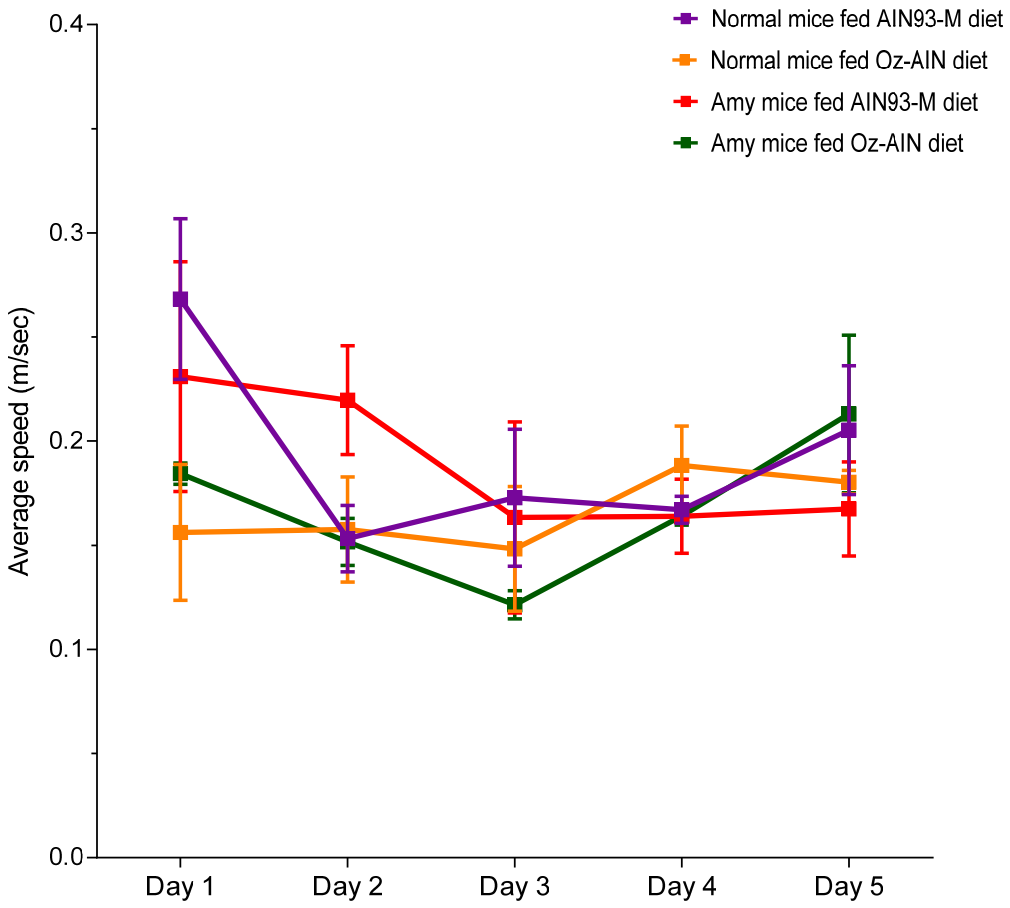


Figure 3C. The average speed (m/s) travelled to a submerged platform on each of the five training days when mice were 12 months old. Normal mice fed the AIN93-M diet (purple line, n=12), normal mice fed the Oz-AIN diet (orange line, n=12), Amy mice fed the AIN93-M diet (red line, n=8), and Amy mice fed the Oz-AIN diet (green line, n=9). Error bars are mean ±SEM.

Between group comparisons: Genotype and diet-type effects on spatial learning abilities of 12 month old.

Overall improved latency (s) to the platform after five training days.

A two-way ANOVA revealed that genotype and the genotype-diet-type interaction only accounted for 0.64% and 1.67% of the overall variance of improvement of latencies to the platform by 12 month old mice ($p=0.61$ and $p=0.41$, respectively, Table 4). Diet-type accounted for 7.68% of the overall variances of improved latencies to the platform by 12 month old mice ($p=0.08$). While not significant at $p<0.05$, it suggests that there may have been trends for diet-type to affect improvements in latency to the platform. However, Bonferroni post tests did not detect trends. While the Amy mice that were fed the Oz-AIN diet made an overall improvement that was 12.03 ±3.91 s greater than that of Amy mice that were fed the AIN93-M diet, this was not significant with Bonferroni post tests ($p=0.58$, Table 4). Similarly, there were no differences between the overall improved latencies to platform made by normal mice that were fed the AIN93-M diet or the Oz-AIN diet ($p>0.99$, Table 4).

Table 4. Genotype and diet-type effects on overall changes in latency (s) for 12 month old mice to reach the submerged platform after five training days.

	Normal mice	**Amy mice**	**Difference (s)**
AIN93-M diet	9.46 ±4.50	8.00 ±1.49	**1.47 ±3.07**
Oz-AIN diet	13.84 ±5.44	20.03 ±3.97	**6.19 ±6.97**
Difference (s)	**4.37 ±4.59**	**12.03 ±3.91**	

All numbers are mean ±SEM. Differences were calculated individually for each mouse and averaged to determine mean ±SEM. Genotype comparisons are made across rows. Diet-type comparisons are made down columns.

Overall improved distance travelled (m) to the platform after five training days.

A two-way ANOVA did not detect significant effects of genotype (p=0.52, Table 5), diet-type (p=0.59, Table 5), or a genotype-diet-type interaction (p=0.45, Table 5). The normal mice that were fed the Oz-AIN diet made the smallest overall improvement of distance travelled, whilst the normal mice that were fed the AIN93-M diet made the greatest improvement of overall distance travelled (Table 5). This suggests that there may have been some effect of diet on improvement of distance travelled by normal mice. However, Bonferroni post tests did not detect significant differences between the two groups (p>0.99, Table 5).

Collectively, these data indicate that genotype, diet-type or the genotype-diet-type interaction do not have significant effects on spatial learning abilities of normal and Amy mice at 12 months.

Table 5. Genotype and diet-type effects on overall changes in the distance travelled (m) by 12 month old mice before reaching the submerged platform after five training days.

	Normal mice	**Amy mice**	**Difference**
AIN93-M diet	3.44 ±1.76	3.04 ±0.74	**0.40 ±1.91**
Oz-AIN diet	1.54 ±1.46	3.37 ±0.83	**-1.83 ±1.90**
Difference	**1.90 ±0.51**	**-0.33 ±0.80**	

All numbers are mean ±SEM. Differences were calculated individually for each mouse and averaged to determine mean ±SEM. Genotype effects are demonstrated by comparisons across rows. Diet-type effects are demonstrated by comparisons down columns.

5.2.2.2. Performance of 15 month old mice throughout the acquisition phase in the Morris Water Maze.

Owing to problems with software, there are no data to describe average speed or distance travelled by 15 month old mice over five training days in the MWM. The following data only describe changes in latency.

Normal mice fed AIN93-M diet.

The normal mice that were fed the AIN93-M diet appeared to make gradual improvements in latency to the platform throughout the acquisition phase (Figure 4A). However, a one-way ANOVA did not detect significant variances ($p=0.56$, Figure 4A). This suggests that normal mice that were fed the AIN93-M diet failed to learn the location of the platform.

Normal mice fed Oz-AIN diet.

The normal mice that were fed the Oz-AIN diet did not appear to improve latency to platform until Day 4, when they made a 5.93 ±1.26 s improvement, relative to day 1. However, this was not significant ($p>0.099$, Figure 4). Furthermore, one-way ANOVA did not detect any significant variance of latency to platform for normal mice that were fed the Oz-AIN diet ($p=0.13$, Figure 4). This indicated that normal mice that were fed the Oz-AIN diet did not learn the location of the submerged platform.

Amy mice fed AIN93-M diet.

Amy mice that were fed the AIN93-M diet failed to make consistent improvements throughout the acquisition phase, suggesting that they were not learning the location of the platform (Figure 4). Accordingly, a one-way ANOVA did not detect significant

differences in latencies travelled by Amy mice that were fed the AIN93-M diet throughout the acquisition phase ($p=0.63$, Figure 4).

Amy mice fed Oz-AIN diet.

Although the Amy mice that were fed the Oz-AIN diet made significant improvements at 9 months of age ($p=0.02$, Figure 3A), they did not make improvements in latency to the platform at 15 months of age ($p=0.34$, Figure 4). This suggests that, similar to all other 15 month old mice, the Amy mice that were fed the Oz-AIN diet did not learn the location of the submerged platform. Collectively, these data indicate that at 15 months of age, neither normal nor Amy mice have intact spatial learning abilities.

Figure 4. Latency (s) for 15 month old normal and Amy mice to reach a submerged platform in the Morris Water Maze over five training days.

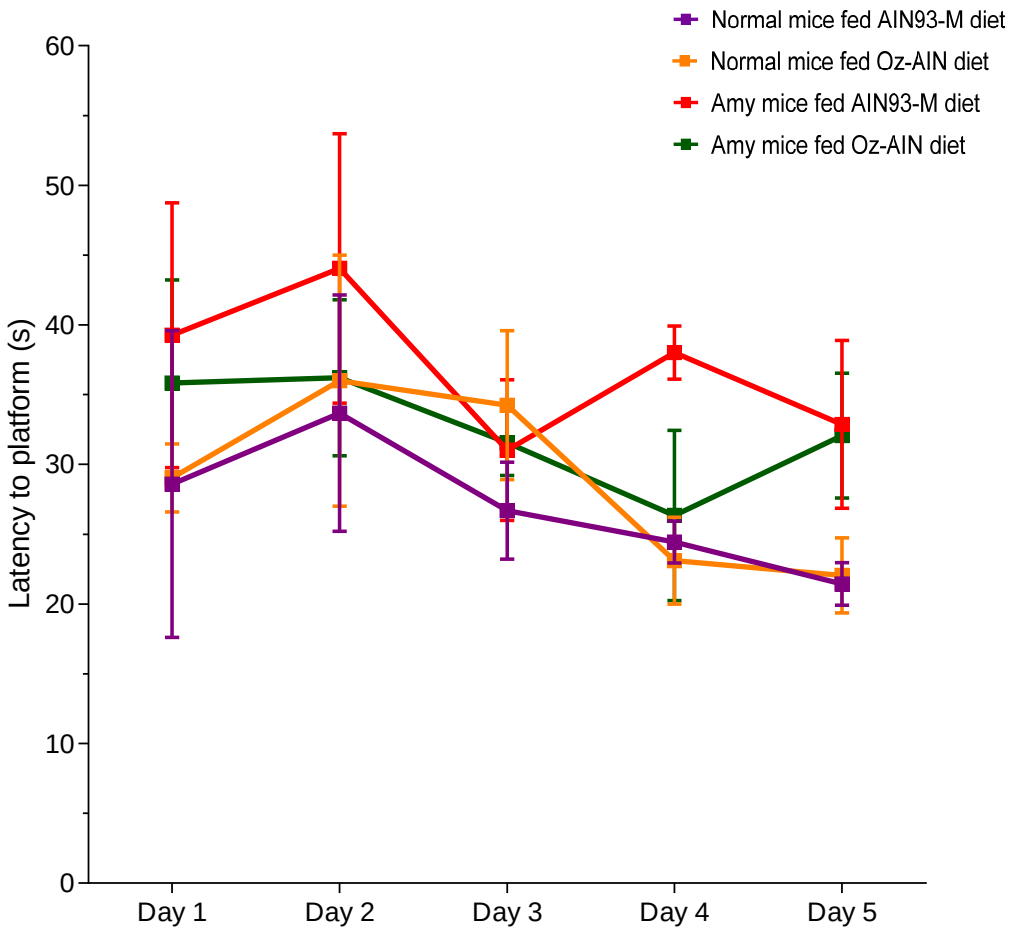


Figure 4. The average latency (s) to reach a submerged platform on each of the five training days when mice were 15 months old. Normal mice fed the AIN93-M diet (purple line, n=12), normal mice fed the Oz-AIN diet (orange line, n=11), Amy mice fed the AIN93-M diet (red line, n=8), and Amy mice fed the Oz-AIN diet (green line, n=9). Error bars are mean ±SEM.

Between treatment group comparisons: Genotype and diet-type effects on spatial learning abilities of 15 month old normal and Amy mice.

Overall improved latency (s) to the platform after five training days.

A two-way ANOVA revealed that neither diet-type nor genotype had a significant effect on overall improvement in latency to a submerged platform when mice were 15 months old. Diet-type accounted for 0.17% of overall variance (p=0.81, Table 6), whilst genotype accounted for 0.24% of overall variance (p=0.77, Table 6). This was expected, owing to the finding that all groups of mice failed to demonstrate spatial learning.

Table 6. Genotype and diet-type effects on overall changes in latency (s) for 15 month old mice to reach the submerged platform after five training days.

	Normal mice	**Amy mice**	**Difference (s)**
AIN93-M diet	7.17 ±9.51	6.40 ±5.03	**0.78 ±5.37**
Oz-AIN diet	6.70 ±0.47	3.77 ±4.23	**3.22 ±4.57**
Difference (s)	**0.19 ±9.85**	**2.63 ±1.26**	

All numbers are mean ±SEM. Differences were calculated individually for each mouse and averaged to determine mean ±SEM. Genotype effects are demonstrated by comparisons across rows. Diet-type effects are demonstrated by comparisons down columns.

Collectively, these data indicate that at 15 months, neither normal nor Amy mice have intact spatial learning abilities; and there are no effects of diet-type or genotype on spatial learning abilities at 15 months.

5.2.2.3. Latency (s) and distance travelled (m) by 18 month old mice before reaching the submerged platform throughout the acquisition phase.

Normal mice fed the AIN93-M diet.

A one-way ANOVA did not detect significant variance in the latency for 18 month old normal mice that were fed the AIN93-M diet to reach the submerged platform throughout the acquisition phase ($p=0.28$, Figure 5A). Furthermore, there were no significant differences detected for distance travelled throughout the training period ($p=0.53$, Figure 5B).

These data indicate that 18 month old normal mice that have been fed the AIN93-M diet do not have intact spatial learning abilities.

Normal mice fed the Oz-AIN diet.

A one-way ANOVA of the variance of latency to the platform for the 18 month old normal mice that were fed the Oz-AIN diet detected significant variances throughout the acquisition phase ($p=0.05$). However, Bonferroni post tests did not detect where these differences lay, as they did not detect significant differences in latency on Day 2 ($p=0.11$, Figure 5A), day 3 ($p=0.11$, Figure 5A), Day 4 ($P>0.99$, Figure 5A) or Day 5 ($p>0.99$, Figure 5A) relative to latency on Day 1.

However, a one-way ANOVA of the average distance travelled each day by normal mice that were fed the Oz-AIN diet revealed that mice swam also different distances throughout the acquisition phase ($p=0.0001$, Figure 5C). Bonferroni post tests revealed the normal mice that were fed the Oz-AIN diet travelled shorter distances on Day 2 ($p=0.004$, Figure 5C), Day 3 ($p=0.04$, Figure 5C) and Day 5 ($p=0.02$, Figure 5C) relative to distance travelled on Day 1. Average speed also declined throughout the acquisition phase, but this was not significant ($p=0.20$, Figure 5B). Collectively,

these data suggest that normal mice that were fed the Oz-AIN diet had intact spatial learning abilities at 18 months of age.

Amy mice fed the AIN93-M diet.

The Amy mice that were fed the AIN93-M diet did not improve latency to the submerged platform throughout the acquisition phase ($p=0.16$, Figure 5A). While there appeared to be a great deal of variance in distances travelled by Amy mice that were fed the AIN93-M diet throughout the training period, this was not significant ($p=0.28$, Figure 5C). Similarly, no significant differences were detected for average speed of Amy mice throughout the training period. Collectively, this suggests that the 18 month old Amy mice that were fed the AIN93-M diet did not have intact spatial learning abilities.

Collectively, these data demonstrate that 18 month old normal and Amy mice have impaired spatial learning abilities in the MWM.

Amy mice fed the Oz-AIN diet.

A one-way ANOVA revealed Amy mice that were fed the Oz-AIN diet did not improve throughout the training period ($p=0.78$, Figure 5A). There were no significant differences in average speed travelled, indicating that the similar latencies each day reflected failure to learn the location of the platform (Figure 5B). Furthermore, Amy mice that were fed the Oz-AIN diet did not travel significantly different distances on any of the five training days ($p=0.42$, Figure 5C).

Collectively, these data indicate that 18 month old Amy mice that were fed the Oz-AIN diet did not have intact spatial learning abilities.

Figure 5A. Latency (s) for 18 month old normal and Amy mice to reach a submerged platform in the Morris Water Maze over five training days.

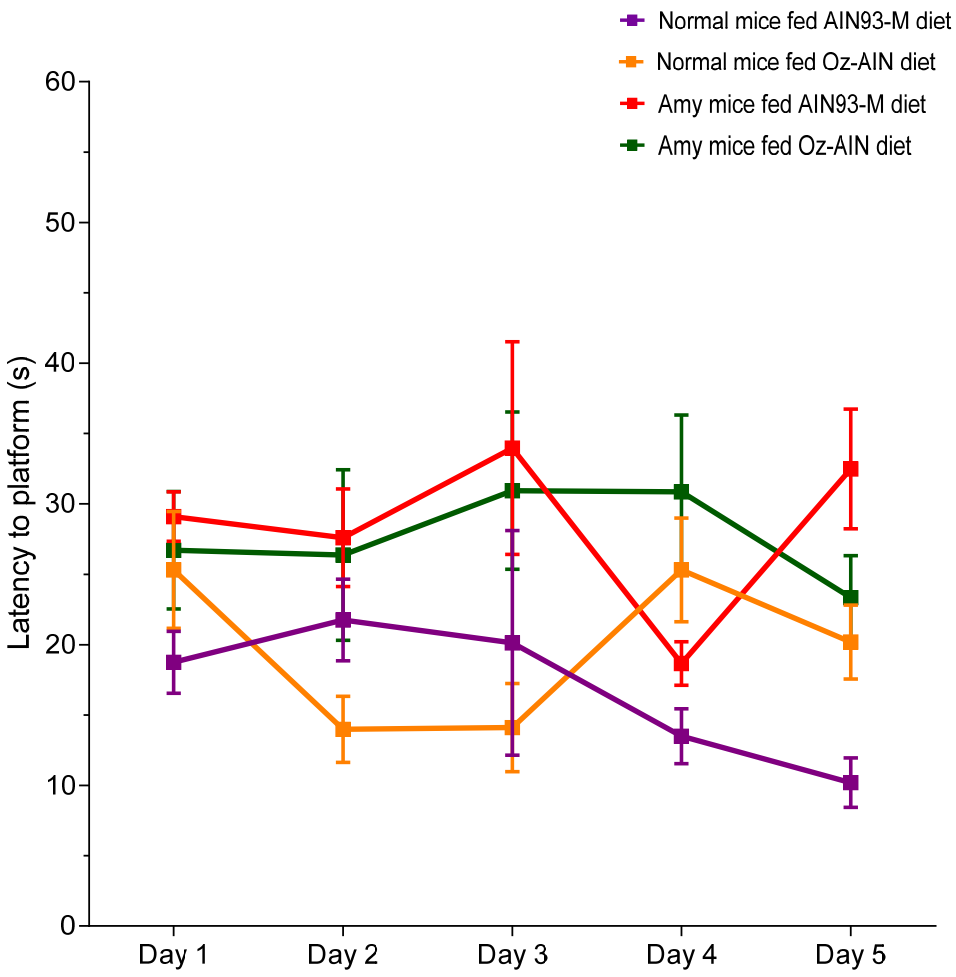


Figure 5A. The average time taken (s) to reach a submerged platform on each of the five training days when mice were 18 months old. Normal mice fed the AIN93-M diet (purple line, n=10), normal mice fed the Oz-AIN diet (orange line, n=6), Amy mice fed the AIN93-M diet (red line, n=8), and Amy mice fed the Oz-AIN diet (green line, n=6). Error bars are mean ±SEM.

Figure 5B. Distance travelled (m) before 18 month old normal and Amy mice reached a submerged platform in the Morris Water Maze over five training days.

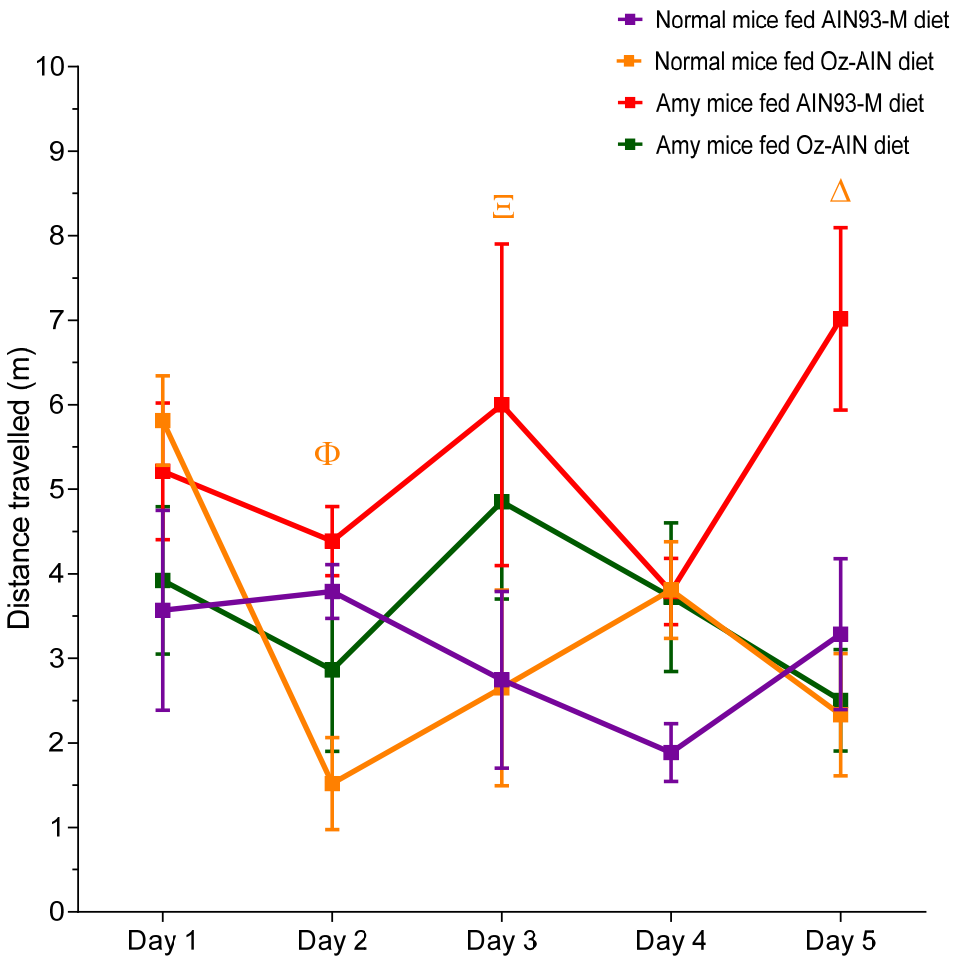


Figure 5B. The average distance travelled (m) during a swim to locate a hidden platform on each of the five training days when mice were 18 months old. Normal mice fed the AIN93-M diet (purple line, n=10), normal mice fed the Oz-AIN diet (orange line, n=6), Amy mice fed the AIN93-M diet (red line, n=8), and Amy mice fed the Oz-AIN diet (green line, n=6). Error bars are mean ±SEM. Symbols indicate significant differences compared to distance travelled on Day 1 within groups that are the same colour as the symbol, and were detected with Bonferroni post tests. (Φ) p=0.004 (orange, normal mice fed the Oz-AIN diet). (Ξ) p=0.04 (orange, normal mice fed the Oz-AIN diet). (Δ) p=0.02 (orange, normal mice fed the Oz-AIN diet).

Figure 5C. Average speed travelled (m/s) by 18 month old normal and Amy mice whilst looking for a submerged platform in the Morris Water Maze over five training days.

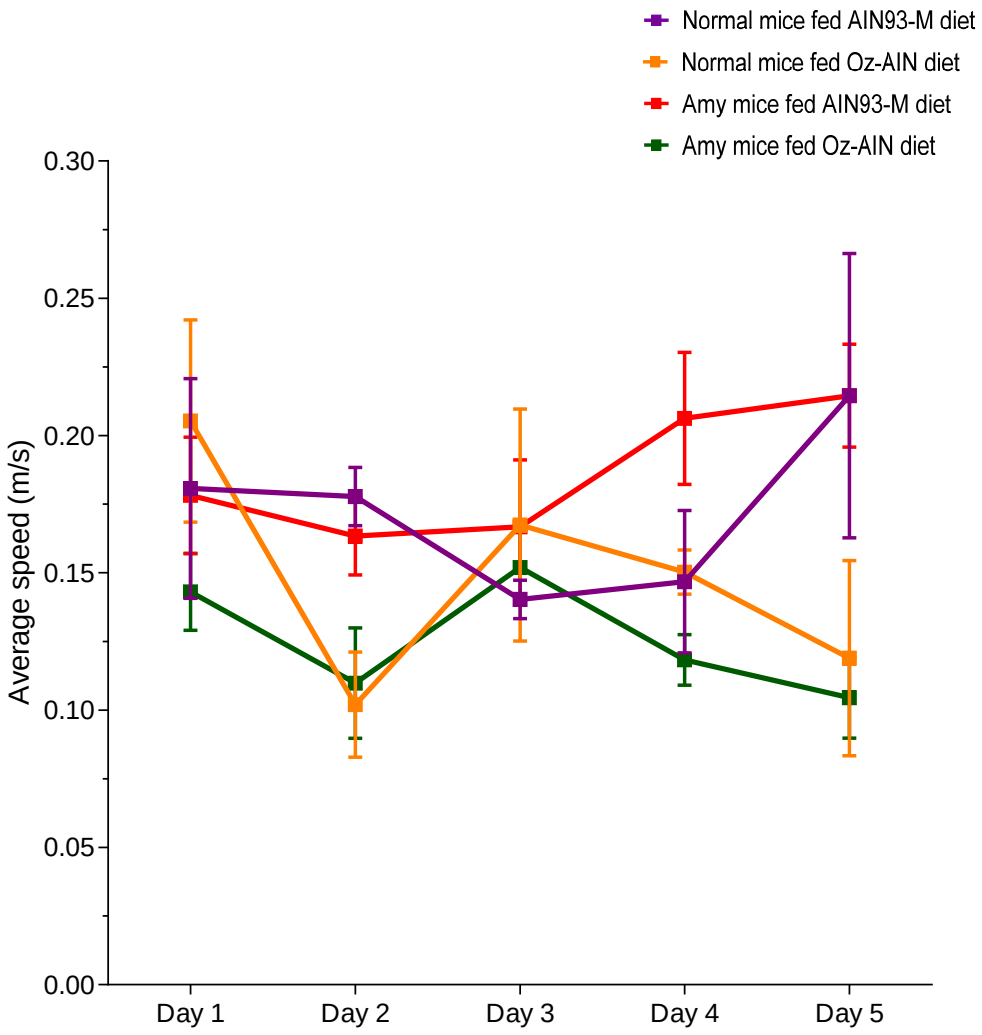


Figure 5C. The average speed (m/s) travelled to a submerged platform on each of the five training days when mice were 18 months old. Normal mice fed the AIN93-M diet (purple line, n=10), normal mice fed the Oz-AIN diet (orange line, n=6), Amy mice fed the AIN93-M diet (red line, n=8), and Amy mice fed the Oz-AIN diet (green line, n=6). Error bars are mean ±SEM.

Between group comparisons: Genotype and diet-type effects on spatial learning abilities of 18 month old normal and Amy mice.

Overall improved latency (s) to the platform after five training days.

A two-way ANOVA revealed that there were significant effects of genotype and a genotype-diet-type interaction that accounted for 24.52% and 14.9% of the variances of improved latencies between normal and Amy mice ($p=0.003$ and $p=0.016$ respectively, Table 7). Diet-type only accounted for 1.73% of variance, and was not significant ($p=0.39$, Table 7).

Normal mice that were fed the AIN93-M diet, normal mice that were fed the Oz-AIN diet and Amy mice that were fed the Oz-AIN diet reduced latencies to locate the submerged platform. However, the Amy mice that were fed the AIN93-M diet increased latency to locate the submerged platform by 3.39 ±2.90 s after five training days (Table 7). This was a significantly poorer performance than normal mice that were fed the AIN93-M diet ($p=0.0006$, Table 7), which may account for the reported genotype effects.

Table 7. Genotype and diet-type effects on overall changes in the time taken (s) by 18 month old mice to reach the submerged platform after five training days.

	Normal mice	**Amy mice**	**Difference**
AIN93-M diet	8.56 ±0.73	-3.39 ±2.90	**11.96 ±2.99** Ξ
Oz-AIN diet	5.11 ±2.28	3.63 ±1.83	**1.75 ±2.92**
Difference	**3.46 ±2.39**	**6.76 ±3.43**	

All numbers are mean ±SEM. Differences were calculated individually for each mouse and averaged to determine mean ±SEM. Genotype effects are demonstrated by comparisons across rows. Diet-type effects are demonstrated by comparisons down columns. (Ξ) $p=0.0006$.

Overall improved distance travelled (m) to the platform after five training days.

A two-way ANOVA revealed that diet-type and genotype accounted for 47.54% and 19.77% of the overall variation in changes in distance travelled by normal and Amy mice ($p<0.001$ and $p=0.0005$ respectively, Table 8).

The mice that were fed the Oz-AN diet made significantly greater improvements in distance travelled than genotype matched mice fed the AIN93-M diet ($p=0.0009$ between normal mice and $p=0.001$ between Amy mice, Table 8). This suggests that the Oz-AIN diet had a beneficial effect on spatial learning at 18 months of age.

The Amy mice tended to make smaller improvements in distance travelled before reaching the platform ($p=0.025$ between mice that were fed the AIN93-M diet, $p=0.10$ between mice that were fed the Oz-AIN diet, Table 8). This was only significant between normal and Amy mice that were fed the AIN93-M diet, and likely to be attributed to the finding that Amy mice that were fed the AIN93-M diet travelled further on Day 5 than they did on Day 1 ($p=0.025$, Table 8).

While a significant effect of diet was detected in the data describing changes of distance travelled, significant differences were not detected in the data describing improvements in latency. It is suggested that this may be attributed to the variations in speed travelled after five training days. If this was the case, then distance travelled may be the better reflection of learning, which suggests that although 18 month old Amy mice have poorer spatial learning abilities than 18 month old normal mice, the Oz-AIN diet may have a beneficial effect on spatial learning abilities.

Table 8. Genotype and diet-type effects on overall changes in the distance travelled (m) by 18 month old mice before reaching the submerged platform after five training days.

	Normal mice	Amy mice	Difference
AIN93-M diet	0.28 ±0.53	-1.80 ±0.61	**0.26 ±0.50** $^{\Delta}$
Oz-AIN diet	3.48 ±0.23	1.42 ±0.32	**2.06 ±0.55**
Difference	**3.20 ±0.58** $^{\Phi}$	**3.22 ±0.69** $^{\Xi}$	

All numbers are mean ±SEM. Differences were calculated individually for each mouse and averaged to determine mean ±SEM. Genotype effects were determined by comparisons between mice that were fed he same diet. Diet-type effects were determined by comparisons between mice that were the same genotype. (Φ) p=0.0009. (Ξ) p=0.001. (Δ) p=0.025.

5.2.3. Spatial memory in the Morris Water Maze.

Spatial memory was assessed during a Test Trial in the MWM. The platform was removed from the arena and mice were given a two minute free swim.

Within groups comparisons of the amount of time that mice spent (s) and distance travelled (m) in the Test Quadrant and the Opposite Quadrant were used to verify spatial memory deficits. Mice that demonstrated intact spatial memory spent significantly more time (s) and travelled significantly further distances (m) in the Test Quadrant than in the Opposite Quadrant.

The number of times that mice passed over the location that the platform had previously been in the pool was also compared between treatment groups to give further indication of genotype and diet-type effects on spatial memory.

5.2.3.1. Performance of 12 month old mice during the Test Trial in the Morris Water Maze.

Normal mice fed the AIN93-M diet.

The 12 month old normal mice that were fed the AIN93-M diet demonstrated intact spatial memory (Figure 6). A Student's *t*-test indicated that normal mice fed the

AIN93-M diet spent significantly longer in the Test Quadrant than in the Opposite Quadrant whilst searching for the removed platform (p=0.0004, Figure 6A). Similarly, they travelled significantly further in the Test Quadrant compared to the Opposite Quadrant (p=0.0008, Figure 6B). This indicates that the 12 month old normal mice that were fed the AIN93-M diet had intact spatial memory.

Normal mice fed the Oz-AIN diet.

A Students *t*-test revealed that the normal mice that were fed the Oz-AIN diet also spent significantly longer in the Test Quadrant than in the Opposite Quadrant whilst searching for the removed platform (p=0.005, Figure 6A). Furthermore, they travelled significantly further in the Test Quadrant whilst searching for the platform (p=0.014, Figure 6B). These data suggest that the normal mice that were fed the Oz-AIN diet had intact spatial memory at 12 months of age.

Amy mice fed the AIN93-M diet.

There were no significant differences in the distance travelled by 12 month old Amy mice that were fed the AIN93-M diet in either the Test Quadrant while searching for the platform (p=0.07, Figure 6B). However, this may not necessarily reflect mice failing to recall where the platform had been. Amy mice that were fed the AIN93-M diet may have intact spatial memory and swam slower in the Test Quadrant while they were searching for the platform. In support of this is the finding that they spent more time in the Test Quadrant than the Opposite Quadrant (p=0.019, Figure 6A) and that they crossed over the platform location with similar frequencies as other mice (Table 9).

Amy mice fed the Oz-AIN diet.

The Amy mice fed the Oz-AIN diet spent significantly more time in the Test Quadrant than the Opposite Quadrant whilst searching for the removed platform ($p=0.011$, Figure 6A), suggesting that they had intact spatial memory at 12 months of age. In support of this is the finding that they travelled significantly further in the Test Quadrant than the Opposite Quadrant ($p=0.033$, Figure 6B).

The finding that mice spent more time (Figure 6A) and travelled further distances (Figure 6B) in the Test Quadrant than the Opposite Quadrant during a 120 s Test Trial suggests that mice searched harder for the platform in the Test Quadrant than the Opposite Quadrant. This demonstrates that all 12 month old mice had intact spatial memory and recalled where the platform should have been.

Figure 6A. Time spent (s) in the Test and Opposite Quadrant during the Test Trial of the Morris Water Maze by 12 month old normal and Amy mice.

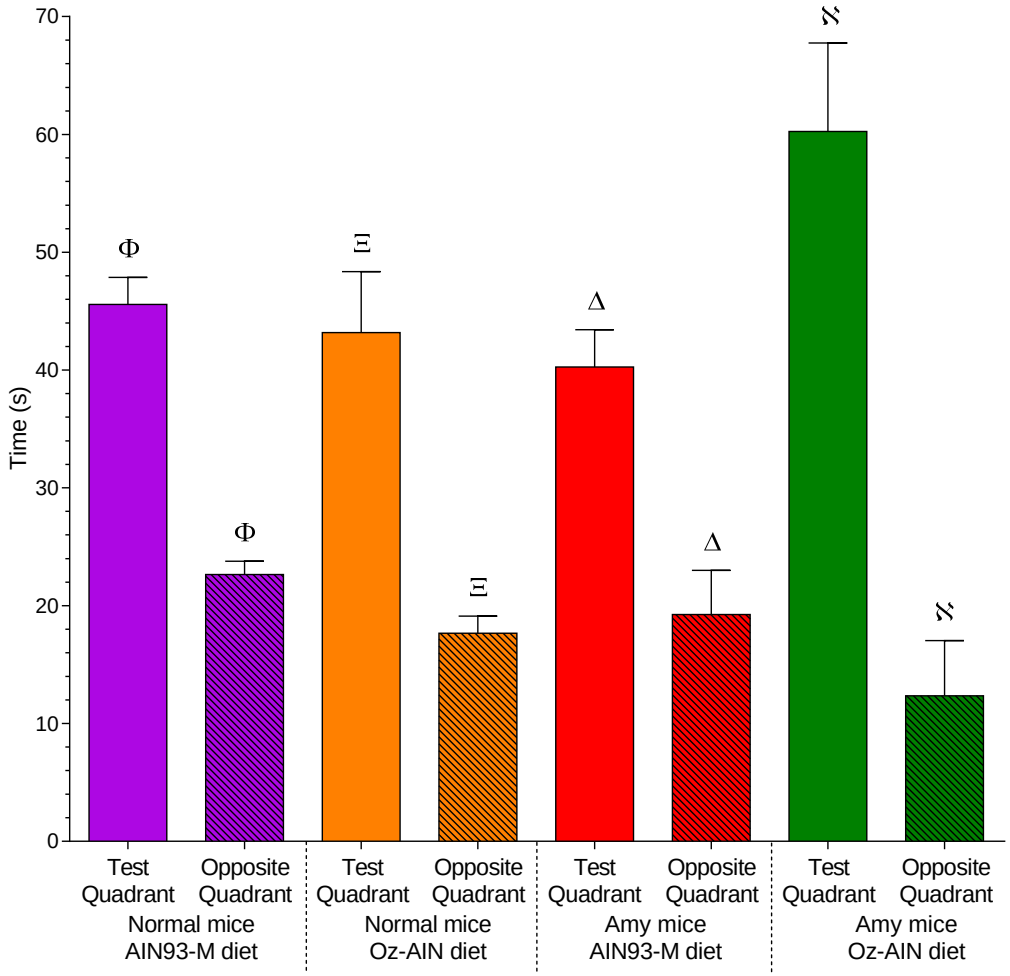


Figure 6A. Comparison of time spent (s) in the Test Quadrant (solid bars) and Opposite Quadrant (striped bars) during the Test Trial in the Morris Water Maze by 12 month old mice. Normal mice fed the AIN93-M diet (purple; n=12), normal mice fed the Oz-AIN diet (orange, n=12), Amy mice fed the AIN93-M diet (red, n=8), and Amy mice fed the Oz-AIN diet (green, n=9). Bars are mean ±SEM. Bars with matching symbols are significantly different with a paired Student's *t*-test. (Φ) p=0.0004. (Ξ) p=0.005. (Δ) p=0.019. (ϰ) p=0.011.

Figure 6B. Distance travelled (m) in the Test and Opposite Quadrant during the Test Trial of the Morris Water Maze by 12 month old normal and Amy mice.

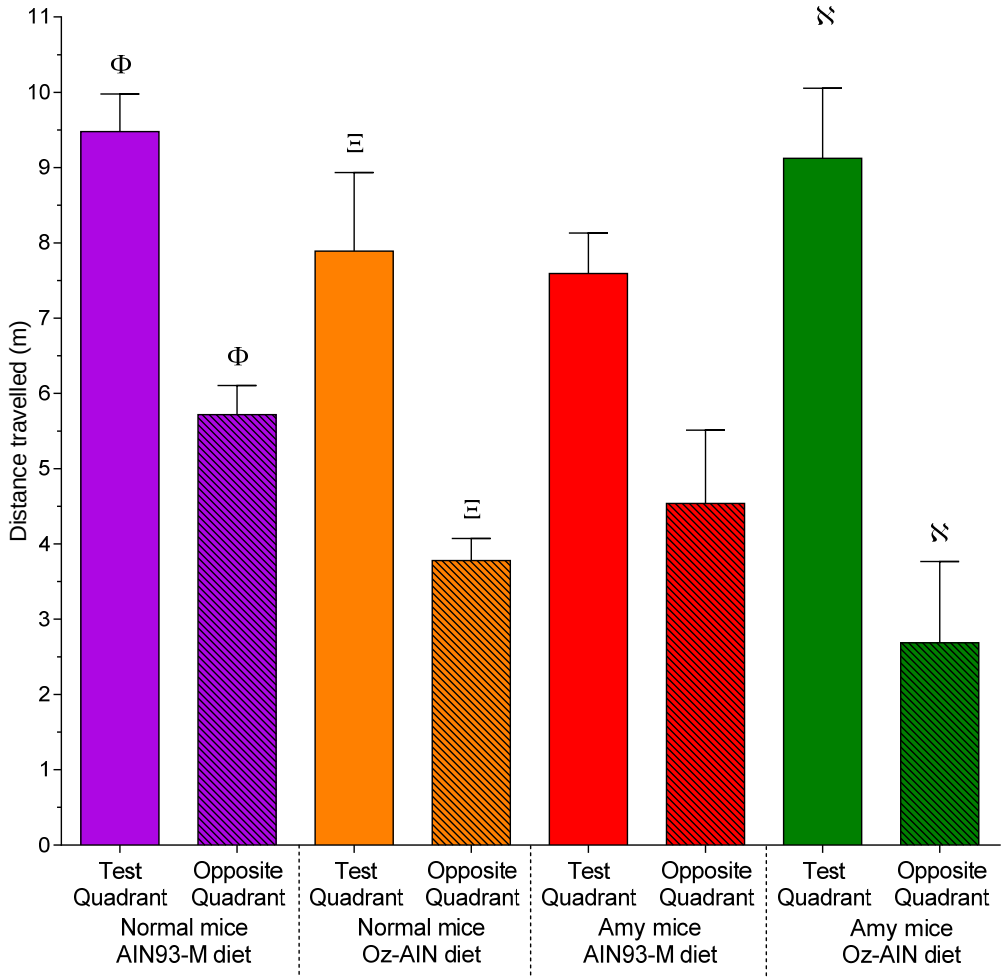


Figure 6B. Comparison of the distance travelled (m) in the Test Quadrant (solid bars) and Opposite Quadrant (striped bars) during the Test Trial in the Morris Water Maze by 12 month old mice. Normal mice fed the AIN93-M diet (purple; n=12), normal mice fed the Oz-AIN diet (orange, n=12), Amy mice fed the AIN93-M diet (red, n=8), and Amy mice fed the Oz-AIN diet (green, n=9). Bars are mean ±SEM. Bars with matching symbols are significantly different with a paired Student's *t*-test. (Φ) p=0.0008. (Ξ) p=0.014. (χ) p=0.033.

Between group comparisons: Percent of time that 12 month old normal and Amy mice spent in the Test Quadrant and Opposite Quadrant.

Percent of time spent in the Test Quadrant.

The percent of time that 12 month old mice spent in the Test Quadrant was significantly affected by diet-type (p=0.006, Table 9), genotype (p=0.0008, Table 9) and a significant diet-genotype interaction (p=0.03, Table 9). A two-way ANOVA revealed that genotype accounted for 20.83% of the overall variance of percent time spent in the Test Quadrant. Diet-type and the diet-genotype interaction accounted for 13.35% and 8.46% of variance, respectively. The genotype and diet-type effects are likely to be due to the high percentage of time spent in the Test Quadrant by 12 month old Amy mice that were fed the Oz-AIN diet (Table 9). Bonferroni post Tests revealed that Amy mice that were fed the Oz-AIN diet spent a significantly greater percentage of time in the Test Quadrant than either Amy mice that were fed the AIN93-M diet (p=0.009, Table 9) or normal mice that were fed the Oz-AIN diet (p=0.0007, Table 9). This suggests that Amy mice that were fed the Oz-AIN diet had better spatial memory than other mice at 12 months of age. There were no differences between normal mice that were fed the AIN93-M diet, normal mice that were fed the Oz-AIN diet or Amy mice that were fed the AIN93-M diet (p>0.99 all comparisons, Table 9).

Collectively, this indicates that while diet and genotype may affect the percentage time that Amy mice spend in the Test Quadrant whilst searching for the platform, they do not affect normal mice (Table 9).

Percent of time spent in the Opposite Quadrant.

The percent of time that mice spent in the Opposite Quadrant was significantly affected by diet (p=0.04, Table 9) but not genotype (p=0.12, Table 9) or a diet-genotype interaction (p=0.73, Table 9). While Bonferroni post tests did not detect significant differences between groups, the normal and Amy mice that were fed the Oz-AIN diet tended to spend less time in the Opposite Quadrant than genotype-matched mice that were fed the AIN93-M diet (Table 9). This suggests that the Oz-AIN diet may have a beneficial effect on spatial memory for 12 month old mice.

Table 9. Percentage of time that 12 month old mice spent in the Test Quadrant and Opposite Quadrant during a 120 s Test Trial.

	Normal mice AIN93-M diet	**Normal mice Oz-AIN diet**	**Amy mice AIN93-M diet**	**Amy mice Oz-AIN diet**
Test Quadrant (%)	37.97 ±1.92	35.98 ±4.33 $^{\Xi}$	33.55 ±2.64$^{\Theta}$	50.21 ±6.26$^{\Xi, \Theta}$
Opposite Quadrant (%)	18.87 ±0.94	14.72 ±1.21	16.04 ±3.13	10.29 ±3.91
Test (%) / Opposite (%)	2.06 ±0.19	2.60 ±0.45	3.11 ±0.76	12.59 ±5.00

All values are reported as mean ±SEM. The Test Quadrant / Opposite Quadrant ratio was calculated for each mouse and then averaged within each group. Numbers with matching symbols are significantly different with Bonferroni post tests. (Ξ) p=0.0007. (Θ) p=0.009.

Between group comparisons: Percent of the distance travelled by 12 month old normal and Amy mice in the Test Quadrant and Opposite Quadrant.

Percent of distance travelled in the Test Quadrant.

The percent of overall distance that the 12 month old mice spent in the Test Quadrant was significantly affected by a diet-genotype interaction (p=0.05), but not by diet-type (p=0.08) or genotype (p=0.23) alone (Table 10). A two-way ANOVA revealed that the diet-genotype interaction accounted for 9.12% of the overall variance of

distance travelled in the Test Quadrant, whilst diet-type and genotype accounted for 7.02% and 3.29% respectively (Table 10). Bonferroni post tests failed to indicate where the significant differences lay. However, the percent of distance travelled in the Test Quadrant was higher for Amy mice that were fed the Oz-AIN diet than either Amy mice that were fed the AIN93-M diet ($p=0.10$, Table 10) or normal mice that were fed the Oz-AIN diet ($p=0.12$, Table 9). There were no differences between normal mice that were fed the AIN93-M diet, the normal mice that were fed the Oz-AIN diet, or the Amy mice that were fed the AIN93-M diet ($p>0.99$ all comparisons). This suggests that the diet-genotype interaction may be due to Amy mice being more susceptible to the effects of diet than normal mice.

Percent of distance travelled in the Opposite Quadrant.

The percent of distance that the 12 month old mice spent in the Opposite Quadrant was significantly affected by diet-type ($p=0.02$, Table 10), but not by genotype ($p=0.09$, Table 10) or a diet-genotype interaction ($p=0.56$, Table 10). While Bonferroni post tests did not detect significant differences between groups, the mice that were fed the Oz-AIN diet tended to have lower percent distances than mice that were fed the AIN93-M diet. This suggests that the mice that were fed the Oz-AIN diet had a lower preference for the Opposite Quadrant whilst searching for the platform than mice that were fed the AIN93-M diet, and therefore had better spatial memory. Furthermore, the percent distance travelled in the Opposite Quadrant by Amy mice that were fed the Oz-AIN diet was smaller than that of normal mice that were fed the Oz-AIN diet, suggesting that they had better spatial memory of the two groups. This is similar to the percent of time that mice spent in the Opposite Quadrant.

Table 10. Percentage of the distance travelled by 12 month old mice in the Test Quadrant and Opposite Quadrant during a 120 s Test Trial.

	Normal mice AIN93-M diet	Normal mice Oz-AIN diet	Amy mice AIN93-M diet	Amy mice Oz-AIN diet
Test Quadrant (%)	35.04 ±1.64	34.07 ±3.89	31.92 ±2.74	46.51 ±5.90
Opposite Quadrant (%)	21.01 ±0.93	16.56 ±1.52	18.17 ±3.63	10.81 ±3.95
Test (%) / Opposite (%)	1.70 ±0.13	2.22 ±0.39 $^{\Theta}$	2.72 ±0.71$^{\Xi}$	9.46 ±3.36$^{\Xi,\Theta}$

All values are reported as mean ±SEM. The Test Quadrant / Opposite Quadrant ratio was calculated for each mouse and then averaged within each group. Numbers with matching symbols are significantly different with Bonferroni post tests. (Ξ) p=0.009. (Θ) p=0.04.

Between groups comparisons: The number of passes that 12 month old mice made over the platform location.

There were no significant effects of diet-type (p=0.99), genotype (p=0.17) or a diet-genotype interaction (p=0.34) on the number of passes over the platform location. A two-way ANOVA indicated that genotype and the diet-genotype interaction accounted for 4.90% and 2.34% of the variation of passes over the platform location, whilst diet-type accounted for less than 1% of variance (Table 11). Similarly, Bonferroni post tests did not detect significant differences between groups. This suggests that all groups of mice searched for the platform with similar of accuracy.

Table 11. Number of times that 12 month old normal and Amy mice passed over the platform location during a 120 s Test Trial.

	Normal mice AIN93-M diet	Normal mice Oz-AIN diet	Amy mice AIN93-M diet	Amy mice Oz-AIN diet
Platform crossings	7.00 ±1.00	8.14 ±1.39	6.50 ±0.96	5.40 ±0.87

All values are reported as mean ±SEM.

Summary of Spatial Memory of 12 month old mice.

Collectively, these data indicate that all 12 month old mice had intact spatial memory and that genotype and diet-type had significant effects on spatial memory. The Oz-AIN diet may conserve spatial memory in Amy mice, as demonstrated by percentage time and distance spent in the Test Quadrant. However, accuracy of recollection of the platform location or search strategy was not conserved, as there were no differences between treatment groups on the number of passes over the platform location. The potentially protective effect of the Oz-AIN diet on spatial memory in 12 month old Amy mice is addressed further in the discussion.

5.2.3.2. Performance of 15 month old mice during the Test Trial in the Morris Water Maze.

Normal mice fed the AIN93-M diet.

A Students *t*-test revealed that the 15 month old normal mice fed the AIN93-M diet spent significantly more time in the Test Quadrant than in the Opposite Quadrant whilst searching for the submerged platform ($p=0.006$, Figure 7A). Similarly, they travelled further in the Test Quadrant than the Opposite Quadrant (7.53 ±0.93 m and 4.88 ±0.74 m respectively, Figure 7B) However, this was not significant ($p=0.12$, Figure 7B).

These data suggest that normal mice that were fed the AIN93-M diet had intact spatial memory at 15 months of age.

Normal mice fed the Oz-AIN diet.

A Student's *t*-test revealed that the 15 month old normal mice that were fed the Oz-AIN diet spent significantly more time in the Test Quadrant than the Opposite

Quadrant (p=0.0001, Figure 7A). Furthermore, the normal mice that were fed the Oz-AIN diet were the only mice that covered a significantly greater distance in the Test Quadrant than in the Opposite Quadrant whilst searching for the platform at 15 months of age (p=0.002, Figure 7B).

These data suggest that normal mice that were fed the Oz-AIN diet had intact spatial memory at 15 months of age.

Amy mice fed the AIN93-M diet.

A Student's *t*-test revealed that the 15 month old Amy mice fed the AIN93-M diet spent significantly more time in the Test Quadrant than in the Opposite Quadrant whilst searching for the removed platform (p=0.005, Figure 7A). While they also swam further in the Test Quadrant than in the Opposite Quadrant, this was not significant (p=0.10, Figure 7B).

These data suggest that Amy mice that were fed the AIN93-M diet had intact spatial memory at 15 months of age.

Amy mice fed the Oz-AIN diet.

A Student's *t*-test did not detect significant differences in the time that Amy mice that were fed the Oz-AIN diet spent in either the Test Quadrant or the Opposite Quadrant (p=0.17, Figure 7A). Furthermore, the Amy mice that were fed the Oz-AIN diet travelled equal distances in the Test and Opposite Quadrants whilst searching for the removed platform (p=0.91, Figure 7B).

These data indicate that 15 month old Amy mice that were fed the Oz-AIN diet did not have intact spatial memory.

Figure 7A. Time spent (s) in the Test and Opposite Quadrant during the Test Trial of the Morris Water Maze by 15 month old normal and Amy mice.

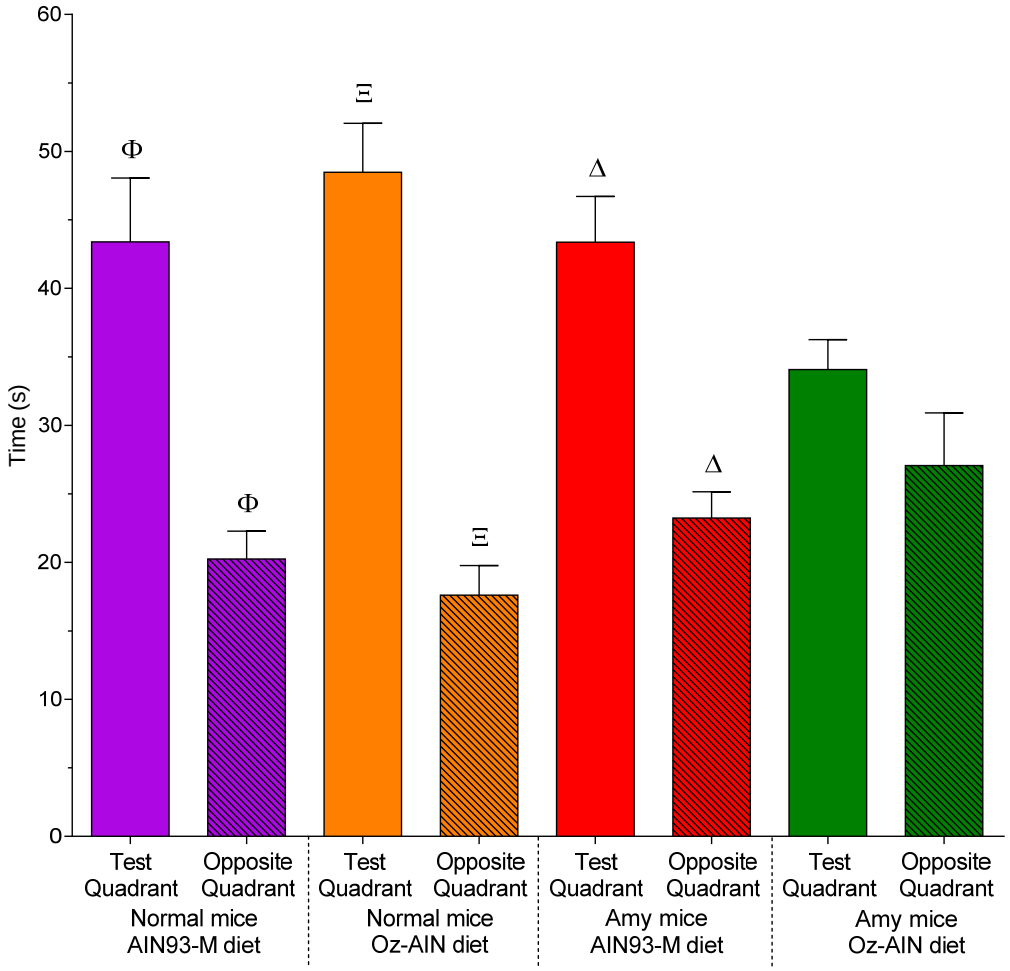


Figure 7A. Comparison of time spent (s) in the Test Quadrant (solid bars) and Opposite Quadrant (striped bars) during the Test Trial in the Morris Water Maze by 15 month old mice. Normal mice fed the AIN93-M diet (purple, n=12), normal mice fed the Oz-AIN diet (orange, n=11), Amy mice fed the AIN93-M diet (red, n=8), and Amy mice fed the Oz-AIN diet (green, n=9). Bars are mean ±SEM. Bars with matching symbols are significantly different with a paired Student's *t*-test. (Φ) p=0.06. (Ξ) p=0.0001. (Δ) p=0.005.

Figure 7B. Distance travelled (m) in the Test and Opposite Quadrant during the Test Trial of the Morris Water Maze by 15 month old normal and Amy mice.

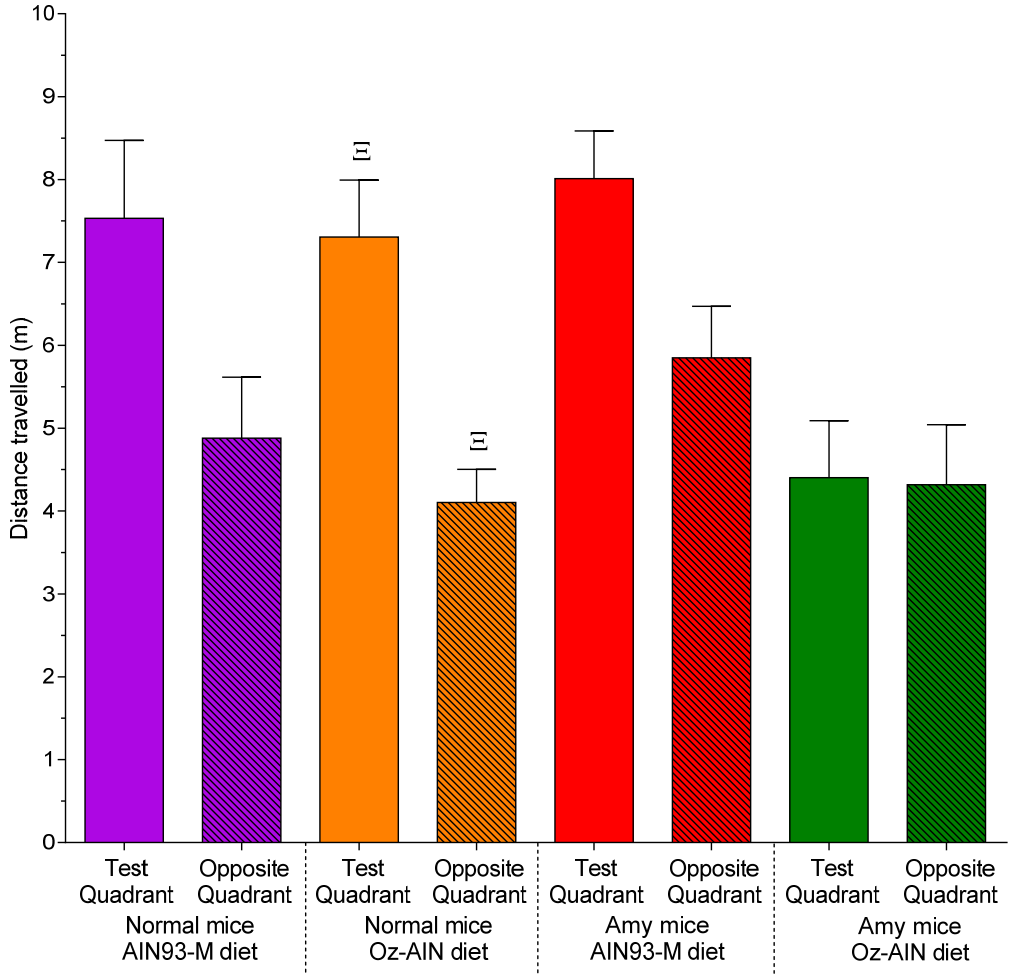


Figure 7B. Comparison of the distance travelled (m) in the Test Quadrant (solid bars) and Opposite Quadrant (striped bars) during the Test Trial in the Morris Water Maze by 15 month old mice. Normal mice fed the AIN93-M diet (purple, n=12), normal mice fed the Oz-AIN diet (orange, n=11), Amy mice fed the AIN93-M diet (red, n=8), and Amy mice fed the Oz-AIN diet (green, n=9). Bars are mean ±SEM. Bars with matching symbols are significantly different with a paired Student's *t*-test. (Ξ) p=0.002.

Between group comparisons: Percent of time that 15 month old normal and Amy mice spent in the Test Quadrant and Opposite Quadrant.

Percent of time spent in the Test Quadrant.

A two-way ANOVA did not detect significant effects of diet ($p=0.59$), genotype ($p=0.07$) or a diet-genotype interaction ($p=0.07$). Bonferroni post tests also did not detect significant differences between groups. However, the Amy mice that were fed the Oz-AIN diet spent less time in the Test Quadrant than normal mice that were fed the Oz-AIN diet, suggesting that they may have poorer memory for the platform location ($p=0.07$, Table 12).

Percent of time spent in the Opposite Quadrant.

A two-way ANOVA detected that genotype accounted for 13.41% of the variance in the amount of time spent in the Opposite Quadrant ($p=0.02$, Table 12). Diet-type and a genotype-diet-type interaction only accounted for 0.12% and 3.61% respectively, and were not significant ($p=0.82$ and $p=0.22$ respectively, Table 12).

Bonferroni post tests failed to indicate significant differences between groups. However, the Amy mice that were fed the Oz-AIN diet spent more time searching the Opposite Quadrant for the platform than normal mice that were fed the Oz-AIN diet ($p=0.08$, Table 12). While this is not significant at $p<0.05$, it would be significant at $p<0.10$, which suggests there may be weak trends for diet-type to have a detrimental effect on spatial memory in Amy mice.

There were no other differences detected between groups of mice, suggesting that Amy mice that were fed the Oz-AIN diet were the only ones to be effected by diet (Table 12).

Table 12. Percentage of time that 15 month old mice spent in the Test Quadrant and Opposite Quadrant during the 120 s Test Trial.

	Normal mice AIN93-M diet	Normal mice Oz-AIN diet	Amy mice AIN93-M diet	Amy mice Oz-AIN diet
Test Quadrant (%)	36.16 ±3.88	40.39 ±2.98	36.15 ±2.79	28.40 ±1.83
Opposite Quadrant (%)	16.87 ±1.70	14.67 ±1.81	19.37 ±1.59	22.56 ±3.2
Test (%) / Opposite (%)	2.55 ±0.66	3.30 ±0.51	2.06 ±0.37	1.47 ±0.23

All values are reported as mean ±SEM. The Test Quadrant / Opposite Quadrant ratio was calculated for each mouse and then averaged within each group.

Between group comparisons: Percent of the distance travelled by 15 month old normal and Amy mice in the Test Quadrant and Opposite Quadrant.

Percent of distance travelled in the Test Quadrant.

A two-way ANOVA did not detect significant effects of genotype ($p=0.46$), diet-type ($p>0.99$) or a genotype-diet-type interaction ($p=0.27$). Genotype accounted for 1.46% of the variance of percent distance travelled, whilst diet-type and the genotype-diet-type interaction accounted for <0.1% and 3.35% respectively (Table 13). Similarly, Bonferroni post tests did not detect significant differences between groups.

Percent of distance travelled in the Opposite Quadrant.

Similar to the findings reported above for the amount of time spent in the Opposite Quadrant, a two-way ANOVA detected a significant effect of genotype on percentage distance travelled in the Opposite Quadrant ($p=0.02$, Table 13). Genotype accounted for 13.75% of the overall variance of percent distance travelled in the Opposite Quadrant. Diet-type and the genotype-diet-type interaction accounted for 2.17% and

2.66% of variation respectively, and were not significant (p=0.33 and p=0.29 respectively).

Bonferroni post tests did not detect significant differences. However, they did reveal non-significant trends between normal and Amy mice that were fed the Oz-AIN diet. The percentage distance travelled in the Opposite Quadrant by Amy mice that were fed the Oz-AIN diet was greater than that of normal mice that were fed the Oz-AIN diet (p=0.10, Table 12). While these trends were weak, they suggest that Amy mice that were fed the Oz-AIN diet were more susceptible to the detrimental effects of the Oz-AIN diet than normal mice.

Table 13. Percentage of the total distance travelled by 15 month old mice in the Test Quadrant and Opposite Quadrant during 120 s Test Trial.

	Normal mice AIN93-M diet	**Normal mice Oz-AIN diet**	**Amy mice AIN93-M diet**	**Amy mice Oz-AIN diet**
Test Quadrant (%)	30.50 ±3.92	33.95 ±2.77	31.69 ±2.70	28.15 ±1.17
Opposite Quadrant (%)	19.17 ±2.53	18.91 ±1.44	22.64 ±2.01	27.82 ±3.56
Test (%) / Opposite (%)	5.03 ±3.21	1.92 ±0.22	1.58 ±0.30	1.14 ±0.17

All values are reported as mean ±SEM. The Test Quadrant / Opposite Quadrant ratio was calculated for each mouse and then averaged within each group.

Between groups comparisons: The number of passes that 15 month old mice made over the platform location.

A two-way ANOVA did not detect significant effects of genotype (p=0.10, Table 14), diet-type (p=0.75, Table 14) or a genotype-diet-type interaction (p=0.78, Table 14) on the number of times that mice passed over the platform location whilst searching for the submerged platform. While Amy mice that were fed the Oz-AIN diet passed over the platform location fewer timed than either normal mice that were fed the Oz-AIN

diet or Amy mice that were fed the AIN93-M diet, this was not significant ($p=0.70$ and $p>0.99$ respectively, Table 14). This suggests that there were no differences in spatial memory abilities of 15 month old mice.

Table 14. Number of times that 15 month old normal and Amy mice passed over the platform location during a 120 s Test Trial.

	Normal mice AIN93-M diet	Normal mice Oz-AIN diet	Amy mice AIN93-M diet	Amy mice Oz-AIN diet
Platform crossings	6.17 ±0.86	6.18 ±0.81	5.25 ±0.49	4.33 ±0.87

All values are reported as mean ±SEM.

Summary of Spatial Memory of 15 month old mice.

Collectively, these data suggest that genotype, but nor diet-type, has an effect on spatial memory in 15 month old mice. All mice searched the Test Quadrant with similar levels of accuracy. However, the Amy mice that were fed the Oz-AIN spent a greater percentage of time and distance in the Opposite Quadrant than normal mice that were fed the Oz-AIN diet, suggesting that they had a greater preference for the incorrect quadrant than normal mice. This genotype effect was specific to mice that were fed the Oz-AIN diet, as no differences were detected between normal and Amy mice that were fed the AIN93-M diet.

Furthermore, the 15 month old Amy mice that were fed the Oz-AIN diet were the only group of mice that failed to demonstrate spatial memory, suggesting that they were the most severely affected by diet-type and genotype.

5.2.3.3. Performance of 18 month old mice during the Test Trial in the Morris Water Maze.

Normal mice fed the AIN93-M diet.

Although the normal mice that were fed the AIN93-M diet spent more time in the Test Quadrant than in the Opposite Quadrant, this failed to achieve significance at $p<0.05$ ($p=0.07$, Figure 8A). The normal mice that were fed the AIN93-M diet also travelled further in the Test Quadrant than the Opposite Quadrant. However this also failed to reach significance ($p=0.09$, Figure 8B).

These data suggest that the normal mice that were fed the AIN93-M diet may have had impaired spatial memory.

Normal mice fed the Oz-AIN diet.

Student's *t*-tests revealed that the normal mice that were fed the Oz-AIN diet spent significantly longer in the Test Quadrant than the Opposite Quadrant whilst searching for the platform ($p=0.005$, Figure 8A). Similarly, normal mice that were fed the Oz-AIN diet travelled significantly further in the Test Quadrant than in the Opposite Quadrant whilst searching for the removed platform ($p=0.005$, Figure 8B).

These data suggest that the normal mice that were fed the Oz-AIN diet had intact spatial memory at 18 months of age.

Amy mice fed the AIN93-M diet.

Student's *t*-tests did not detect significant differences in the amount of time the Amy mice that were fed the AIN93-M diet spent in the Test and Opposite Quadrant ($p=0.62$, Figure 8A). Furthermore, Amy mice that were fed the AIN93-M diet travelled equal distances in both quadrants whilst searching for the removed platform

(p=0.68, Figure 8B). These data suggest that Amy mice that were fed the AIN93-M diet did not have intact spatial memory at 18 months of age.

Amy mice fed the Oz-AIN diet.

Amy mice that were fed the Oz-AIN diet spent significantly longer in the Test Quadrant than the Opposite Quadrant whilst searching for the removed platform (p=0.024, Figure 8A). Furthermore, the Amy mice that were fed the Oz-AIN diet swam further in the Test Quadrant than the Opposite Quadrant. However, this was not significant (p=0.08, Figure 8B). These data suggest that 18 month old Amy mice fed the Oz-AIN diet may have intact spatial memory.

Figure 8A. Time spent (s) in the Test and Opposite Quadrant during the Test Trial of the Morris Water Maze by 18 month old normal and Amy mice.

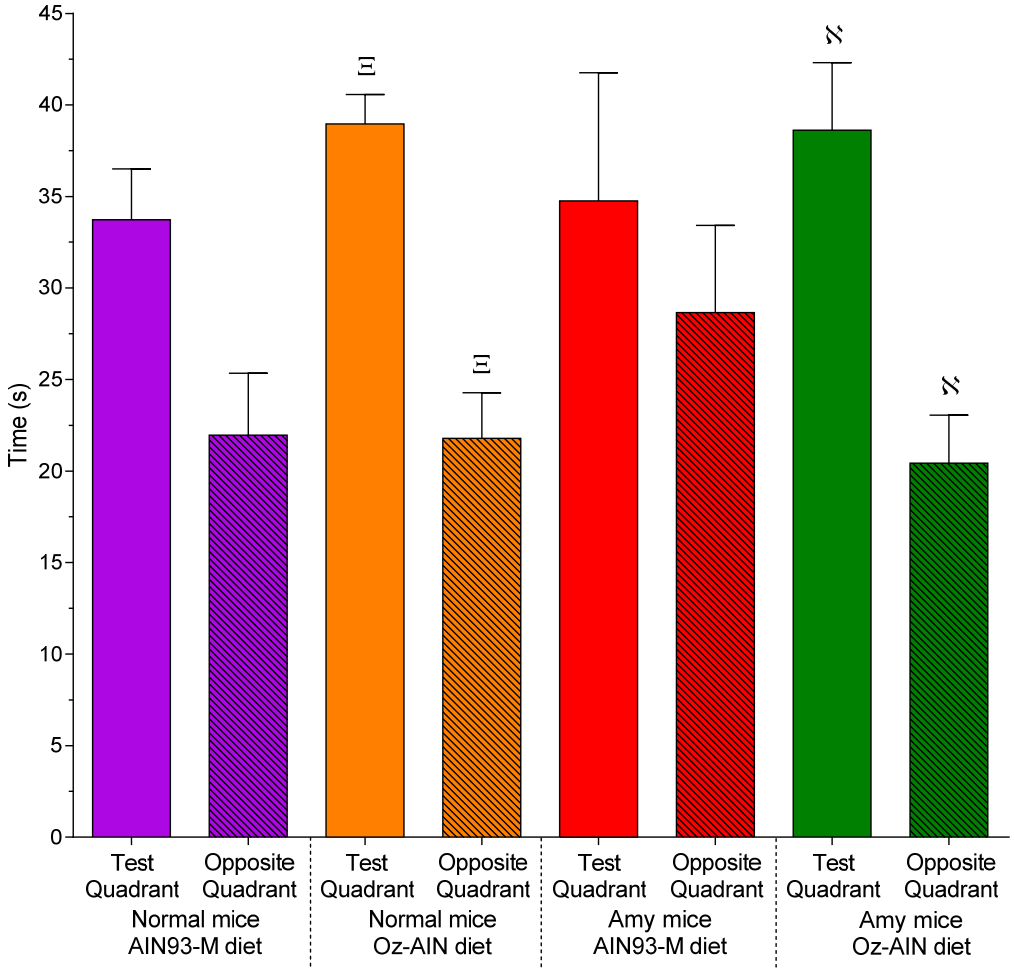


Figure 8A. Comparison of time spent (s) in the Test Quadrant (solid bars) and Opposite Quadrant (striped bars) during the Test Trial of the Morris Water Maze by 18 month old mice. Normal mice fed the AIN93-M diet (purple, n=10), normal mice fed the Oz-AIN diet (orange, n=6), Amy mice fed the AIN93-M diet (red; n=8), and Amy mice fed the Oz-AIN diet (green, n=6). Bars are mean ±SEM. Bars with matching symbols are significantly different with a paired Student's *t*-test. (Ξ) p=0.005. (χ) p=0.024.

Figure 8B. Distance travelled (m) in the Test and Opposite Quadrant during the Test Trial of the Morris Water Maze by 18 month old normal and Amy mice.

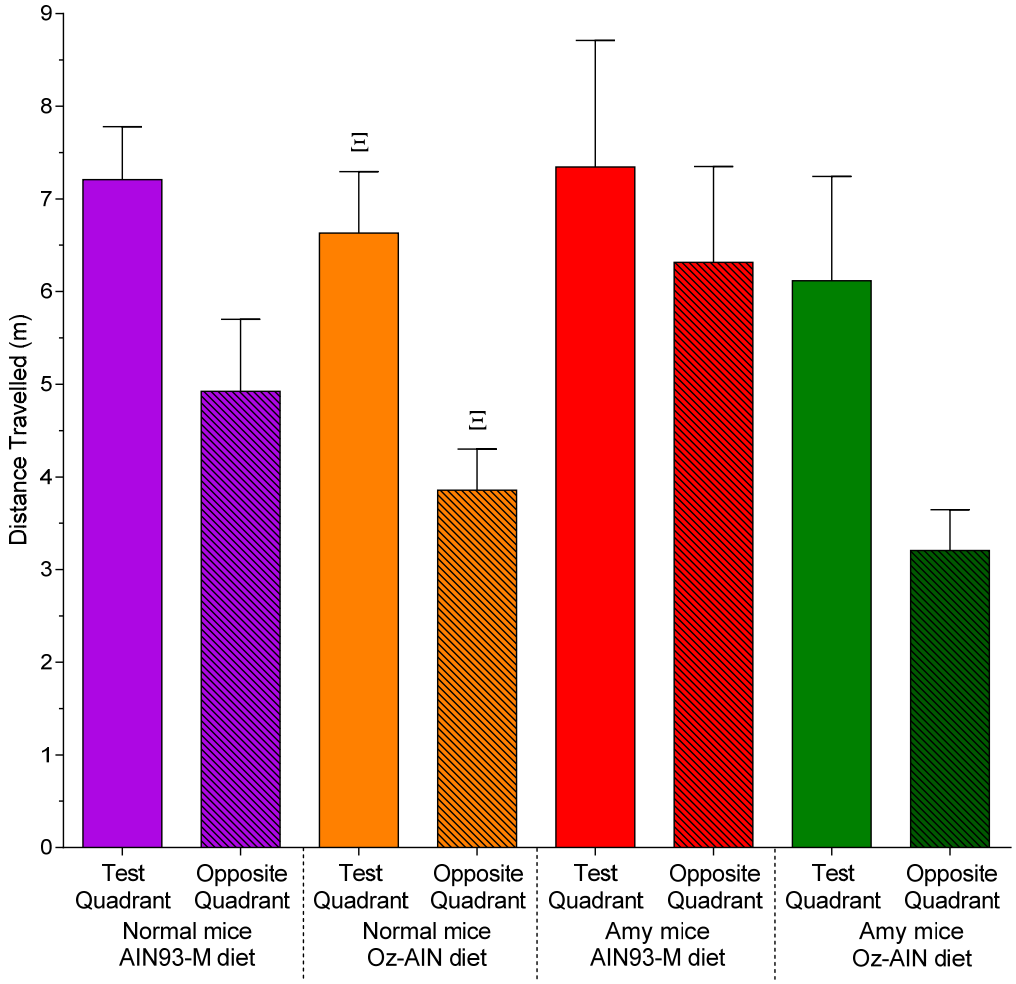


Figure 8B. Comparison of the distance travelled in the Test Quadrant (solid bars) and Opposite Quadrant (striped bars) during the Test Trial of the Morris Water Maze by 18 month old mice. Normal mice fed the AIN93-M diet (purple, n=10), normal mice fed the Oz-AIN diet (orange, n=6), Amy mice fed the AIN93-M diet (red; n=8), and Amy mice fed the Oz-AIN diet (green, n=6). Bars are mean ±SEM. Bars with matching symbols are significantly different with a paired Student's *t*-test. (Ξ) p=0.005.

Between group comparisons: Percent of time that 18 month old normal and Amy mice spent in the Test Quadrant and Opposite Quadrant.

Percent of time spent in the Test Quadrant.

A two-way ANOVA did not detect significant effects of genotype (p=0.94, Table 15), diet-type (p=0.33, Table 15), or a genotype-diet-type interaction (p=0.88, Table 15) on the percent of time that 18 month old mice spent in the Test Quadrant. Diet-type accounted for the greatest degree of variance, but this was only 3.63%. Genotype and the genotype-diet-type interaction only accounted for 0.02% and 0.83% respectively (Table 15). This suggests that neither diet-type nor genotype influenced accuracy for recollection of the platform location.

Bonferroni post tests also did not detect significant differences between groups, supporting the finding above, that neither genotype nor diet-type had an effect on the percent time that mice spent searching the correct quadrant for the removed platform.

Percent of time spent in the Opposite Quadrant.

A two-way ANOVA did not detect significant effects of genotype (p=0.44, Table 15), diet-type (p=0.22, Table 15) or a genotype-diet-type interaction (p=0.44, Table 15). The Amy mice that were fed the Oz-AIN diet a lower percentage of time in the Opposite Quadrant than other mice (17.03 ±2.19%, Table 15). In contrast to this, the Amy mice that were fed the AIN93-M diet spent the highest percentage of time in the Opposite Quadrant, compared to other mice (23.89 ±3.96%, Table 15). While this may suggest that diet-type had an effect on preference for the incorrect quadrant, the difference between the two was not significant (p=0.69).

Collectively, these data suggest that genotype, diet-type or the genotype-diet-type interaction do not affect the percentage of time spent in either the Test Quadrant or the Opposite Quadrant whilst mice search for the removed platform.

Table 15: Percentage of time that 18 month old mice spent in the Test Quadrant and Opposite Quadrant of the Morris Water Maze during a 120 s Test Trial.

	Normal mice AIN93-M diet	Normal mice Oz-AIN diet	Amy mice AIN93-M diet	Amy mice Oz-AIN diet
Test Quadrant (%)	28.11 ±2.31	32.47 ±1.34	28.97 ±5.83	32.18 ±3.08
Opposite Quadrant (%)	18.31 ±2.81	18.25 ±2.08	23.89 ±3.96	17.03 ±2.19
Test (%) / Opposite (%)	2.05 ±0.44	1.92 ±0.24	50.58 ±49.63	2.33 ±0.75

All values are reported as mean ±SEM.

Between group comparisons: Percent of the distance travelled by 12 month old normal and Amy mice in the Test Quadrant and Opposite Quadrant.

Percent of distance travelled in the Test Quadrant.

A two-way ANOVA did not detect significant effects of genotype ($p=0.82$, Table 16) diet-type ($p=0.24$, Table 16) or a genotype-diet-type interaction ($p=0.90$, Table 16) on the percentage distance travelled in the Test Quadrant by normal and Amy mice. Diet-type accounted for the greatest degree of variance, but this was only 3.71%. Genotype and the genotype-diet-type interaction only accounted for 0.14% and 0.04% respectively (Table 16). This suggests that neither diet-type nor genotype influenced accuracy for recollection of the platform location.

Bonferroni post tests also did not detect differences between groups ($p>0.99$ for all comparisons), supporting the findings above that neither diet-type nor genotype have

an effect on the percent of time spent in the Test Quadrant whilst searching for the removed platform (Table 16).

Percent of distance travelled in the Opposite Quadrant.

A two-way ANOVA did not detect significant effects of genotype (p=0.44, Table 16), diet-type (p=0.22, Table 16) or a genotype-diet-type interaction (p=0.31, Table 16) on the percent distance travelled in the Opposite Quadrant. Similar to the data describing percent time spent in the Opposite Quadrant, the Amy mice that were fed the Oz-AIN diet had a lower percentage of distance travelled in the Opposite Quadrant compared to other mice, and Amy mice that were fed the AIN93-M diet had the highest percentage of distance in the Opposite Quadrant (Table 16). While this suggests that diet-type had an effect on the distance that Amy mice travelled in the Opposite Quadrant, the difference between these two groups was not significant (Table 16).

Table 16: Percentage of the total distance travelled by 18 month old mice in the Test Quadrant and Opposite Quadrant of the Morris Water Maze during a 120 s Test Trial.

	Normal mice AIN93-M diet	**Normal mice Oz-AIN diet**	**Amy mice AIN93-M diet**	**Amy mice Oz-AIN diet**
Test Quadrant (%)	28.01 ±2.48	31.51 ±1.10	28.36 ±4.79	32.66 ±4.79
Opposite Quadrant (%)	19.23 ±3.24	18.55 ±2.04	24.80 ±4.29	17.79 ±2.57
Test (%) / Opposite (%)	1.95 ±0.40	1.83 ±0.24	44.89 ±43.96	2.28 ±0.81

All values are reported as mean ±SEM.

Between groups comparisons: The number of passes that 18 month old mice made over the platform location.

A two-way ANOVA revealed that there were no effects of genotype (p=0.30, Table 17), diet-type (p=0.20, Table 17) or a genotype-diet-type interaction (p=0.58, Table 17) on the number of times that 18 month old mice passed over the platform location whilst searching for the removed platform. This is consistent with the results above that report that neither genotype nor diet-type have an effect on spatial memory in 18 month old normal and Amy mice.

Table 17: Number of times that 18 month old normal and Amy mice passed over the platform location during a 120 s Test Trial.

	Normal mice AIN93-M diet	Normal mice Oz-AIN diet	Amy mice AIN93-M diet	Amy mice Oz-AIN diet
Platform crossings	5.90 ±0.64	6.67 ±1.23	4.25 ±1.11	6.17 ±0.91

All values are reported as mean ±SEM.

Summary of Spatial Memory of 18 month old mice.

Collectively, these data indicate there are no genotype, diet-type or genotype-diet-type interaction effects on spatial memory in 18 month old mice. However, irrespective of genotype, the mice that were fed the Oz-AIN diet were the only mice that demonstrated intact spatial memory, suggesting that the Oz-AIN diet may have a protective effect on spatial memory in 18 month old mice.

5.2.4. Summary of Results.

The following tables present a summary of the results from the MWM. The discussion section corresponds with the tables below.

Table 18. Genotype effects on spatial learning and spatial memory in the MWM

Spatial learning	*12 months:* Genotype did not have an effect on overall improvements made by 12 month old normal and Amy mice that were fed the AIN93-M diet. Amy mice fed the Oz-AIN diet were the only mice to make significant improvements in latency and distance travelled, suggesting that genotype *may* affect spatial learning of mice fed the Oz-AIN diet. *15 months*: Genotype did not have an effect on spatial learning abilities between 15 month old mice fed either the AIN93-M or the Oz-AIN diet. *18 months:* Genotype had an effect on spatial learning ability between 18 month old mice that were fed the AIN93-M diet. Normal mice fed the Oz-AIN diet were the only mice to make significant improvements throughout the training period, suggesting that genotype *may* affect spatial learning abilities of mice fed the Oz-AIN diet. However, there were no significant differences in overall improvement.
Spatial memory	*12 months:* Genotype did not have an effect on spatial memory between 12 month old mice fed either the AIN93-M diet or the Oz-AIN diet. *15 months:* Genotype did not have an effect on spatial memory between 15 month old mice the AIN93-M diet. Genotype did have an effect on spatial memory between 15 month old mice that were fed the Oz-AIN diet. Normal mice fed the Oz-AIN diet had better spatial memory than Amy mice fed the Oz-AIN diet. *18 months:* There were no effects of genotype between 18 month old mice that were fed the AIN93-M diet or 18 month old mice that were fed the Oz-AIN diet.

Table 19. Diet-type effects on spatial learning and spatial memory.

Spatial learning	*12 months* Diet-type did not have an effect on spatial learning abilities of normal mice. Amy mice fed the Oz-AIN diet were the only mice to make significant improvements in latency and distance travelled, suggesting that diet-type *may* affect spatial learning of mice fed the Oz-AIN diet. *15 months* Diet-type did not have an effect on spatial learning abilities of 15 month old normal or Amy mice. *18 months* Diet-type had an effect on spatial learning. Normal and Amy mice that were fed the Oz-AIN diet made greater improvements than genotype matched mice fed the AIN93-M diet, suggesting that the Oz-AIN diet may conserve spatial learning abilities in 18 month old mice.
Spatial memory	*12 months* Diet-type effects were genotype dependent, and only affected spatial memory abilities of Amy mice. The Oz-AIN diet may have had a protective effect on spatial memory in 12 month old Amy mice. *15 months* Diet-type did not have an effect on spatial memory between 15 month old normal mice. Diet-type had an effect on spatial memory between Amy mice. Amy mice that were fed the Oz-AIN diet failed to demonstrate spatial memory. *18 months* There were no significant diet-type effects on spatial memory of normal or Amy mice. However, irrespective of genotype, mice fed the Oz-AIN diet were the only mice to demonstrate spatial memory. This suggests that diet-type effects did exist and that the Oz-AIN diet conserves spatial memory in 18 month old mice.

5.3. Discussion.

Consistent with the aims of this chapter, the effects of genotype and diet-type on spatial learning and spatial memory were assessed in 12, 15 and 18 month old normal and Amy mice.

The 12 month old Amy mice that were fed the Oz-AIN diet were the only mice that demonstrated intact spatial learning. At no other age did any of the treatment groups demonstrate significant spatial learning abilities in the MWM. This makes it hard to demonstrate significant effects of diet-type and genotype on spatial learning. Nonetheless, changes in latencies and distance throughout the acquisition period will be compared and discussed in further detail, with possible explanations as to why spatial learning was not observed.

Despite mice failing to demonstrate spatial learning at any age, mice demonstrated intact spatial memory abilities. While spatial learning and spatial memory are both hippocampal dependent, they are independent processes and may not reflect similar neurobiological dysfunction [155]. Improvements throughout the acquisition phase reflect spatial learning and plasticity, de Fiebre *et al*. suggest that performance in the Test Trial may reflect differences in efficiency to reach the platform. As a consequence, a mouse may have poor spatial learning abilities, but demonstrate what appears to be better spatial memory due to superior problem solving and path efficiency [155]. While mice in the current study failed to demonstrate spatial learning, it is still valuable to evaluate the effects of genotype and diet-type on spatial memory, as this may involve different neural pathways.

Genotype effects: Spatial learning in the Morris Water Maze.

Consistent with the first aim of this chapter, genotype effects on spatial learning abilities of normal and Amy mice were assessed in the MWM at 12, 15 and 18 months.

The finding that at 12 months, the Amy mice fed the Oz-AIN diet were the only group of mice that had intact spatial learning abilities, contradicts reports from others that Amy mice develop spatial learning deficits by the time they are 8 months old [192], especially considering that high-fat diets impair spatial learning and memory in AD-type mice [472, 473]. However, many research groups also point out that amyloid pathology and behavioural deficits do not always correlate in AD-mouse models. The Amy mouse model, for example, does not reflect AD *per se*, but is a mouse model of amyloid over-expression, which is a common feature in AD. Hardy and Selkoe point out that one of the greatest concerns with amyloid mouse models is that there is no clear correlation with amyloid load and cognitive decline in either amyloid mouse models or AD patients, and that AD-mouse models that do not have deficits in amyloid expression often show more severe cognitive decline [474]. The current findings suggest that a sub-optimal, high-fat diet (the Oz-AIN diet) may have had deleterious effects on learning abilities of 12 month old normal mice, but that it may also have beneficial effects on learning abilities of Amy mice, so that Amy mice do not lose spatial learning abilities as young as they would on a standard diet. This suggests that response to diet may be genotype dependent.

A phenotype-dependent response to dietary nutrients was first reported by Minihane *et al.* [475]. They compared ApoE allele status with response to fish oil in treatment of dyslipidemia. Carriers of the Ape E4 allele (which is also know to increase AD risk) not only had lower baseline high-density lipoprotein levels, but fish oil treatment

resulted in a significant increase in total cholesterol and a reduction of high-density lipoproteins, compared to people with an ApoE3/ApoE3 phenotype [475]. The importance of this is that it demonstrates that although some nutrients may be considered beneficial for the general population, they are in fact detrimental to a select population of a given phenotype. In the context of the current thesis it is possible that the reverse has happened. Dietary nutrients that are considered detrimental for spatial learning abilities of the normal population (ie. normal mice) may actually benefit spatial learning abilities of a select population (ie. Amy mice).

Further insight as to how fats may have protected spatial learning abilities of 12 month old Amy mice may come from comparison with other mouse studies. Using a different AD mouse model to the Amy model used here, Avdesh *et al*. demonstrated that AD-type mice have better reference memory and similar working memory than normal mice [476]. The mice used by Avdesh *et al.* were ApoE knock-out mice. ApoE is involved in cholesterol transport and metabolism and also plays a role in obesity. Expression of the ApoE4 allele, which is a poorer transporter of cholesterol than other ApoE species, increases risk for developing AD. This suggests that poorer cholesterol transport may play a role in AD behavioural deficits, and may explain why AD-type mice are reportedly more susceptible to high-fat diet induced memory impairments than normal mice. However, the results of Avdesh *et al*. and the current study suggest otherwise.

In light of the similarities between spatial learning abilities of the ApoE mice used by Avdesh *et al.* and the Amy mice in the current study, it is possible that altered fat or cholesterol metabolism may play a protective role in spatial learning abilities of 12 month old AD-type mice. ApoE mice have impaired cholesterol transport and may be susceptible to weight gain [477]. The Amy mice that were used in the current study

were more susceptible to diet-induced weight gain and adiposity than normal mice. While the mechanism was not defined, this suggests that altered fat metabolism is also a characteristic of the Amy mouse phenotype. Both groups of mice (Amy mice and ApoE4 mice) demonstrated intact memory [476].

The current study has not conducted mechanistic studies to explain how obesity or high-fat diet consumption may affect neurobiological processes involved in cognitive deficits in AD. The wealth of literature reporting that a high-fat diet has detrimental effects on spatial learning abilities in AD mice indicates that there may have been other factors to explain the significant improvements of Amy mice that were fed the Oz-AIN diet, but not in other mice [82, 478, 479]. For example, it is possible that the apparent conserved learning abilities of 12 month old Amy mice may have been related diet-associated behaviours in AD mice. This is addressed further on page 333-334, in the discussion on the effects of diet on spatial learning. However, in light of the studies by Avdesh *et al.* it is possible that the intact spatial learning abilities of 12 month old Amy mice that were fed the Oz-AIN diet may be due to undefined alterations of fat metabolism that are specific to Amy mice.

Significant genotype effects on overall improvements in latency (s) or distance travelled (m) were apparent at 18 months of age, but not earlier. This was unexpected as Amy mice typically demonstrate spatial learning deficits in the MWM by the time they are 8 months old [159] whilst significant spatial learning deficits are not apparent in normal mice until they are 24 months old [155]. The lack of genotype effect at younger ages may be due to accelerated spatial learning impairments in normal mice or protection against spatial learning deficits in Amy mice, or a combination of both.

It is possible that impaired performance in 12 month old normal mice may be reflective of other age-associated factors such as decreased mobility or reduced ability

to respond to visual cues [155]. However, in the current studies, cued trials demonstrated that normal and Amy mice were able to respond to visual cues. This is supported by reports from others that normal mice and Amy mice do not develop age related impairments such as decreased visual ability, until very old age [155]. Nonetheless, this does not rule out the possibility that individual mice may have had impaired visual abilities which would have reduced their performance throughout the MWM.

Genotype effects: Spatial memory in the Morris Water Maze.

Consistent with the first aim of this chapter, genotype effects on spatial memory of normal and Amy mice was assessed in the MWM at 12, 15 and 18 months.

Genotype effects on spatial memory were not apparent between normal and Amy mice that were fed the AIN93-M diet at 12, 15 or 18 months of age. This indicates that when mice are fed an ideal diet, genotype does not have an effect on spatial memory. This contradicts reports of others that genotype effects occur in Amy mice as young as 8 months, and that normal mice retain learning abilities for a much longer period [128, 159].

Genotype did have an effect on spatial memory of mice that were fed a sub-optimal diet (Oz-AIN diet) from weaning for 15 months. Spatial memory deficits were more pronounced in Amy mice than normal mice that have been fed the Oz-AIN diet. Although these genotype effects occurred later than reported by previous research [128, 159], these findings are consistent with others that a high-fat diet impairs spatial memory in AD-type mice [473].

Collectively, these data have not clearly identified genotype effects on spatial learning or spatial memory in normal and Amy mice. Current literature reports that Amy mice

demonstrate impaired spatial learning and spatial memory abilities at 8 to 11 months of age [128, 159], and that a high-fat diet impairs spatial learning and memory in normal [479] and Amy mice [472]. However, in the current study, the 12 month old Amy mice that were fed the Oz-AIN diet were apparently the only mice to have intact spatial learning abilities, suggesting that the Oz-AIN diet may conserve learning abilities of 12 month old Amy mice. The failure to observe spatial learning in other treatment groups may have been due to the late age that mice were tested. By the time assessments in the MWM started, spatial learning and spatial memory deficits of normal mice were starting to decline, which may have reduced the chance of observing possible genotype effects. Therefore, future studies should begin assessments of spatial learning and spatial memory start at a younger age. This would also provide the opportunity to evaluate change associated with aging.

Diet-type effects: Spatial learning in the Morris Water Maze.

Consistent with the second aim of this chapter, diet-type effects on spatial learning abilities in normal and Amy mice that were fed either an optimal diet (AIN93-M diet) or a sub-optimal diet (Oz-AIN diet) were assessed in the MWM at 12, 15 and 18 months.

These results suggest that not only does a high-fat diet *not* affect spatial learning in normal mice, but that it may actually *enhance* learning abilities of 12 month old Amy mice. This contrasts with the current school of thought that high-fat, sub-optimal diets impair spatial learning abilities in normal mice [82, 167, 186, 469, 472, 478-481] and Amy mice [82, 204, 478, 479].

The majority of studies reporting that high-fat diets impair spatial learning abilities of normal mice have assessed cognitive abilities of mice younger than 10 months old [82, 472, 478, 479]. It is well established that aging impairs hippocampal dependent tasks in mice [155]. It is possible that by the time that the mice in the current study were tested, age-related spatial learning deficits in normal mice were already present. Therefore, even if the high-fat diet was to impair spatial learning abilities of normal mice, age-related spatial learning deficits in mice that were fed optimal diets may have already set in. The consequence of this is that any diet-induced changes would be masked by the detrimental effect of age.

There are a range of diet-related behaviours that may have partially played a role in the seemingly protective effects of the Oz-AIN diet on spatial learning in 12 month old AD-type mice. For example, Maesako *et al*. report that increased environmental enrichment and exercise prevent high-fat diet induced behavioural deficits [167, 369]. It was observed throughout the study that mice that were fed the Oz-AIN diet interacted with their food more than mice that were fed the AIN93-M diet. i.e. they

spent longer periods of time eating, carried pieces of Oz-AIN diet around the cage, and played in the food bowls. While the increased activity was not quantified, it is possible that this may have inadvertently increased environmental enrichment in these cages, and subsequently improved spatial learning. If this was the case the reason that this was observed in Amy mice and not normal mice may be due to differences in locomotor activity and interaction with the environment between the two strains of mice. Walker *et al.* used the Barnes Maze to investigate locomotor activity in young and aged AD-type mice. They report that, irrespective of age, AD-type mice demonstrate increased locomotor activity compared to normal mice [482]. While these may not be the sole contributing factors to spatial learning abilities of Amy mice, they are possible examples of how factors associated with the high-fat diet and Amy genotype may influence spatial learning.

At 15 months of age, spatial learning in the MWM was not consistent for any dietary group. It was observed that Amy mice that were fed the Oz-AIN diet made improvements that were almost half that of Amy mice that were fed the AIN93-M diet. This contradicts the suggestions that the Oz-AIN diet may have been protective of spatial memory at 12 months of age. If the beneficial effects of Oz-AIN diet were indeed present at 12 months, then the high levels of oxidative stress and neuro-inflammation that are brought on by a high-fat diet [483] may have started to have a detrimental effect in the Amy mice by the time they were 15 months old. However, the data describing the performances of mice at 15 months of age were limited, and may not be meaningful.

Diet-type effects: Spatial memory in the Morris Water Maze.

Consistent with the second aim of this chapter, diet-type effects on spatial memory in normal and Amy mice that were fed either an optimal diet (AIN93-M diet) or a sub-optimal diet (Oz-AIN diet) were assessed in the MWM at 12, 15 and 18 months.

Diet did not have an effect on spatial memory in 12 or 15 month old normal mice. This is in support of findings from Mielke *et al*. that high-fat diets do not impair hippocampal dependent processes such as spatial memory in normal aging mice [308]. However, diet affected spatial memory of 12 and 15 month old Amy mice. The 12 month old Amy mice that were fed a sub-optimal high-fat diet (the Oz-AIN diet) had better spatial memory than Amy mice that are fed an optimal diet.

Fitz *et al.* investigated the effect of a high-fat diet on spatial learning and memory of 12-14 month old mice using the MWM [473]. In contrast to the current findings, Fitz *et al*. report that a high-fat diet impairs spatial memory in AD-type mice, but not normal mice [473]. A possible reason for the differences between the findings of Fitz *et al.* and the current study is that in the current study, mice were fed mice their test diets from weaning, whereas Fitz *et al.* fed their diets over the 4 months leading up to behavioural testing. Boitard *et al*. demonstrated that the age that mice receive dietary manipulations has a significant effect on its effects on spatial memory [484]. This suggests that the length of time that the mice in the current study compared to that of Fritz *et al.* may be the cause of differences in findings. Boitard *et al*. also demonstrated that high-fat diet feeding from a young age has a detrimental effect on spatial memory [484].

Alternately, the benefits of the Oz-AIN diet on spatial memory in 12 month old Amy mice may be due to an increase in astrocyte activity and function. Astrocytes play a

large role in AD neuropathology and AD-type mice undergo astrocyte cytoskeletal atrophy and consequent impaired astrocyte functioning [485]. High-fat diets alleviate neurological deficits caused by dysfunctional or sub-optimal astrocyte lipid metabolism [486]. It is therefore possible that the high-fat content of the Oz-AIN diet may have enhanced activity in the astrocytes in the brains of Amy mice, which may have offered more support to neurons and prevent cognitive deficits. The current study has not investigate astrocytes in the brains of Amy mice that were 12 months old, and therefore there is no data to confirm whether or not changes in astrocyte activity or population size are responsible for the improved spatial memory of 12 month old Amy mice. However, this warrants further investigation.

In contrast to the performance of the 12 month old Amy mice, the 15 month old Amy mice that were fed the Oz-AIN diet were the only mice that did not demonstrate spatial memory. This supports the findings from the spatial learning tests that by 15 months of age, any beneficial effects of the Oz-AIN diet in Amy mice have been overcome by other factors.

Irrespective of genotype, by the time mice were 18 months old the Oz-AIN diet appeared to have a protective effect on spatial memory. Normal mice and Amy mice that were fed the Oz-AIN diet demonstrated intact spatial memory, whilst genotype-matched mice that were fed the AIN93-M diet did not.

Collectively these data indicate that diet-type does not affect spatial learning or spatial memory in normal mice until old age. On the other hand, Amy mice appear much more susceptible to the effects of diet on spatial learning and spatial memory. Consistent with the reports of others, the high-fat sub-optimal diet had a detrimental effect on spatial learning and memory of 15 month old mice. However, at 12 and 18 months, the Oz-AIN diet had a beneficial effect on spatial learning and spatial

memory in Amy mice. While the mechanisms behind this are unclear, this could be further investigated by establishing spatial learning and spatial memory abilities of younger mice that are 6 months old.

5.4. Conclusion.

In conclusion this study has found that while genotype appeared to have very little influence over spatial learning and spatial memory abilities of normal and Amy mice, diet-type effects were observed. Amy mice may be more susceptible to the effects of diet than normal mice, and these effects appeared to change with age. In particular, at 15 months of age, the Oz-AIN diet had a detrimental effect on spatial learning and memory in Amy mice, but at 12 and 18 months the Oz-AIN diet appeared to have a conservative effect.

Chapter 6: The effect of dietary supplementation on spatial learning and spatial memory in Amy mice.

6. Background.

Two behavioural deficits that are observed in AD are impaired spatial learning and spatial memory [159, 460, 482]. Chapter 5 set out to demonstrate how spatial learning and spatial memory are affected by genotype and diet-type in the Amy mouse model. Genotype did not have strong effects on spatial memory or spatial learning until mice were 15 and 18 months old respectively. Diet-type, on the other hand, had a significant effect on both spatial learning and memory in Amy mice. This suggests that a sub-optimal diet has an effect on spatial learning and spatial memory of Amy mice before the effects of genotype are apparent.

The present chapter set out to further explore the effect of diet on spatial learning and spatial memory in Amy mice by determining whether or not diet and genotype-induced deficits can be ameliorated by nutrient supplements. Furthermore the current chapter set out to determine how these cognitive abilities change with age in Amy mice, and whether or not this could also be prevented by nutritional supplementation.

The aims of the study described in this chapter are to:

1. **Establish the effect of aging on spatial learning and spatial memory in normal and Amy mice.**

   To achieve this aim, the MWM was used to measure and compare the spatial learning and spatial memory abilities of normal and Amy mice at 6, 12 and 15 months.

2. **Determine whether nutrient supplements can prevent genotype effects on spatial learning and memory in Amy mice (Figure 1A).**

   Two sets of comparisons were made to achieve this aim.

   (i) The spatial learning and memory abilities of normal mice and Amy mice that were fed the Oz-AIN diet were evaluated in the MWM and compared to demonstrate any genotype effects at 12 and 15 months.

   (ii) The spatial learning and memory abilities of Amy mice that were fed the Oz-AIN Supp diet were evaluated in the MWM and compared with those of normal mice and Amy mice that were fed the Oz-AIN diet to determine whether or not nutrient supplements can prevent genotype effects at 12 or 15 months.

3. **Determine whether or not nutrient supplements can prevent diet-type effects on spatial learning and memory in Amy mice (Figure 1B).**

   Two sets of comparisons were made to achieve this aim:

   (i) The spatial learning and memory abilities of Amy mice that were fed the AIN93-M diet and Amy mice that were fed the Oz-AIN diet were evaluated in the MWM and compared to demonstrate any diet-type effects in Amy mice at 12 and 15 months.

   (ii) The spatial learning and memory abilities of Amy mice that were fed the Oz-AIN Supp diet were evaluated in the MWM and compared with those of Amy mice that were fed either the AIN93-M diet or the Oz-AIN diet to determine whether or not nutrient supplements can prevent diet-type effects at 12 or 15 months.

There are three hypotheses for the studies in this chapter. First, it is hypothesised that age will have an effect on spatial learning and spatial memory abilities in normal and

Amy mice at 6, 12 and 15 months old. Second, it is hypothesised that genotype will have an effect on spatial learning and spatial memory in 12 and 15 month old Amy mice, and that this can be prevented with nutrient supplements. Third, it is hypothesised that diet-type will have an effect on spatial learning and spatial memory in 12 and 15 month old Amy mice and that this can be prevented with nutrient supplements.

The MWM procedures that were used to assess spatial learning and memory in the current chapter are the same as those used in Chapter 4 (pp. 273-275). A description of the study design and data analysis that were used for the current study are provided in the methods section of this chapter.

The principal findings of this study were that age has an effect on spatial learning and spatial memory abilities in normal and Amy mice. While spatial learning abilities decreased with age, spatial memory abilities of normal and Amy mice appeared to improve. However, this may have been a consequence of the repetitive testing in the MWM. Genotype and diet-type impaired spatial learning of Amy mice at 15 months of age, and this was prevented with nutrient supplements.

Despite failing to demonstrate spatial learning at 15 months, Amy mice that were fed the Oz-AIN diet demonstrated intact spatial memory. In contrast to this, Amy mice that were fed the AIN93-M diet failed to demonstrate spatial memory but demonstrated intact spatial learning abilities. This suggests that at 15 months of age spatial learning and spatial memory deficits are independent processes. This conclusion is similar to proposals from other studies that report that spatial learning and spatial memory are independent processes in mice [155]. Collectively, these results indicate that spatial learning and spatial memory are separate processes in mice and are independently affected by age, genotype, and diet.

6.1. Methods.

6.1.1. Animals.

All experiments were approved by the Commonwealth Scientific and Industrial Research Organisation (CSIRO) Animal Welfare Committee, Australia in accordance with National Health and Medical Research Council guidelines.

Female Amy (APPswe/PSEN1dE9) mice and their female normal (C57bl/6) littermates were bred at and provided by Flinders University Animal Facility, Bedford Park, South Australia. Genotype was confirmed by PCR and agarose gel electrophoresis, as described in Appendix I.

Amy mice were divided into three dietary groups and were fed the AIN93-M diet, the Oz-AIN diet or the Oz-AIN Supp diet from weaning. Normal mice were fed the Oz-AIN diet from weaning and were used as a control. All mice were fed their respective diets until 15 months of age. Mice were housed (n<6) in cages lined with sawdust, and had free access to food and water.

Mice were tested in the MWM at 6, 12 and 15 months of age (Figure 1). Mice had to be removed from the study at 15 months due to difficulties managing over grooming. Therefore, group sizes at 15 months are smaller than those at the start of the study. The numbers of mice in each group at 6, 12 and 15 months are reported in Table 1. Group sizes between five and twelve have been reliably used to demonstrate cognitive changes in mice using the MWM [86, 470, 471].

Table 1. The numbers of mice in each treatment group that were tested in the MWM at 6, 12 and 15 months of age.

	Normal mice fed the Oz-AIN diet	Amy mice fed the AIN93-M diet	Amy mice fed the Oz-AIN diet	Amy mice fed the Oz-AIN Supp diet
6 months	n = 12	n = 11	n = 15	n = 16
12 months	n = 12	n =11	n = 15	n = 16
15 months	n = 8	n = 11	n = 13	n = 12

6.1.2. Study design.

Aim 1: To investigate the effect of AGING on spatial learning and spatial memory.

To establish the effects of aging on spatial learning and spatial memory in normal and Amy mice, all comparisons were made within groups. The performance of mice was compared at 6, 12 and 15 months of age.

The data describing the performance of mice in the MWM at 6 months were not included in studies of genotype or diet-type. These data were only required to demonstrate that normal and Amy mice had intact spatial learning and memory abilities as young adults in the studies of age-related effects (Figure 1A).

Aim 2: To investigate the ability for nutrient supplements to PREVENT GENOTYPE EFFECTS on spatial learning and spatial memory.

The effect of genotype on spatial learning and spatial memory was established through comparisons between 12 and 15 month old normal mice and Amy mice that had been fed the Oz-AIN diet (Figure 1B(i)).

A second set of comparisons was made between normal and Amy mice that were fed the Oz-AIN diet and Amy mice that were fed the Oz-AIN Supp diet to investigate whether or not nutrient supplements can prevent genotype induced changes in learning and memory in the Amy mouse (Figure 1B(ii)).

Aim 3. To investigate the ability of nutrient supplements to PREVENT DIET-TYPE EFFECTS on spatial learning and spatial memory in Amy mice.

The effect of the diet-type on spatial learning and spatial memory in 12 and 15 month old Amy mice was established by comparisons between Amy mice that were fed the AIN93-M diet, the Oz-AIN diet or the Oz-AIN Supp diet.

Comparisons between mice that were fed the AIN93-M diet and the Oz-AIN diet were made to demonstrate the effects of the Oz-AIN diet on spatial learning and spatial memory in Amy mice. Comparisons between Amy mice that were fed the Oz-AIN Supp diet and Amy mice that had been fed either the AIN93-M diet or the Oz-AIN diet were made, to demonstrate whether or not nutrient supplements could prevent diet-type effects on spatial learning and spatial memory in 12 and 15 month old mice (Figure 1C(ii)).

Figure 1A. The study design used to investigate the effect of aging on spatial learning and spatial memory in normal and Amy mice.

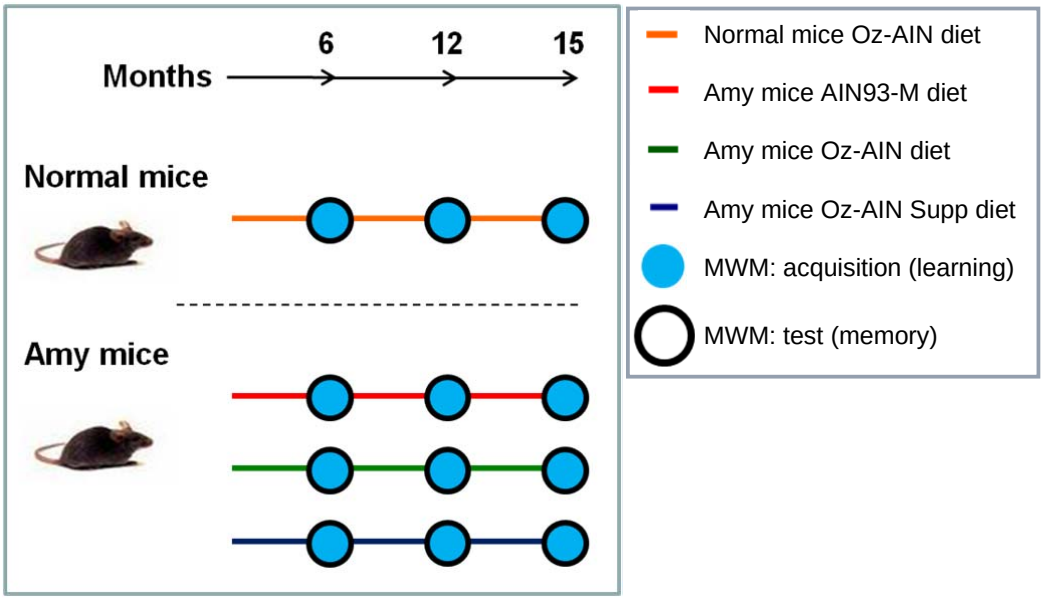


Figure 1A. The Morris Water Maze (MWM) was used to assess spatial learning (blue dots) and spatial memory (black circles). Mice underwent assessments at 6, 12, and 15 months.

Aging effects on spatial learning and spatial memory were assessed by comparing learning and memory abilities at 6, 12 and 15 months of age for normal mice that were fed the Oz-AIN diet (yellow line), Amy mice that were fed the AIN93-M diet (red line), Amy mice that were fed the Oz-AIN diet (green line) and Amy mice that were fed the Oz-AIN Supp diet (blue line).

Figure 1B. The study design used to investigate the potential ability for nutrient supplements to prevent genotype effects on spatial learning and spatial memory in normal and Amy mice.

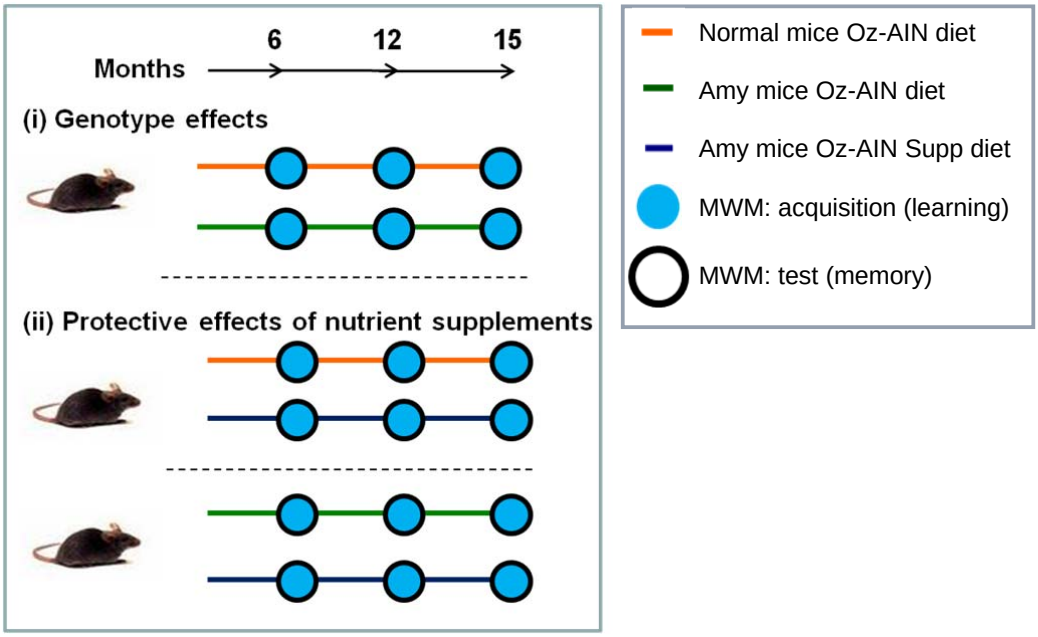


Figure 1B. The Morris water Maze (MWM) was used to assess whether or not nutrient supplements could prevent genotype effects on spatial learning (blue dots) and spatial memory (black circles) abilities of 12 and 15 month old normal and Amy mice.

The ability for nutrient supplements to prevent genotype effects on spatial learning and memory were demonstrated over three comparisons.

(i) Normal mice (yellow line) and Amy mice (green line) that were fed the Oz-AIN diet were compared to demonstrate genotype effects on spatial learning and spatial memory.

(ii) Amy mice that were fed the Oz-AIN Supp diet (blue line) were compared with either normal mice that were fed the Oz-AIN diet or Amy mice that were fed the Oz-AIN diet to determine whether or not nutrient supplements prevented the genotype effects.

Figure 1C. The study design used to investigate the potential ability for nutrient supplements to prevent diet-type effects on spatial learning and spatial memory in Amy mice.

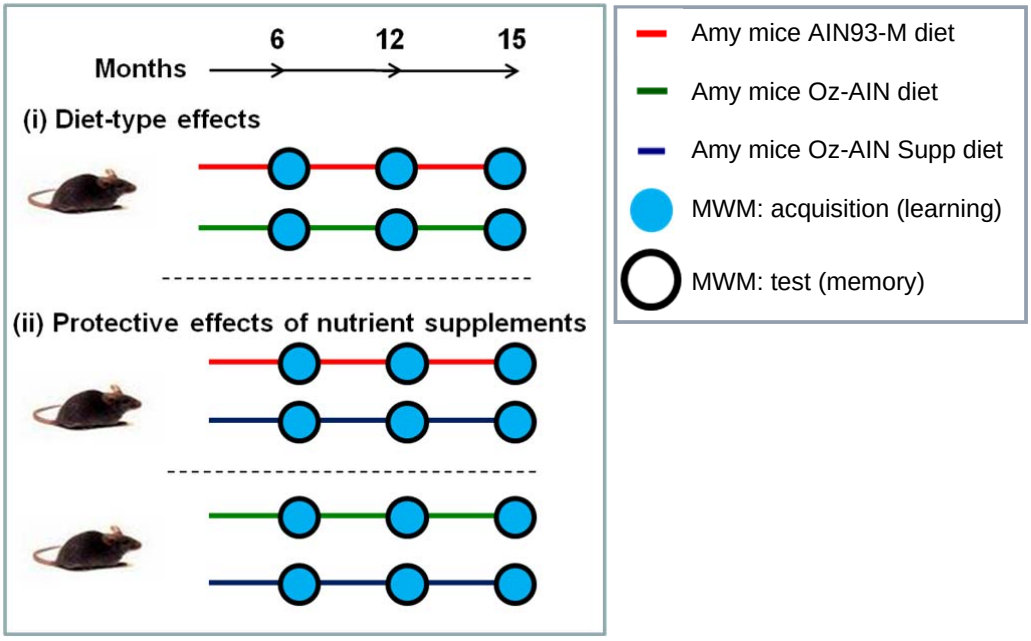


Figure 1C. The Morris water Maze (MWM) was used to assess whether or not nutrient supplements could prevent genotype effects on spatial learning (blue dots) and spatial memory abilities (black circles) of 12 and 15 month old normal and Amy mice.

The ability for nutrient supplements to prevent diet-type effects on spatial learning and memory were demonstrated over three comparisons.

(i) Amy mice that were fed the AIN93-M diet (red line) and Amy mice (green line) that were fed the Oz-AIN diet were compared to demonstrate diet-type effects on spatial learning and spatial memory.

(ii) Amy mice that were fed the Oz-AIN Supp diet (blue line) were compared with Amy mice that were fed either the AIN93-M diet or the Oz-AIN diet to determine whether or not nutrient supplements prevented the diet-type effects.

6.1.3. Apparatus.

The apparatus set up that was used to conduct the MWM for the studies in the current chapter is the same as described in Chapter 5 (5.1.3., pp. 273-275).

6.1.4. Protocol.

The protocols that were used to conduct the MWM for the studies in the current chapter are the same as those described in Chapter 5 (5.1.4., p. 275).

6.1.5. Data collection.

Consistent with the protocols used in Chapter 5, latency (s), distance travelled (m), average speed (m/s) and the number of "platform passes" that mice made were recorded using ANYmaze software (Stoelting Co., Wood Dale, USA). All data was stored in excel files and analysed using GraphPad Prism^{R} Software (Prism 5 for Windows, version 5.04, GraphPad Software Inc., CA, USA). All data is reported as mean ±SEM.

Similar to Chapter 4, latencies to platform were also timed manually from video footage to confirm that mice were accurately detected by ANYmaze software (Stoelting Co.). Any swims where manual latencies and the latencies recorded by ANYmaze differed by >2 s were deemed to not have detected mice accurately. For these data, only the manual latencies were used and distance and average speed were excluded from analysis.

6.1.6. Data analysis.

Improvements in time taken (s) and total distance travelled (m) before reaching the submerged platform over the five training days were the primary indicators of spatial learning in the MWM.

Aging effects, spatial learning and between treatment group comparisons of spatial memory were analysed using one-way ANOVA and Bonferroni multiple comparison post tests. The within group comparisons of spatial memory were analysed using Student's *t* tests. For all comparisons statistical significance was set at $p<0.05$.

Aging effect.

All comparisons were made within groups at 6, 12 and 15 months of age.

Spatial learning.

Spatial learning *within groups* was established through comparison of latency (s) and distance travelled (m) on each day of training relative to the first day of training.

Between groups comparisons were made to establish genotype or diet-type effects on overall improvement in latency (s) and distance travelled (m) after five training days. Genotype effects were established by comparisons between normal mice that were fed the Oz-AIN diet and Amy mice that were fed the Oz-AIN diet or the Oz-AIN Supp diet. Diet-type effects were established by comparisons between Amy mice that were fed the AIN93-M diet, the Oz-AIN diet or the Oz-AIN Supp diet.

Spatial memory.

The number of passes over the position that the submerged platform had previously been located was used as a measure of variance of spatial memory *between treatment groups*. The amount of time (s) and total distance travelled (m) within the Test Quadrant compared to the Opposite quadrant was used as the primary indicator of degree to which mice recalled the location of the platform.

6.2. Results.

6.2.1. Validation that all mice could respond to visual cues.

A cued trial was used to validate that mice could respond to visual cues to locate a submerged platform (see Chapter 4, p. 282, for methods).

6 months of age.

At 6 months of age, all mice were able to respond to visual cues to locate the submerged platform (Table 2, Table 3). All mice were able to locate the cued platform within 25 seconds (Table 2) and there were no significant differences between treatment groups for the distance travelled before reaching the platform. This demonstrates that irrespective of genotype or diet type, all 6 month old mice were able to use visual cues to locate a submerged platform in the MWM.

12 months of age.

At 12 months of age, all mice were able to respond to visual cues to locate the submerged platform (Table 2, Table 3). A one-way ANOVA did not detect significant differences in the time taken to reach the visible platform by normal mice fed the Oz-AIN diet, the Amy mice fed the Oz-AIN diet, the Amy mice fed the AIN93-M diet or the Amy mice fed the Oz-AIN Supp diet ($p=0.89$, Table 2). Similarly, total distance travelled (m) before reaching the platform did not differ between groups ($p=0.95$, Table 3). This confirms that at 12 months of age, all treatment groups were able to respond to visual cues to locate the submerged platform in the MWM.

15 months of age.

At 15 months of age, one-way ANOVA revealed that diet had a significant effect on time taken to reach the visible platform (p=0.04, Table 2). Bonferroni multiple comparison post tests revealed that Amy mice that were fed the Oz-AIN diet took longer to reach the platform than Amy mice that were fed the AIN93-M diet (p=0.03, Table 2). This does not suggest that the 15 month old Amy mice that were fed the Oz-AIN diet were unable to use visual cues to locate the platform, as they were still able to reach the platform within 21.12 ±3.39 s. At 15 months of age, Amy mice that were fed the Oz-AIN diet were, on average, at least 10 g heavier than mice in other treatment groups (see Chapter 3, pp. 130-133 and 154-157), which may have resulted in these mice swimming slower.

There were no differences between total distance travelled (m) before reaching the platform, which supports the finding that at 15 months of age all mice were able to use visual cues to locate the submerged platform (p=0.45, Table 3).

Table 2. Time taken (s) to reach to the visible platform during the cued trial in the Morris Water Maze at 6, 12, and 15 months of age.

	6 months old	12 months old	15 months old
Normal mice Oz-AIN diet	18.53 ±3.25	16.54 ±3.82	16.55 ±3.10
Amy mice AIN93-M diet	22.61 ±4.05	16.58 ±7.35	11.13 ±2.56$^{\Xi}$
Amy mice Oz-AIN diet	22.27 ±2.80	19.31 ±2.72	21.12 ±3.39$^{\Xi}$
Amy mice Oz-AIN Supp diet	21.35 ±2.77	20.91 ±5.36	13.25 ±1.18

All values are reported as mean ±SEM. Numbers with matching symbols are significantly different with Bonferroni post tests. (Ξ) p=0.04.

Table 3. Distance travelled (m) to the visible platform during the cued trial in the Morris Water Maze at 6, 12, and 15 months of age.

	6 months old	12 months old	15 months old
Normal mice Oz-AIN diet	3.67 ±0.59	4.52 ±1.72	2.56 ±0.45
Amy mice AIN93-M diet	3.42 ±2.20	5.82 ±2.90	2.75 ±0.70
Amy mice Oz-AIN diet	4.53 ±0.57	5.10 ±1.93	3.24 ±0.62
Amy mice Oz-AIN Supp diet	3.14 ±0.38	4.28 ±1.21	2.33 ±0.31

All values are reported as mean ±SEM.

6.2.2. Effect of age on spatial learning and spatial memory.

To investigate the effect of aging on spatial learning and spatial memory abilities of normal mice, performance of mice in the acquisition phase and the test swim in the MWM were compared at 6, 12 and 15 months of age (see Chapter 5, pp. 275-276 for protocols).

6.2.2.1. Spatial learning.

Normal mice fed the Oz-AIN diet.

Comparison of improved latency to platform for normal mice that were fed the Oz-AIN diet suggests that aging does not affect spatial learning in normal mice. There were no significant differences in the improved latency to platform after five training days when mice were 6, 12 or 15 months old ($p=0.60$, Figure 2A). Similarly, improved distance travelled to platform was not significantly different ($p=0.14$, Figure 2B). However, when normal mice that were fed the Oz-AIN diet were 15

months old, they made smaller improvements compared to improved distances at 12 months (p=0.23, Figure 2B) or 6 months (p=0.29, Figure 2B). Although not significant, this trend suggests that age had a negative effect on spatial learning abilities of normal adult mice, but not until late adulthood (15 months).

Amy mice fed the AIN93-M diet.

The improvements in latency to the platform for Amy mice that were fed the AIN93-M diet were greater at 6 months compared to 12 months (p=0.07, Figure 2A) and 15 months (p=0.03, Figure 2A). This suggests that aging has a detrimental effect on ability to learn the location of a submerged platform, but this is not significant until late adulthood.

Improvements in distance travelled also appeared to decrease with age (Figure 2B). However, this was not significant, perhaps due the degree of variation in distance travelled at 6 and 12 months (Figure 2B). When mice were 15 months old, they improved distance travelled by 6.16 ±1.36 m after five training days. This was a much smaller improvement than that made at 6 months (10.94 ±3.21 m) or 12 months (10.18 ±3.61 m). Collectively, these data suggest that age has a negative effect on spatial learning abilities of Amy mice that are fed the AIN93-M diet, but that, similar to normal mice that are fed the Oz-AIN diet, aging deficits are not significant until mice reach very old age (15 months).

Amy mice fed the Oz-AIN diet.

Aging had a significant negative effect on changes in latency to the platform for Amy mice that were fed the Oz-AIN diet (p=0.001, Figure 2A). At 15 months of age, Amy mice that were fed the Oz-AIN diet made significantly smaller improvements in latency to platform than when they were 12 months (p=0.002, Figure 2A) or 6 months

(p=0.004, Figure 2A). Similarly, improvements in distance travelled were significantly smaller when Amy mice that were fed the Oz-AIN diet were 15 months old compared to when they were 12 months (p=0.008, Figure 2B) or 6 months (p=0.02, Figure 2B). This demonstrates that age significantly impairs the spatial learning abilities of Amy mice that were fed the Oz-AIN diet.

Amy mice fed the Oz-AIN Supp diet.

Aging had a significant negative effect on improved latencies for Amy mice that were fed the Oz-AIN Supp diet (p=0.03, Figure 2A). The improvements in latency to platform after five training days were smaller at 12 months than at 6 months of age (Figure 2A). While this was not significant at $p<0.05$, it would be significant at $p<0.1$. This suggests that there were trends for aging to have an effect on improved latency to platform. The improvements in distance travelled to the platform after five training days reduced significantly with age (p=0.003, Figure 2B). At 15 months Amy mice that were fed the Oz-AIN Supp diet made significantly smaller improvements in distance travelled compared to when they were 6 months (p=0.003, Figure 2B).

Taken together, these results demonstrate that age affects spatial learning abilities of normal and Amy mice and suggest that nutrient supplements do not prevent or delay age related decline in spatial learning abilities in Amy mice.

Figure 2A. The effect of age on change in latency (s) to reach the submerged platform.

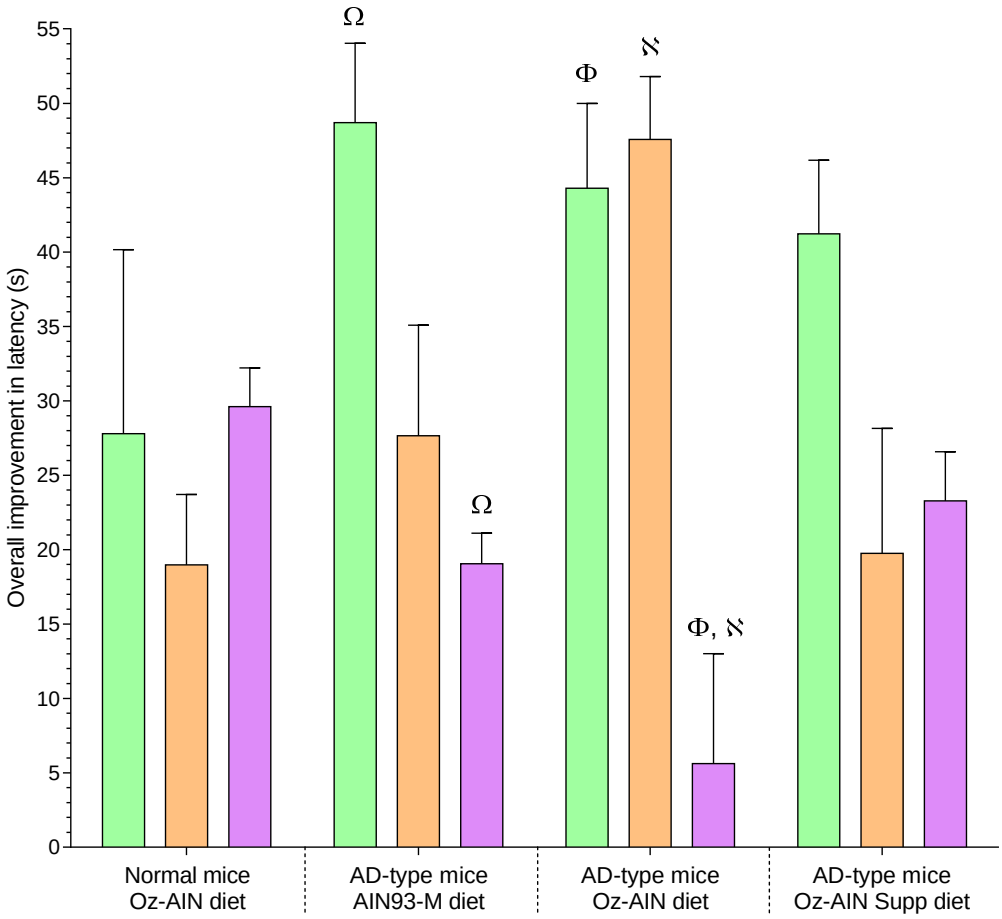


Figure 2A. Comparison of the change in latency to the platform when mice were 6 months old (green bars), 12 months old (orange bars) and 15 months old (purple bars). Normal mice fed the Oz-AIN diet (6 months n=12; 12 months n=12; 15 months n=8). Amy mice fed the AIN93-M diet (6 months n=11; 12 months n=11; 15 months n=11). Amy mice fed the Oz-AIN diet (6 months n=15; 12 months n=15; 15 months n=13). Amy mice fed the Oz-AIN Supp diet (6 months n=16; 12 months n=16; 15 months n=12). Bars are mean ±SEM. Bars with matching symbols are significantly different with Bonferroni post tests. (Φ) p=0.004. (ϰ) p=0.002. (Ω) p=0.03.

Figure 2B. The effect of age on change and distance travelled (m) before reaching the submerged platform.

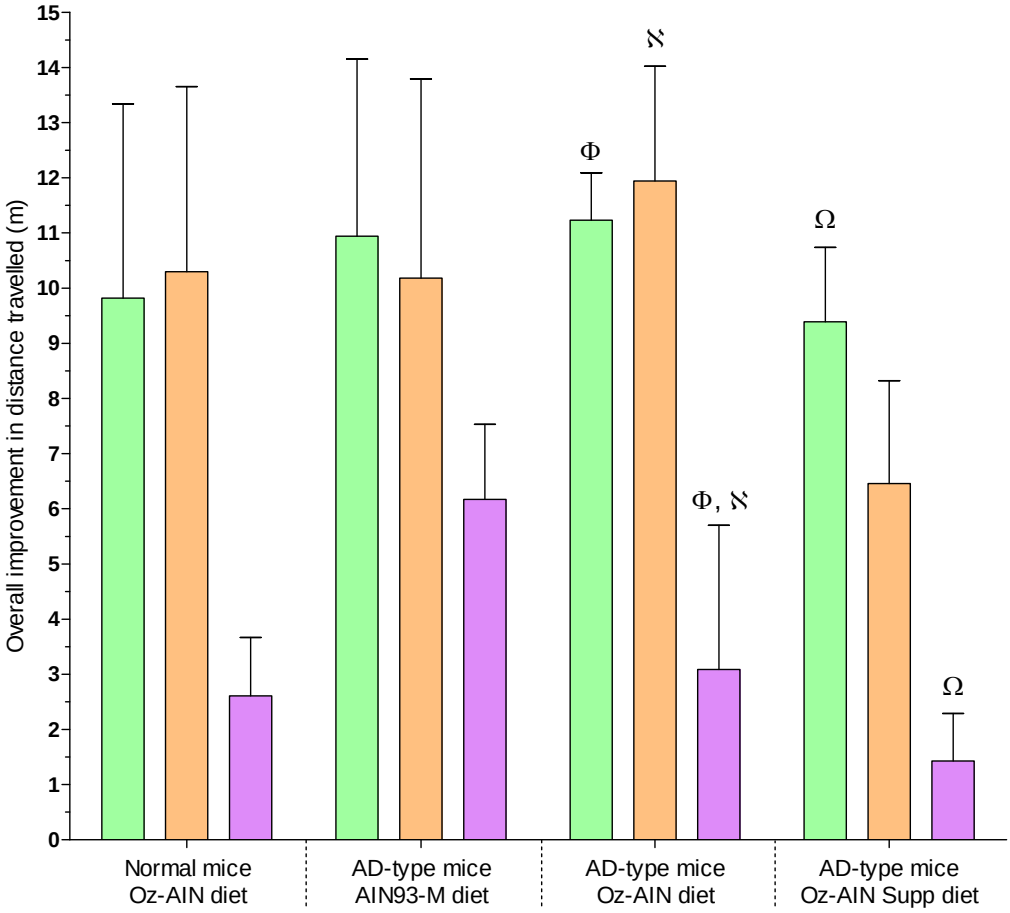


Figure 2B. Comparison of the change in distance to the platform when mice were 6 months old (green bars), 12 months old (orange bars), and 15 months old (purple bars). Normal mice fed the Oz-AIN diet (6 months n=12; 12 months n=12; 15 months n=8). Amy mice fed the AIN93-M diet (6 months n=11; 12 months n=11; 15 months n=11). Amy mice fed the Oz-AIN diet (6 months n=15; 12 months n=15; 15 months n=13). Amy mice fed the Oz-AIN Supp diet (6 months n=16; 12 months n=16; 15 months n=12). Bars are mean ±SEM. Bars with matching symbols are significantly different with Bonferroni post tests. (Φ) p=0.02. (ϰ) p=0.0008. (Ω) p=0.003.

6.2.2.2. Spatial memory.

Normal mice fed the Oz-AIN diet.

The percentage of time that the normal mice that were fed the Oz-AIN diet spent in the Test Quadrant was not affected by age (p=0.62, Table 4). The normal mice that were fed the Oz-AIN diet spent a similar percentage of time in the Test Quadrant at 6, 12 and 15 months, suggesting that their preference for this quadrant did not change as they aged. However, there were significant differences between the percentages of time that normal mice spent in the Opposite Quadrant at 6, 12 and 15 months old (p=0.04, Table 4). The 6 month old mice that were fed the Oz-AIN diet spent a significantly greater percentage of time (p=0.05, Table 4) and distance (p=0.03, Table 5) in the Opposite Quadrant compared to when they were 15 months. This suggests that age affects spatial memory of normal mice.

Differences in percentage of time spent and distance travelled in the Opposite Quadrant may not reflect decline in spatial memory with aging, but may reflect improved search strategies. There were no changes in the percent time spent in the Test Quadrant with aging, suggesting that memory for the platform location had not changed. However, mice may have had a reduced preference for the incorrect quadrant. This is discussed further in the discussion section of this chapter.

Table 4. Comparison of the percent time that normal mice that were fed the Oz-AIN diet spent in the Test and Opposite Quadrants at 6, 12 and 15 months during the test trial.

	6 months old	12 months old	15 months old
Test Quadrant (%)	31.85 ±3.70	37.69 ±3.08	34.86 ±7.50
Opposite Quadrant (%)	22.54 ±2.11$^{\Theta}$	16.14 ±2.21	13.30 ±3.39$^{\Theta}$
Test (%) / Opposite (%)	1.65 ±0.36	3.57 ±1.21	4.20 ±1.47

All values are reported as mean ±SEM. Numbers with matching symbols are significantly different with Bonferroni post tests. (Θ) p=0.05.

Table 5. Comparison of the percent distance that normal mice that were fed the Oz-AIN diet spent in the Test and Opposite Quadrants at 6, 12 and 15 months during the test trial.

	6 months old	12 months old	15 months old
Test Quadrant (%)	30.66 ±3.58	29.98 ±2.41	36.93 ±5.84
Opposite Quadrant (%)	23.37 ±1.75$^{\Theta}$	22.56 ±1.83	14.81 ±3.32$^{\Theta}$
Test (%) / Opposite (%)	1.56 ±0.33	1.46 ±0.23	3.48 ±1.34

All values are reported as mean ±SEM. Numbers with matching symbols are significantly different with Bonferroni post tests. (Θ) p=0.03.

Amy mice fed the AIN93-M diet.

There was no significant effect of aging on percentage of time spent (p=0.51, Table 6) or distance travelled (p=0.13, Table 7) in the Test Quadrant by 6, 12, or 15 month old Amy mice that were fed the AIN93-M diet. This suggests that aging does not affect spatial memory for the correct quadrant in aging Amy mice.

Aging did have a significant effect on percentage of time spent (p=0.002, Table 6) and distance travelled (p=0.02, Table 7) in the Opposite Quadrant. When Amy mice that were fed the AIN93-M diet were 12 months, they spent a significantly smaller percentage of time overall in the Opposite Quadrant compared to 6 months (p=0.002,

Table 6) or 15 months (p=0.02, Table 6). However, there was no significant differences in percent time (p=0.64) or distance travelled (p=0.32, Table 7) in the Opposite Quadrant at 6 or 15 months. This suggests that the memory abilities of young and old Amy mice are similar, and that they may be poorer than those of adult Amy mice.

Table 6. Comparison of the percent time that Amy mice that were fed the AIN93-M diet spent in the Test and Opposite Quadrants at 6, 12 and 15 months during the test trial.

	6 months old	12 months old	15 months old
Test Quadrant (%)	28.41 ±3.17	34.61 ±4.83	30.67 ±3.11
Opposite Quadrant (%)	25.98 ±3.55$^{\Omega}$	12.33 ±2.24$^{\Omega\,\Theta}$	22.70 ±1.39$^{\Theta}$
Test (%) / Opposite (%)	1.39 ±0.34	5.87 ±2.35	1.44 ±0.20

All values are reported as mean ±SEM. Numbers with matching symbols are significantly different with Bonferroni post tests. (Θ) p=0.02. (Ω) p=0.002.

Table 7. Comparison of the percent distance that Amy mice that were fed the AIN93-M diet spent in the Test and Opposite Quadrants at 6, 12 and 15 months during the test trial.

	6 months old	12 months old	15 months old
Test Quadrant (%)	24.37 ±3.48	36.42 ±5.54	28.77 ±2.79
Opposite Quadrant (%)	32.28 ±5.73$^{\Theta}$	16.56 ±2.81$^{\Theta}$	24.44 ±1.52
Test (%) / Opposite (%)	1.16 ±0.28	3.90 ±1.52	1.25 ±0.17

All values are reported as mean ±SEM. Numbers with matching symbols are significantly different with Bonferroni post tests. (Θ) p=0.02.

Amy mice fed the Oz-AIN diet.

Percent of time spent in the Test Quadrant increased with age for the Amy mice that were fed the Oz-AIN diet, although this finding was non-significant (p=0.24, Table 8). The percent of time that Amy mice that were fed the Oz-AIN diet spent in the

Opposite Quadrant decreased significantly with age (p=0.008, Table 8). Bonferroni post tests revealed that when the Amy mice that were fed the Oz-AIN diet were 12 months old, they spent significantly less time in the Opposite Quadrant compared to when they were 6 months old (p=0.008, Table 8). Furthermore, there were non-significant trends for mice to travel less in the Opposite Quadrant at 15 months compared to 6 months (p=0.08, Table 9). This suggests that as Amy mice that were fed the Oz-AIN diet aged their spatial memory abilities may have improved. This is an unexpected finding, as literature reports a decline in spatial memory with age in mice [155, 158, 159, 460]. It is possible that these improvements reflect an effect of repeated testing, which is discussed further in the discussion section (pp. 405-406).

Table 8. Comparison of the percent time that Amy mice that were fed the Oz-AIN diet spent in the Test and Opposite Quadrants at 6, 12 and 15 months during the test trial.

	6 months old	**12 months old**	**15 months old**
Test Quadrant (%)	29.02 ±1.78	37.37 ±5.00	36.66 ±4.02
Opposite Quadrant (%)	22.80 ±1.65$^{\Omega}$	13.89 ±2.46$^{\Omega}$	15.99 ±1.87
Test (%) / Opposite (%)	1.47 ±0.27	4.33 ±1.12	4.21 ±2.11

All values are reported as mean ±SEM. Numbers with matching symbols are significantly different with Bonferroni post tests. (Ω) $p<0.01$.

Table 9. Comparison of the percent distance that Amy mice that were fed the Oz-AIN diet spent in the Test and Opposite Quadrants at 6, 12 and 15 months during the test trial.

	6 months old	**12 months old**	**15 months old**
Test Quadrant (%)	28.26 ±1.45	35.73 ±5.60	37.59 ±3.54
Opposite Quadrant (%)	23.61 ±1.46	19.16 ±2.81	16.88 ±1.80
Test (%) / Opposite (%)	1.32 ±0.17	2.35 ±0.81	3.89 ±1.89

All values are reported as mean ±SEM

Amy mice fed the Oz-AIN Supp diet.

The Amy mice that were fed the Oz-AIN Supp diet increased the percentage of overall time that they spent in the Test Quadrant as they aged ($p=0.07$, Table 10). While these increases were not significant, mice also reduced the percentage of time that they spent in the Opposite Quadrant ($p=0.16$, Table 11). This suggests that as Amy mice that are fed the Oz-Ain Supp diet aged, their spatial learning abilities improved. This is supported by comparisons of the changes in percentage distance travelled in either quadrant with aging (Table 11). The distance travelled in the Test or Opposite Quadrant by Amy mice that were fed the Oz-AIN Supp diet changed significantly with age ($p=0.04$ for both quadrants, Table 11). At 15 months, Amy mice that were fed the Oz-AIN Supp diet travelled further in the Test Quadrant compared to when they were 6 months ($p=0.07$, Table 11). While this is not significant at $p<0.05$, it would be significant at $p<0.1$. This suggests that there may have been non-significant trends for Amy mice that were fed the Oz-AIN Supp diet to improve spatial memory with aging. This is supported by the finding that at 15 months, Amy mice that were fed the Oz-AIN Supp diet travelled significantly less in the Opposite Quadrant compared to when they were 6 months ($p=0.03$, Table 11).

Table 10. Comparison of the percent time that Amy mice that were fed the Oz-AIN Supp diet spent in the Test and Opposite Quadrants at 6, 12 and 15 months during the test trial.

	6 months old	**12 months old**	**15 months old**
Test Quadrant (%)	25.24 ±3.32	32.71 ±1.96	32.86 ±2.00
Opposite Quadrant (%)	21.86 ±2.43	17.49 ±2.09	16.07 ±1.59
Test (%) / Opposite (%)	1.32 ±0.22	2.97 ±0.79	3.56 ±1.65

All values are reported as mean ±SEM.

Table 11. Comparison of the percent distance that Amy mice that were fed the Oz-AIN Supp diet spent in the Test and Opposite Quadrants at 6,12 and 15 months during the test trial.

	6 months old	12 months old	15 months old
Test Quadrant (%)	24.66 ±2.62	32.06 ±2.21	32.62 ±2.09
Opposite Quadrant (%)	24.98 ±1.98$^{\Theta}$	22.06 ±1.83	17.60 ±1.67$^{\Theta}$
Test (%) / Opposite (%)	1.20 ±0.21	1.76 ±0.42	2.84 ±1.09

All values are reported as mean ±SEM. Numbers with matching symbols are significantly different. (Θ) $p<0.05$.

Table 12: Comparison of the number of times that Oz-AIN diet crossed the platform position at 6, 12 and 15 months.

	6 months old	12 months old	15 months old
Normal mice Oz-AIN diet	5.92 ±1.11	3.70 ±0.73	5.12 ±1.80
Amy mice AIN93-M diet	4.75 ±0.97	5.00 ±0.80	4.89 ±0.96
Amy mice Oz-AIN diet	3.73 ±0.64	5.13 ±1.08	5.42 ±0.87
Amy mice Oz-AIN Supp diet	4.00 ±0.68	3.60 ±0.50	4.17 ±0.61

All values are reported as mean ±SEM.

Effect of age on accuracy searching for the platform.

There were no significant differences in passes over the platform location for normal mice that were fed the Oz-AIN diet ($p=0.36$, Table 12), Amy mice that were fed the AIN93-M diet ($p=0.98$, Table 12), Amy mice that were fed the Oz-AIN diet ($p=0.36$, Table 12) or Amy mice that were fed the Oz-AIN Supp diet ($p=0.80$, table 12). This

suggests that accuracy for searching for the platform, once mice were in the correct quadrant, did not change with age.

These data have demonstrated no significant evidence for age-related decline in spatial memory as there were no significant changes in percent of time or distance travelled in the Test Quadrant or the numbers of passes over the platform location. However, there were non-significant trends for Amy mice fed either the Oz-AIN or the Oz-AIN Supp diet to improve spatial memory over time. There was also a significant reduction in the percent of time spent and distance travelled by mice in the incorrect side of the pool. Collectively, this suggests that while mice may not have recalled exactly where the platform was, there was a reduced preference to search the wrong side of the pool as mice aged.

6.2.3. Spatial learning in the Morris Water Maze.

Spatial learning was assessed during an acquisition phase (see Chapter 5, p. 282 for protocol).

6.2.3.1. Performance of 6 month old mice throughout the acquisition phase in the Morris Water Maze.

Changes in latency (s) and distance travelled (m) to platform.

All groups of mice were able to learn the location of the submerged platform when they were 6 months old. The latency to reach the platform significantly improved throughout the acquisition phase for 6 month old normal mice that were fed the Oz-AIN diet ($p=0.03$, Figure 3A), Amy mice that were fed the AIN93-M diet ($p=0.006$, Figure 3A), Amy mice that were fed the Oz-AIN diet ($p=0.0004$, Figure 3A) and Amy mice that were fed the Oz-AIN Supp diet ($p=0.002$, Figure 3A). There were no

differences in speed travelled throughout the acquisition phase, confirming that improved latencies reflected spatial learning (Figure 3C). All groups significantly reduced the distance travelled before reaching the platform throughout the training period, confirming that they had intact spatial learning abilities (Figure 3B).

The rate of learning was also the same between all groups at 6 months of age. Significant improvements in the MWM were first apparent on Day 4 for normal mice that were fed the Oz-AIN diet ($p=0.01$), Amy mice that were fed the AIN93-M diet ($p=0.01$), Amy mice that were fed the Oz-AIN diet ($p=0.03$) and Amy mice that were fed the Oz-AIN Supp diet ($p=0.01$).

Figure 3A. Latency (s) for 6 month old normal and Amy mice to reach a submerged platform in the Morris Water Maze.

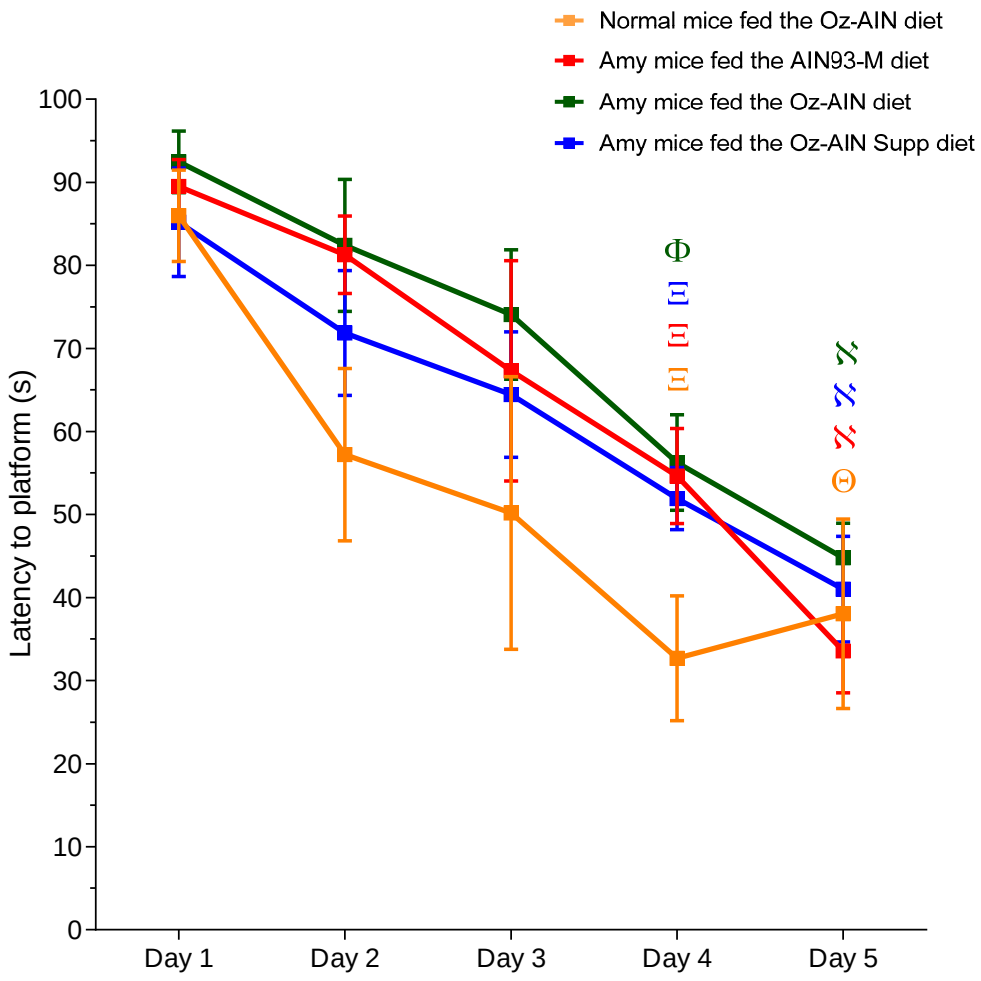


Figure 3A. Average time taken (s) to reach a submerged platform on each of the five training days when mice were 6 months old. Normal mice fed the Oz-AIN diet (orange line, n=12), Amy mice fed the AIN93-M diet (red line, n=11), Amy mice fed the Oz-AIN diet (green line, n=15), and Amy mice fed the Oz-AIN Supp diet (blue line, n=16). Error bars are mean ±SEM. Symbols indicate significant differences compared to distance travelled on Day 1 within groups that are the same colour as the symbol, and were detected with Bonferroni post tests. (Θ) p=0.03 (orange, normal mice fed the Oz-AIN diet). (Ξ) p=0.01 (orange, normal mice fed the Oz-AIN diet) (red, Amy mice fed the AIN93-M diet) (blue, Amy mice fed the Oz-AIN Supp diet). (Φ) p=0.003 (green, Amy mice fed the Oz-AIN diet). (ϰ) p<0.001 (red, Amy mice fed the AIN93-M diet) (green, Amy mice fed the Oz-AIN diet) (blue, Amy mice fed the Oz-AIN Supp diet).

Figure 3B. Distance travelled (m) by 6 month old normal and Amy mice whilst searching for a submerged platform in the Morris Water Maze

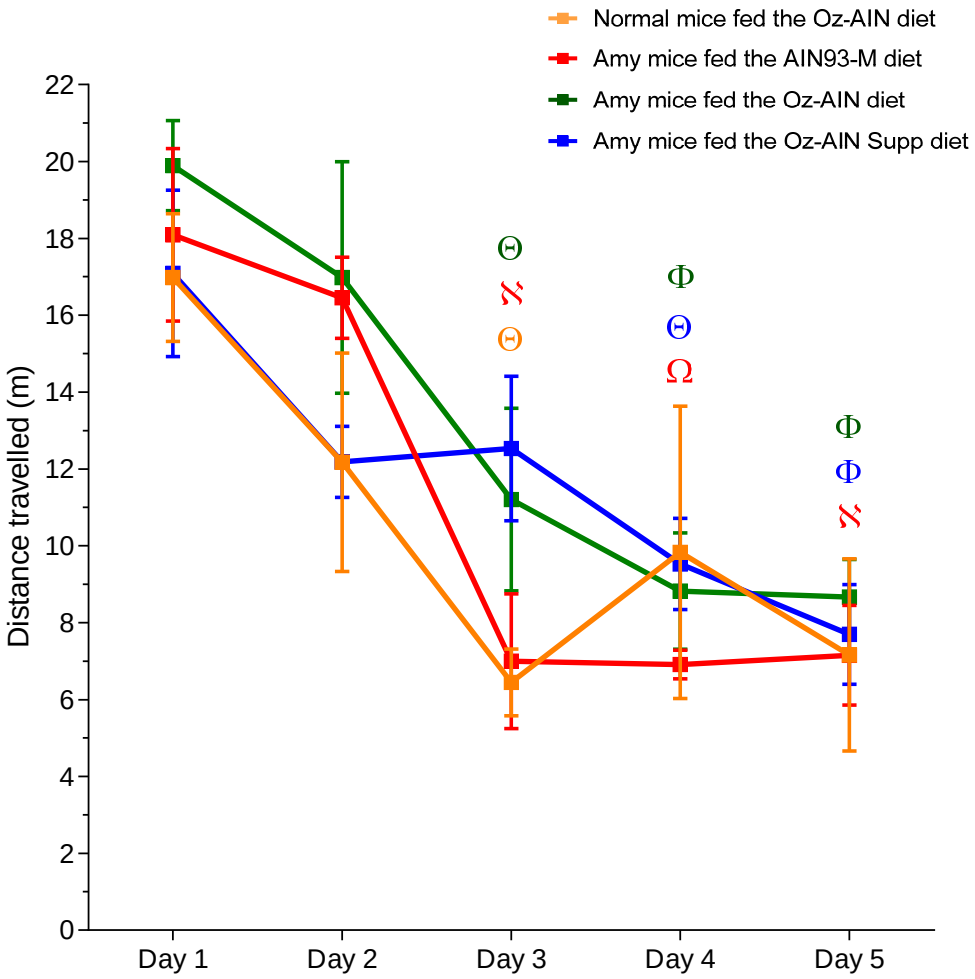


Figure 3B. Average distance travelled (m) during a swim to locate a hidden platform on each of the five training days when mice were 6 months old. Normal mice fed the Oz-AIN diet (orange line, n=12), Amy mice fed the AIN93-M diet (red line, n=11), Amy mice fed the Oz-AIN diet (green line, n=15), and Amy mice fed the Oz-AIN Supp diet (blue line, n=16). Error bars are mean ±SEM. Symbols indicate significant differences compared to distance travelled on Day 1 within groups that are the same colour as the symbol, and were detected with Bonferroni post tests. (Θ) $p<0.05$ (orange, normal mice fed the Oz-AIN diet) (green, Amy mice fed the Oz-AIN diet) (blue, Amy mice fed the Oz-AIN Supp diet). (Φ) $p<0.01$ (green, Amy mice fed the Oz-AIN diet) (blue, Amy mice fed the Oz-AIN Supp diet). (ϰ) $p=0.0004$ (red, Amy mice fed the AIN93-M diet).

Figure 3C. Average speed (m/s) travelled by 6 month old mice whilst searching for a submerged platform in the Morris Water Maze.

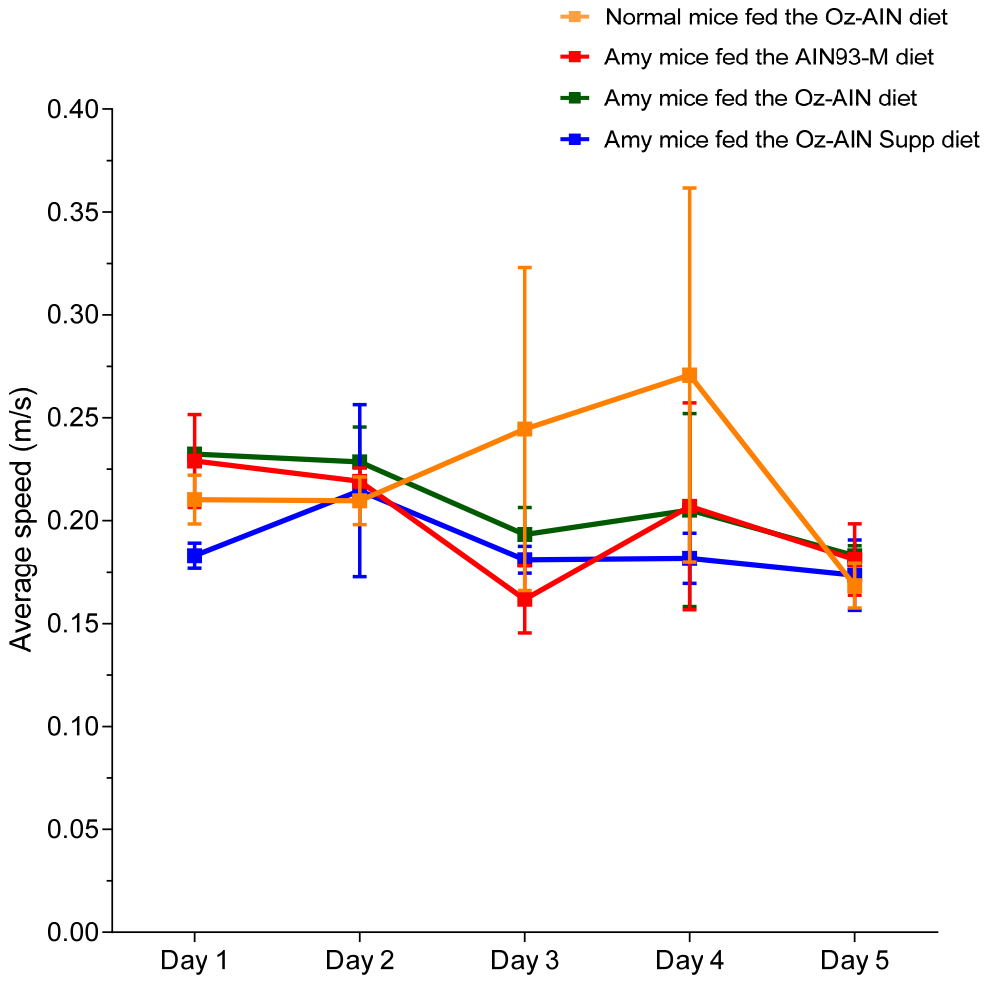


Figure 3C. Average speed (m/s) travelled to a submerged platform on each of the five training days when mice were 6 months old. Normal mice fed the Oz-AIN diet (orange line, n=12), Amy mice fed the AIN93-M diet (red line, n=11), Amy mice fed the Oz-AIN diet (green line, n=15), and Amy mice fed the Oz-AIN Supp diet (blue line, n=16). Error bars are mean ±SEM.

6.2.3.2. Performance of 12 month old mice throughout the acquisition phase in the Morris Water Maze.

Changes in latency (s) and distance travelled (m) to platform.

At 12 months of age, the Amy mice that were fed the Oz-AIN diet made significant improvements in latency (s) to platform ($p=0.0003$, Figure 4A). These significant improvements were made on Day 3 (36.21 ±4.49 s improvement, $p=0.002$, Figure 4A), Day 4 (43.24 ±5.45 s improvement, $p=0.0005$, Figure 4A) and Day 5 (47.57 ±4.22 s improvement, $p=0.0002$, Table 13, Figure 4A). The normal mice that were fed the Oz-AIN diet and the Amy mice that were fed the AIN93-M diet or the Oz-AIN Supp diet also improved latency (s) to the platform after five training days (Figure 4A). However, one-way ANOVA's did not detect significant changes for any of these three groups ($p=0.31$, $p=0.18$ and $p=0.23$ respectively, Figure 4A) There were no significant differences in average speed over the five training days, supporting the interpretation of these data as reflecting spatial learning (Figure 4C).

All 12 month old mice made significant improvements in distance travelled to the platform after five training days (Figure 4B). Consistent with their improved latencies, the Amy mice that were fed the Oz-AIN diet travelled significantly shorter distances on Day 3 ($p=0.008$), Day 4 ($p=0.002$) and Day 5 ($p=0.002$) of the acquisition period (Figure 4B). Amy mice that were fed the Oz-AIN Supp diet made the next fastest improvement in distance travelled, and travelled significantly shorter distances to the platform on Days 3 ($p=0.02$) and on Day 5 ($p=0.01$). Normal mice that were fed the Oz-AIN diet made significant improvements on Day 4 ($p=0.04$) and Day 5 ($p=0.02$). Amy mice that were fed the AIN93-M diet did not travel significantly shorter distances to the platform until the last day of the acquisition

phase ($p=0.05$), suggesting that it took Amy mice that were fed the AIN93-M diet the longest to learn the location of the platform (Figure 4B).

The improved latencies (s) and distances travelled (m) to the submerged platform throughout the acquisition period demonstrate that at 12 months of age all mice had intact spatial learning ability.

Figure 4A. Latency (s) for 12 month old normal and Amy mice to reach a submerged platform in the Morris Water Maze.

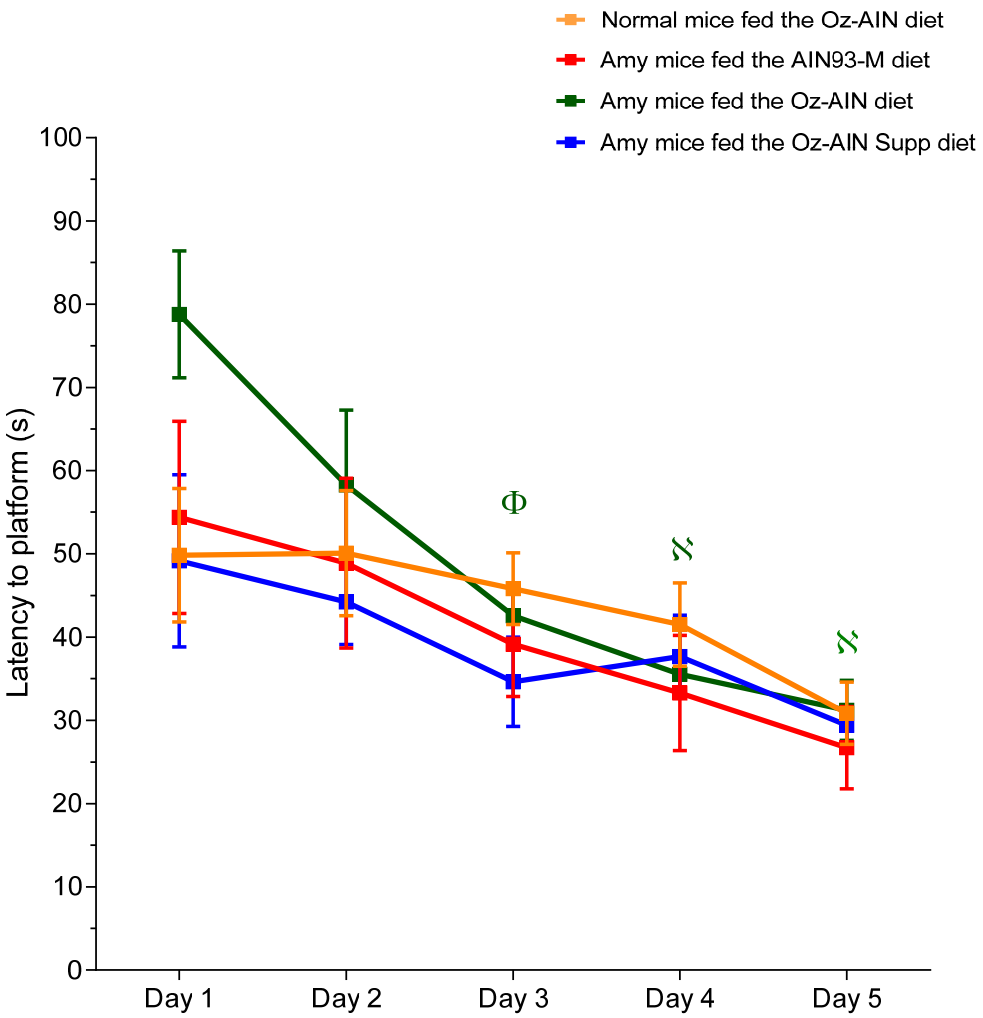


Figure 4A. Average time taken (s) to reach a submerged platform on each of the five training days when mice were 12 months old. Normal mice fed the Oz-AIN diet (orange, n=12), Amy mice fed the AIN93-M diet (red, n=11), Amy mice fed the Oz-AIN diet (green, n=15), and Amy mice fed the Oz-AIN Supp diet (blue, n=16). Error bars are mean ±SEM. Symbols indicate significant differences compared to distance travelled on Day 1 within groups that are the same colour as the symbol, and were detected with Bonferroni post tests. (Φ) $p<0.01$ (green, Amy mice fed the Oz-AIN diet). (ϰ) $p<0.001$ (green, Amy mice fed the Oz-AIN diet).

Figure 4B. Distance travelled (m) by 12 month old normal and Amy mice whilst searching for a submerged platform in the Morris Water Maze

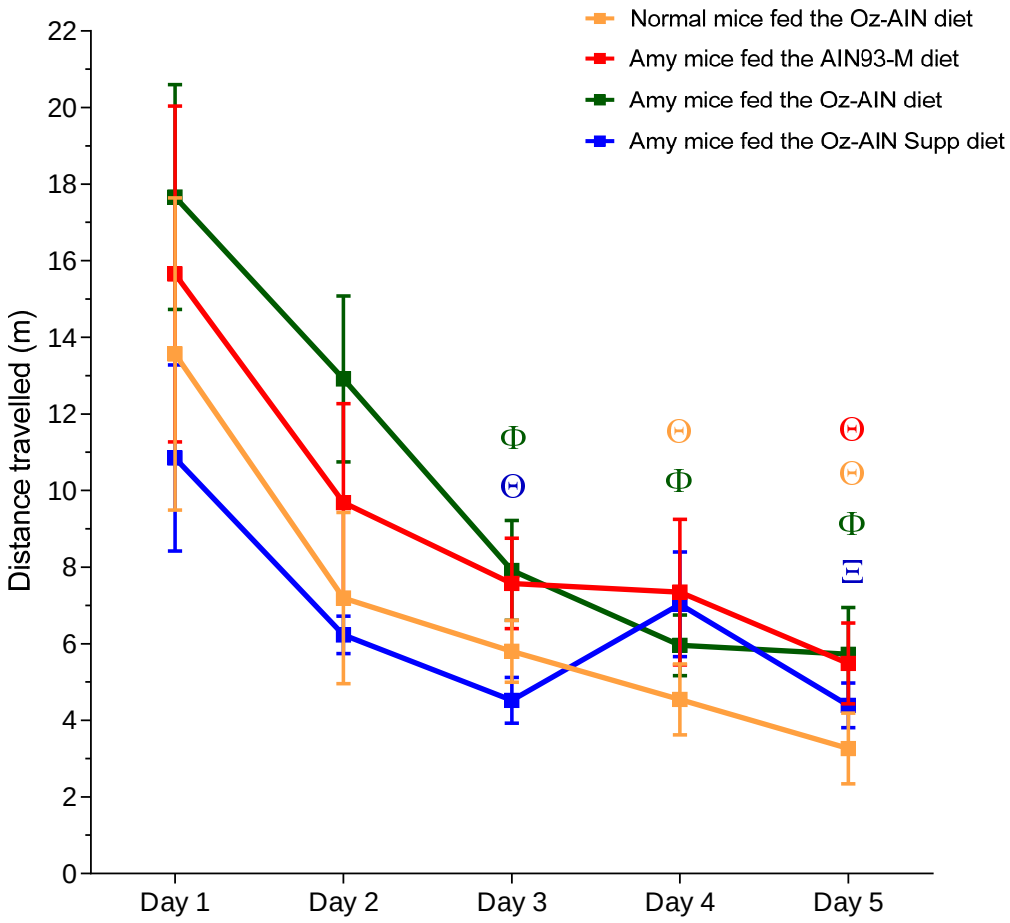


Figure 4B. Average distance travelled (m) during a swim to locate a hidden platform on each of the five training days when mice were 12 months old. Normal mice fed the Oz-AIN diet (orange, n=12), Amy mice fed the AIN93-M diet (red, n=11), Amy mice fed the Oz-AIN diet (green, n=15), and Amy mice fed the Oz-AIN Supp diet (blue, n=16). Error bars are mean ±SEM. Symbols indicate significant differences compared to distance travelled on Day 1 within groups that are the same colour as the symbol, and were detected with Bonferroni post tests. (Θ) $p<0.05$ (red, Amy mice fed the AIN93-M diet) (orange, normal mice fed the Oz-AIN diet). (Φ) $p<0.01$ (green, Amy mice fed the Oz-AIN diet). (Ξ) $p=0.01$ (blue, Amy mice fed the Oz-AIN Supp diet).

Figure 4C. Average speed (m/s) travelled by 12 month old mice whilst searching for a submerged platform in the Morris Water Maze.

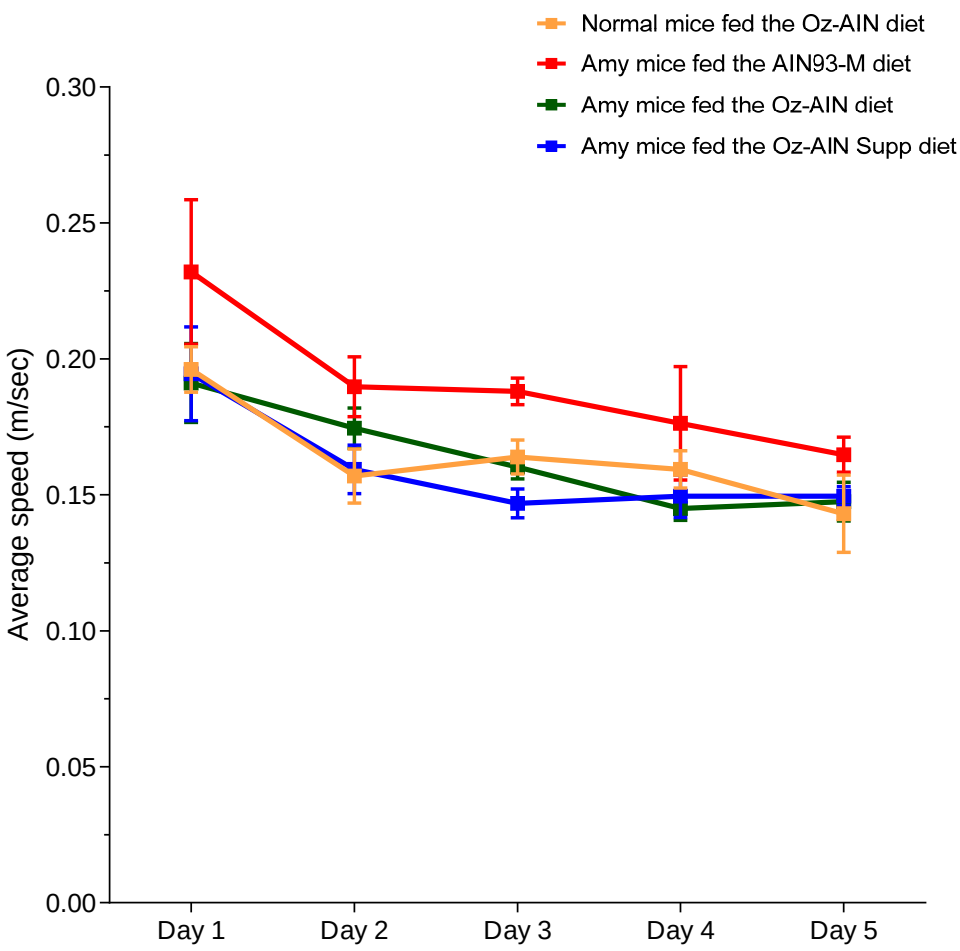


Figure 4C. Average speed (m/s) travelled to a submerged platform on each of the five training days when mice were 12 months old. Normal mice fed the Oz-AIN diet (orange, n=12), Amy mice fed the AIN93-M diet (red, n=11), Amy mice fed the Oz-AIN diet (green, n=15), and Amy mice fed the Oz-AIN Supp diet (blue, n=16). Error bars are mean ±SEM.

Between treatment group comparisons: Genotype effect on improved latency (s) and distance travelled (m) to platform by 12 month old mice after five training days.

A one-way ANOVA detected significant differences in the overall improvements made by normal mice that were fed the Oz-AIN diet, Amy mice that were fed the Oz-AIN diet and Amy mice that were fed the Oz-AIN Supp diet ($p=0.014$, Table 13). Bonferroni post tests revealed that the 12 month old Amy mice that were fed the Oz-AIN diet made a significantly greater improvement in latency than normal mice that were fed the Oz-AIN diet ($p=0.03$, Table 13). This indicates that genotype affects spatial learning abilities of mice and that Amy mice had better spatial learning skills than normal mice.

Bonferroni post tests also indicated that the Amy mice that were fed the Oz-AIN Supp diet made smaller improvements in latency than Amy mice that were fed the Oz-AIN diet ($p=0.03$, Table 13). Furthermore, there were no differences in improved latencies of normal mice that were fed the Oz-AIN diet and Amy mice that were fed the Oz-AIN Supp diet ($p>0.99$, Table 13). This suggests that nutrient supplements prevented the beneficial effect of genotype on improvement of latency to the submerged platform in the MWM.

However, a one-way ANOVA revealed that genotype did not affect improved distance travelled to the platform ($p=0.33$, Table 14). Bonferroni post tests indicated that normal mice and Amy mice that were fed the Oz-AIN diet made similar improvements in distance travelled ($p>0.99$, Table 14). The Amy mice that were fed the Oz-AIN Supp diet made smaller, *albeit* statistically similar improvements than both normal mice that were fed the Oz-AIN diet ($p=0.93$, Table 14) and the Amy mice that were fed the Oz-AIN diet ($p=0.47$, Table 14). The finding that these

differences were not significant suggests that nutrient supplements do not affect improvements in distance travelled over a five day training period in the MWM.

Despite the significant differences in improved latencies throughout the acquisition period, these data do not provide evidence for a genotype effect of diet on spatial learning abilities of 12 month old mice. There were no differences between treatment groups for improvements in distance travelled suggesting that normal and Amy mice equally learned the location of the platform over the five day training period.

Table 13. The ability of nutrient supplements to prevent genotype effects on improvement in latency by 12 month old mice to reach the submerged platform after five training days.

	Normal mice Oz-AIN diet Vs Amy mice Oz-AIN diet	**Normal mice Oz-AIN diet Vs Amy mice Oz-AIN Supp diet**	**Amy mice Oz-AIN diet Vs Amy mice Oz-AIN Supp diet**
Normal mice Oz-AIN diet	18.97 ±4.74	18.97 ±4.74	-------
Amy mice Oz-AIN diet	47.57 ±4.22	-------	47.57 ±4.22
Amy mice Oz-AIN Supp diet	-------	19.75 ±8.40	19.75 ±8.40
Difference	**-28.60 ±3.84 Θ**	**-0.78 ±4.52**	**27.82 ±5.18 Θ**

Improvement of latency (s) to the submerged platform after the five day acquisition phase in the MWM. All values are reported as mean ±SEM. Bold values describe the difference between the improved latencies of the two treatment groups in the same column. Statistical significance was detected between treatment groups in the same column with Bonferroni post tests. (Θ) p=0.03

Table 14. The ability of nutrient supplements to prevent genotype effects on the improvement of distance travelled by 12 month old mice before reaching the submerged platform after five training days.

	Normal mice Oz-AIN diet Vs Amy mice Oz-AIN diet	**Normal mice Oz-AIN diet Vs Amy mice Oz-AIN Supp diet**	**Amy mice Oz-AIN diet Vs Amy mice Oz-AIN Supp diet**
Normal mice Oz-AIN diet	10.30 ±3.35	10.30 ±3.35	-------
Amy mice Oz-AIN diet	11.94 ±2.08	-------	11.94 ±2.08
Amy mice Oz-AIN Supp diet	-------	6.46 ±1.86	6.46 ±1.86
Difference (m)	**-1.64 ±1.52**	**3.84 ±1.91**	**5.48 ±1.54**

Improvement of distance travelled (m) before reaching the submerged platform after the five day acquisition phase in the MWM. All values are reported as mean ±SEM. Bold values describe the difference between the improved distances of the two treatment groups in the same column.

Between treatment group comparisons: Diet-type effect on improved latency (s) and distance travelled (m) to platform by 12 month old mice after five training days.

A one-way ANOVA revealed significant differences in the improvements in latency to the submerged platform after five training days by Amy mice that were fed the AIN93-M diet, the Oz-AIN diet or the Oz-AIN Supp diet ($p<0.049$, Table 15). The 12 month old Amy mice that were fed the Oz-AIN diet made an overall improvement that was 19.91±63 s greater than that of mice that were fed the AIN93-M diet (Table 15). This suggests that the Oz-AIN diet may have had a beneficial effect on spatial learning abilities of 12 month old Amy mice. Amy mice that were fed the Oz-AIN Supp diet had poorer spatial learning abilities than Amy mice that were fed the Oz-AIN diet. Although this suggests that nutrient supplements have a detrimental effect

on spatial learning abilities, the finding was only marginally significant ($p=0.06$, Table 15). There were no significant differences in the improved latencies of Amy mice that were fed either the AIN93-M diet or the Oz-AIN Supp diet, suggesting that they had similar spatial learning abilities (Table 15).

Improvements in distance travelled before reaching the platform were equal between Amy mice that were fed the AIN93-M diet or the Oz-AIN diet, suggesting that diet-type does not affect spatial learning abilities of Amy mice ($p=0.37$, Table 16). While the Amy mice that were fed the Oz-AIN Supp diet made smaller improvements in distance travelled to the platform than either Amy mice that were fed the AIN93-M diet or the Oz-AIN diet, this was not significant with Bonferroni post tests ($p>0.99$ and $p=0.53$ respectively, Table 16). These data suggest that nutrient supplements do not improve distance travelled while searching for a submerged platform in the MWM.

The changes in latencies and distances travelled over five training days by the 12 month old Amy mice that were fed different diets indicate that diet has an effect on spatial learning ability. While broad characteristics of diet such as fat content and energy content may not affect spatial learning abilities in 12 month old Amy mice, specific dietary elements such as those within the nutrient supplement mix may affect spatial learning abilities of 12 month old Amy mice (Table 16).

Table 15. The ability of nutrient supplements to prevent diet-type effects on the improvement in latency for 12 month old Amy mice to reach the submerged platform after five training days.

	Amy mice AIN93-M diet Vs Amy mice Oz-AIN diet	**Amy mice AIN93-M diet Vs Amy mice Oz-AIN Supp diet**	**Amy mice Oz-AIN diet Vs Amy mice Oz-AIN Supp diet**
Amy mice AIN93-M diet	27.66 ±7.43	27.66 ±7.43	-------
Amy mice Oz-AIN diet	47.57 ±4.22	-------	47.57 ±4.22
Amy mice Oz-AIN Supp diet	-------	19.75 ±8.40	19.75 ±8.40
Difference (s)	**-19.91 ±6.26**	**7.91 ±4.24**	**27.82 ±5.18**

Improvement of latency (s) to the submerged platform after the five day acquisition phase in the MWM. All values are reported as mean ±SEM. Bold values describe the difference between the improved latencies of the two dietary groups in the same column.

Table 16. The ability of nutrient supplements to prevent the diet-type effects on the improvement of distance travelled by 12 month old Amy mice before reaching the submerged platform after five training days.

	Amy mice AIN93-M diet Vs Amy mice Oz-AIN diet	**Amy mice AIN93-M diet Vs Amy mice Oz-AIN Supp diet**	**Amy mice Oz-AIN diet Vs Amy mice Oz-AIN Supp diet**
Amy mice AIN93-M diet	10.18 ±3.61	10.18 ±3.61	-------
Amy mice Oz-AIN diet	11.94 ±2.08	-------	11.94 ±2.08
Amy mice Oz-AIN Supp diet	-------	6.46 ±1.86	6.46 ±1.86
Difference (m)	**-1.77 ±2.03**	**3.72 ±1.93**	**5.48 ±1.54**

Improvement of distance travelled (m) before reaching the submerged platform after the five day acquisition phase in the MWM. All values are reported as mean ±SEM. Bold values describe the difference between the improved distances of the two treatment groups in the same column.

6.2.3.3. Performance of 15 month old mice throughout the acquisition phase in the Morris Water Maze.

Changes in latency (s) and distance travelled (m) to platform.

A one-way ANOVA did not detect any significant differences in the latencies to reach the platform throughout the acquisition phase for the 15 month old Amy mice that were fed the AIN93-M diet ($p=0.07$). However, there were trends to suggest that the 15 month old Amy mice that had been fed the AIN93-M diet learned where the platform was. They reached the platform in less than half the time on Day 4 compared to latencies on Day 1 (20.48 ±3.22 s compared to 43.55 ±7.38 s, $p=0.15$, Figure 5A). Furthermore, 15 month old Amy mice that were fed the AIN93-M diet swam less than a third of the distance on Day 4 than they had on Day 1 (2.01 ±0.46 m compared to 9.37 ±2.89 m, $p=0.11$, Figure 5B). These data suggest that in the latter stages of the acquisition phase, Amy mice that were fed the AIN93-M diet learned the location of the submerged platform, even though they were not significant at $p=0.05$.

A one-way ANOVA revealed that the latencies to the submerged platform varied significantly throughout the acquisition phase for normal mice that were fed the Oz-AIN diet ($p=0.002$, Figure 5A). Bonferroni post tests indicated that the normal mice that were fed the Oz-AIN diet reached the platform with shorter latencies on Day 2 ($p=0.02$), Day 3 ($p=0.02$), Day 4 ($p=0.02$) and Day 5 ($p=0.0005$) relative to latencies on Day 1. This suggests that the normal mice that were fed the Oz-AIN diet had intact spatial learning abilities. A one-way ANOVA did not detect significant variance in distance travelled by normal mice that were fed the Oz-AIN diet throughout the acquisition phase ($p=0.33$, Figure 5C). However, the distance that they travelled reduced on each day of the training period, and on Day 5, they travelled 2.61 ±1.06 m

less before reaching the platform. This suggests that they may have still had intact spatial learning abilities at 15 months of age.

Amy mice that were fed the Oz-AIN Supp diet appeared to have similar spatial learning abilities to normal mice that were fed the Oz-AIN diet (Figure 5). Both groups of mice made improvements in latency to the platform on Day 2 and Day 4 of the acquisition period. By Day 2, Amy mice that were fed the Oz-AIN Supp diet had made a 14.88 ±3.15 s improvement ($p=0.12$, Figure 5A), whilst normal mice that were fed the Oz-AIN diet had improved by 18.96 ±3.64 s ($p=0.02$, Figure 5A). By Day 5, the normal mice that were fed the Oz-AIN diet and the Amy mice that were fed the Oz-AIN Supp diet reached the platform with similar latencies (17.87 ±2.84 s and 22.02 ±4.47 s respectively, Figure 5A), and both had made significant improvements relative to Day 1 ($p=0.0005$ and $p=0.03$ respectively, Figure 5A).

Unlike all other treatment groups the 15 month old Amy mice that were fed the Oz-AIN diet did not reduce latency (s) or distance travelled (m) to the submerged platform during the acquisition phase. On the first day of training, Amy mice that were fed the Oz-AIN diet took 40.32 ±10.55 s to reach the platform (Figure 5A) and travelled 7.58 ±2.91 m (Figure 5B). These latencies and distances did not improve over the five training days. By Day 5, these mice took 34.69 ±3.96 s to reach the platform ($p=>0.99$, Figure 5A) and travelled 4.67 ±0.41 m ($p=0.76$, Figure 5B). This indicates that at 15 months of age, Amy mice that were fed the Oz-AIN diet did not have intact spatial learning ability.

Average speed did not differ significantly throughout the acquisition period, indicating that latencies reflect spatial learning abilities (Figure 5C).

Figure 5A. Latency (s) for 15 month old normal and Amy mice to reach a submerged platform in the Morris Water Maze.

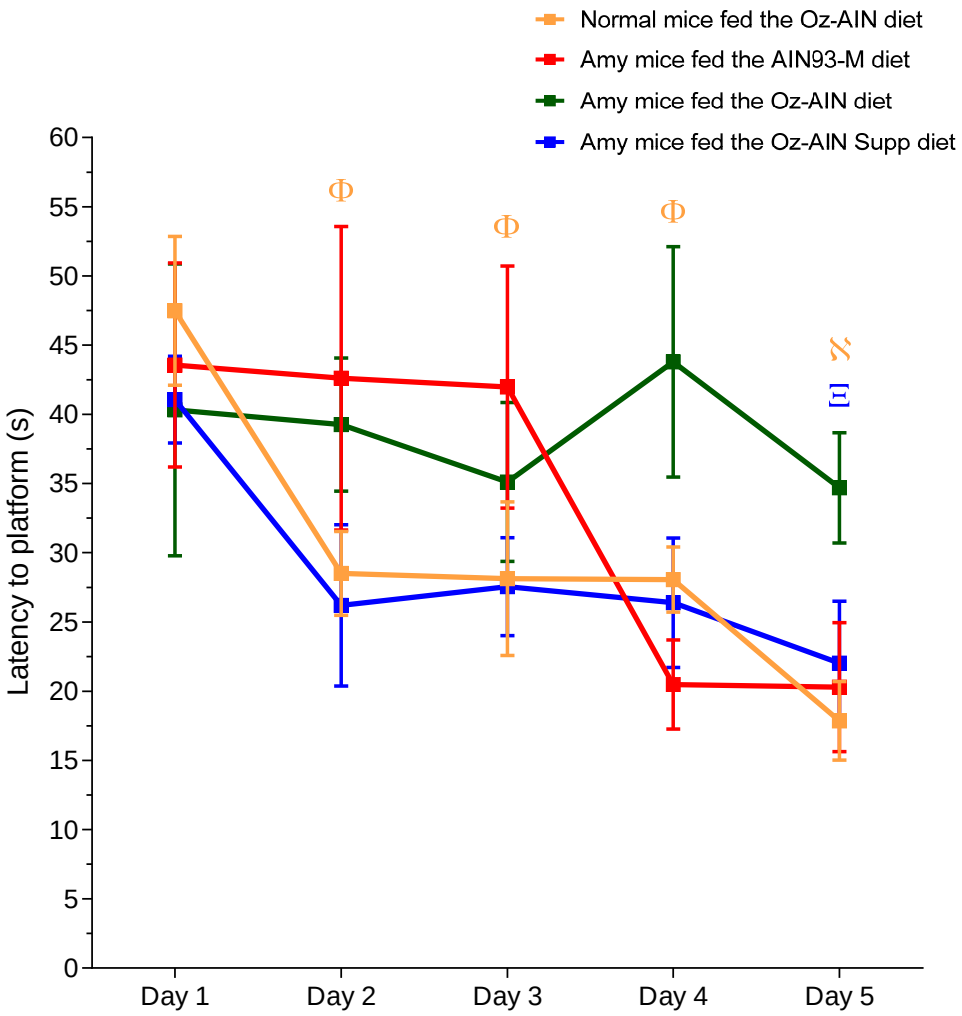


Figure 5A. The average time taken (s) to reach a submerged platform on each of the five training days when mice were 15 months old. Normal mice fed the Oz-AIN diet (orange line, n=8), Amy mice fed the AIN93-M diet (red line, n=11), Amy mice fed the Oz-AIN diet (green line, n=13), and Amy mice fed the Oz-AIN Supp diet (blue line, n=12). Error bars are mean ±SEM. Symbols indicate significant differences compared to distance travelled on Day 1 within groups that are the same colour as the symbol, and were detected with Bonferroni post tests. (Φ) p=0.02 (orange, normal mice fed the Oz-AIN diet). (ϰ) p=0.0005 (orange, normal mice fed the Oz-AIN diet). (Ξ) p=0.03 (blue, Amy mice fed the Oz-AIN Supp diet).

Figure 5B. Distance travelled (m) by 15 month old normal and Amy mice whilst searching for a submerged platform in the Morris Water Maze

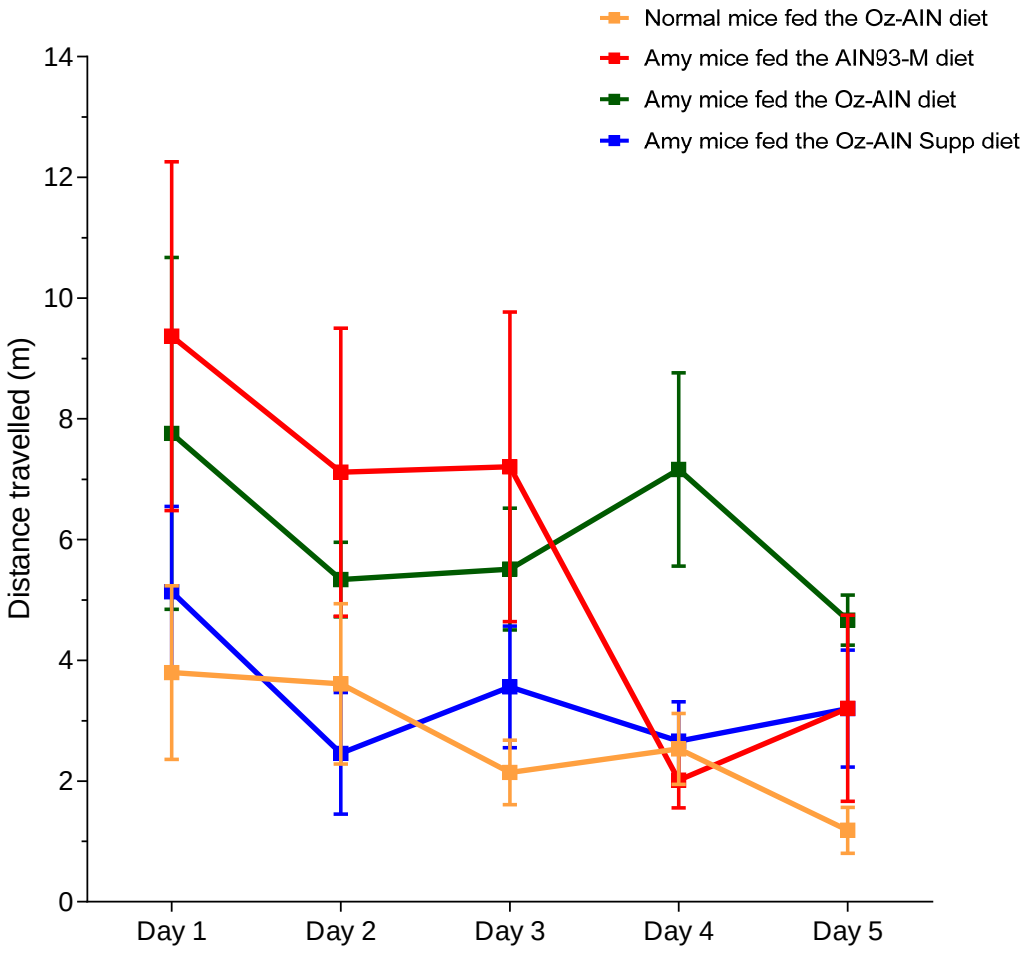


Figure 5B. Average distance travelled (m) during a swim to locate a hidden platform on each of the five training days when mice were 15 months old. Normal mice fed the Oz-AIN diet (orange line, n=8), Amy mice fed the AIN93-M diet (red line, n=11), Amy mice fed the Oz-AIN diet (green line, n=13), and Amy mice fed the Oz-AIN Supp diet (blue line, n=12). Error bars are mean ±SEM.

Figure 5C. Average speed (m/s) travelled by 15 month old mice whilst searching for a submerged platform in the Morris Water Maze.

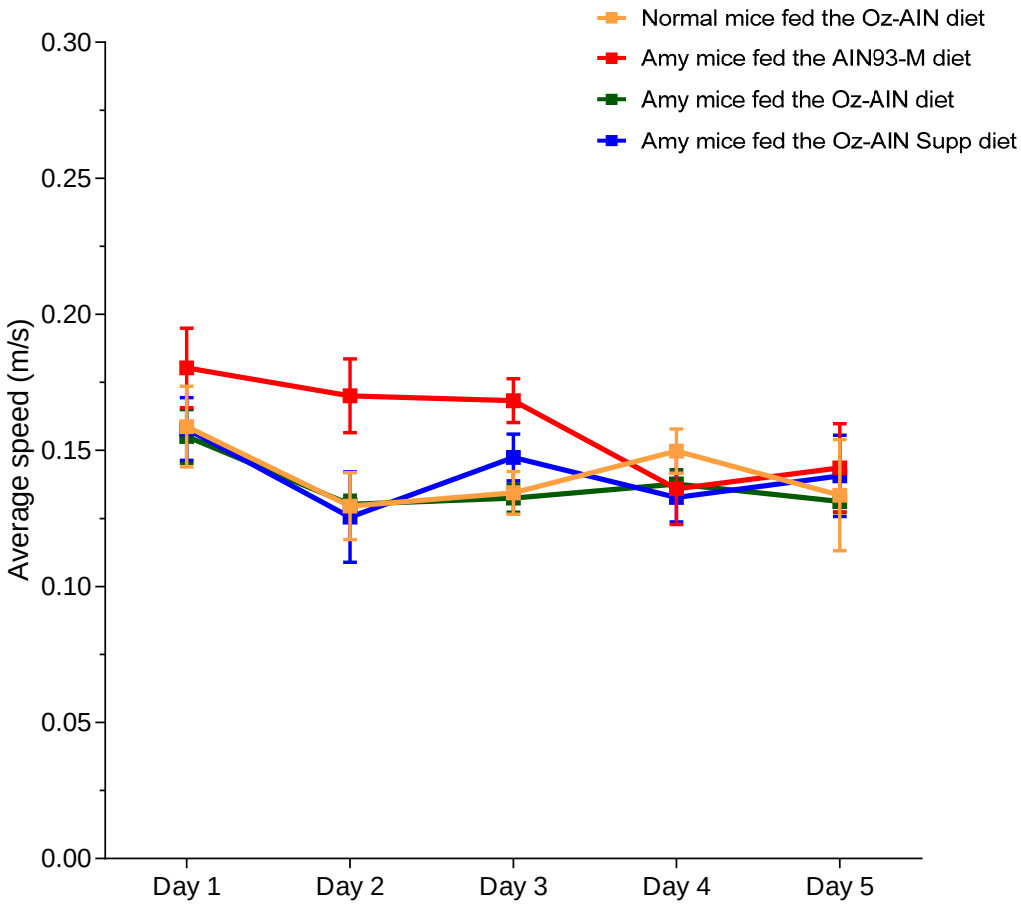


Figure 5C. Average speed (m/s) travelled to a submerged platform on each of the five training days when mice were 15 months old. Normal mice fed the Oz-AIN diet (orange line, n=8), Amy mice fed the AIN93-M diet (red line, n=11), Amy mice fed the Oz-AIN diet (green line, n=13), and Amy mice fed the Oz-AIN Supp diet (blue line, n=12). Error bars are mean ±SEM.

Between treatment group comparisons: Genotype effect on improved latency (s) and distance travelled (m) to platform by 15 month old mice after five training days.

A one-way ANOVA revealed that genotype had an effect on latency to reach the submerged platform when mice were 15 months old (p=0.02, Table 17). The 15 month old Amy mice that were fed the Oz-AIN diet made a significantly smaller improvement than normal mice that were fed the Oz-AIN diet (p=0.02, Table 17, Figure 5A), indicative of genotype effects.

The genotype effect may have been partially alleviated nutritional supplementation. The 15 month old Amy mice that had been fed the Oz-AIN Supp diet made an improvement that was not significantly different to that of normal mice that were fed the Oz-AIN diet (p=0.43, Table 17), suggesting that the nutrient supplements prevented the genotype effect on improved latencies in the MWM. Furthermore, the Amy mice that were fed the Oz-AIN Supp diet made an overall improvement that was almost three times greater than that of Amy mice that were fed the Oz-AIN diet. However, this was not significant (p=0.22, Table 17). This suggests that while nutrient supplements may have only been able to partially reduce the genotype effect on improvement of latency in the MWM.

A one-way ANOVA did not detect significant differences in the improvements of distance travelled by normal mice that were fed the Oz-AIN diet, Amy mice that were fed the Oz-AIN diet and Amy mice that were fed the Oz-AIN Supp diet (p=0.78, Table 18). Failure to achieve significance may have been due to the large degree of variation within groups.

Table 17. Genotype effects on overall changes in the time taken (s) by 15 month old mice to reach the submerged platform after five training days.

	Normal mice Oz-AIN diet Vs Amy mice Oz-AIN diet	**Normal mice Oz-AIN diet Vs Amy mice Oz-AIN Supp diet**	**Amy mice Oz-AIN diet Vs Amy mice Oz-AIN Supp diet**
Normal mice Oz-AIN diet	29.61 ±2.61	29.61 ±2.61	-------
Amy mice Oz-AIN diet	5.63 ±7.39	-------	5.63 ±7.39
Amy mice Oz-AIN Supp diet	-------	19.05 ±2.06	19.05 ±2.06
Difference (s)	**23.98 ±4.93** $^{\Theta}$	**10.56 ±4.40**	**-13.42 ±9.31**

Improvement for latency (s) to platform after the five day acquisition phase in the MWM. All values are reported as mean ±SEM. Bold values describe the difference between the improved latencies of the two dietary groups in the same column. Statistical significance was detected between dietary groups in the same column. (Θ) p=0.02.

Table 18. Genotype effects on overall changes in the distance travelled (m) by 15 month old mice to reach the submerged platform after five training days.

	Normal mice Oz-AIN diet Vs Amy mice Oz-AIN diet	**Normal mice Oz-AIN diet Vs Amy mice Oz-AIN Supp diet**	**Amy mice Oz-AIN diet Vs Amy mice Oz-AIN Supp diet**
Normal mice Oz-AIN diet	2.61 ±1.06	2.61 ±1.06	-------
Amy mice Oz-AIN diet	3.09 ±2.61	-------	3.09 ±2.61
Amy mice Oz-AIN Supp diet	-------	1.43 ±0.86	1.43 ±0.86
Difference (s)	**-0.48 ±1.62**	**1.66 ±3.12**	**1.18 ±1.58**

Improvement of distance travelled (m) before reaching the submerged platform after the five day acquisition phase in the MWM. All values are reported as mean ±SEM. Bold values describe the difference between the improved distances of the two treatment groups in the same column.

These data suggest that there is a genotype effect on spatial learning abilities of 15 month old Amy mice, and that this is prevented by nutrient supplements. While data

describing distance travelled to platform do not fully support this, they may have been compromised by the large degree of variation within groups.

Between treatment group comparisons: Diet-type effect on improved latency (s) and distance travelled (m) to platform by 15 month old mice after five training days.

A one-way ANOVA did not detect significant differences between the improved latencies of 15 months old Amy mice that were fed either the AIN93-M diet, Oz-AIN diet or the Oz-AIN Supp diet ($p=0.07$, Table 19). While this is not significant at $p<0.05$, it would be significant at $p<0.1$, suggesting that there may be trends for diet-type to affect improvements of latency to the submerged platform. Further support from this comes from the finding that the Amy mice that were fed the AIN93-M diet made an improvement that was almost four times greater than that of Amy mice that were fed the Oz-AIN diet ($p=0.09$, Table 19). Similarly, the improvement made by Amy mice that were fed the Oz-AIN Supp diet was almost four times greater than that of Amy mice that were fed the Oz-AIN diet ($p=0.24$, Table 19). Collectively, this suggests that there may be weak trends for diet-type to affect improvement of latency to the platform, and these trends may be prevented with nutritional supplements.

Comparison of improved distance travelled throughout the acquisition phase also suggests that there are weak trends for diet-type effects on spatial learning abilities of 15 month old Amy mice (Table 20). Amy mice that were fed the AIN93-M diet made greater improvements in the distance travelled to the platform than either the Amy mice that were fed the Oz-AIN diet or the Amy mice that were fed the Oz-AIN Supp diet (Table 20). Despite making significant improvements in latency after five training days, the Amy mice that were fed the Oz-AIN Supp diet made the smallest improvements in distance travelled to the platform. However, this is possibly

because the Amy mice that were fed the Oz-AIN Supp diet travelled shorter distances than other mice on Day 1, and therefore there was less 'room for improvement'.

Collectively, the changes in latency (s) and distance travelled (m) to reach the submerged platform after five training days suggest that diet-type may have an effect on spatial learning. Improvements in latency suggest that the Oz-AIN diet has a detrimental effect on spatial learning in 15 month old Amy mice, and that this can be alleviated by nutrient supplements.

Table 19. Diet-type effects on overall changes in the time taken (s) by 15 month old Amy mice to reach the submerged platform after five training days.

	Amy mice AIN93-M diet Vs Amy mice Oz-AIN diet	**Amy mice AIN93-M diet Vs Amy mice Oz-AIN Supp diet**	**Amy mice Oz-AIN diet Vs Amy mice Oz-AIN Supp diet**
Amy mice AIN93-M diet	23.27 ±3.32	23.27 ±3.32	-------
Amy mice Oz-AIN diet	5.63 ±7.39	-------	5.63 ±7.39
Amy mice Oz-AIN Supp diet	-------	19.05 ±2.06	19.05 ±2.06
Difference (s)	**17.64 ±4.10**	**4.22 ±5.21**	**-13.42 ±9.31**

Improvement for latency (s) to platform after the five day acquisition phase in the MWM. All values are reported as mean ±SEM. Bold values describe the difference between the improved latencies of the two dietary groups in the same column. Statistical significance was detected between dietary groups in the same column.

Table 20. Diet-type effects on overall changes in the distance travelled (m) by 15 month old Amy mice to reach the submerged platform after five training days.

	Amy mice AIN93-M diet Vs Amy mice Oz-AIN diet	**Amy mice AIN93-M diet Vs Amy mice Oz-AIN Supp diet**	**Amy mice AIN93-M diet Vs Amy mice Oz-AIN Supp diet**
Amy mice AIN93-M diet	6.17 ±1.36	6.17 ±1.36	-------
Amy mice Oz-AIN diet	3.09 ±2.61	-------	3.09 ±2.61
Amy mice Oz-AIN Supp diet	-------	1.43 ±0.86	1.43 ±0.86
Difference (s)	**3.08 ±1.27**	**4.73 ±1.87**	**1.66 ±3.12**

Improvement of distance travelled (m) before reaching the submerged platform after the five day acquisition phase in the MWM. All values are reported as mean ±SEM. Bold values describe the difference between the improved distances of the two treatment groups in the same column.

6.2.4. Spatial memory in the Morris Water Maze.

Spatial memory was assessed in a Test Trial the day following the acquisition phase (see Chapter 5, page 276).

6.2.4.1. Performance of 12 month old mice during the Test Trial in the Morris Water Maze.

Normal mice fed the Oz-AIN diet.

The normal mice that were fed the Oz-AIN diet spent more significantly more time in the Test Quadrant than in the Opposite Quadrant ($p=0.0001$, Figure 6A). This suggests that at 12 months of age, normal mice that were fed the Oz-AIN diet had intact spatial memory. Normal mice that were fed the Oz-AIN diet also travelled further in the Test Quadrant than the Opposite Quadrant, but this was not significant ($p=0.08$, Figure 6B).

Amy mice fed the AIN93-M diet.

The Amy mice that were fed the AIN93-M diet spent significantly more time in the Test Quadrant than in the Opposite Quadrant ($p=0.005$, Figure 6A), indicating that they had intact spatial memory. Similarly, these mice swam significantly further in the Test Quadrant then in the Opposite Quadrant ($p=0.032$, Figure 6B), confirming that they had intact spatial memory at 12 months.

Amy mice fed the Oz-AIN diet.

The Amy mice that were fed the Oz-AIN diet spent significantly more time in the Test Quadrant than in the Opposite Quadrant ($p=0.004$, Figure 6A), indicating that they had intact spatial memory. The Amy mice that were fed the Oz-AIN diet also travelled further in the Test Quadrant than the Opposite Quadrant, this was not significant ($p=0.06$, Figure 6B). While this is not significant at $p<0.05$, it would be significant at $p<0.10$, suggesting there were trends for Amy mice that were fed the Oz-AIN diet to swim further in the Test Quadrant whilst searching for the platform, and hence had intact spatial memory.

Amy mice fed the Oz-AIN Supp diet.

The Amy mice that were fed the Oz-AIN Supp diet spent significantly more time in the Test Quadrant than in the Opposite Quadrant ($p=0.0003$, Figure 6A). At 12 months, Amy mice that were the Oz-AIN Supp diet also swam significantly further in the Test Quadrant then in the Opposite Quadrant, confirming that these mice had intact spatial memory ($p=0.02$, Figure 6B). The current finding that the 12 month old mice spent more time (s) and travelled further distances (m) in the Test Quadrant than in the Opposite Quadrant suggests that at 12 months of age, all mice had intact spatial memory (Figure 6).

Figure 6A. Time spent (s) by 12 month old normal and Amy mice in the Test and Opposite Quadrant during the Test Trial of the Morris Water Maze.

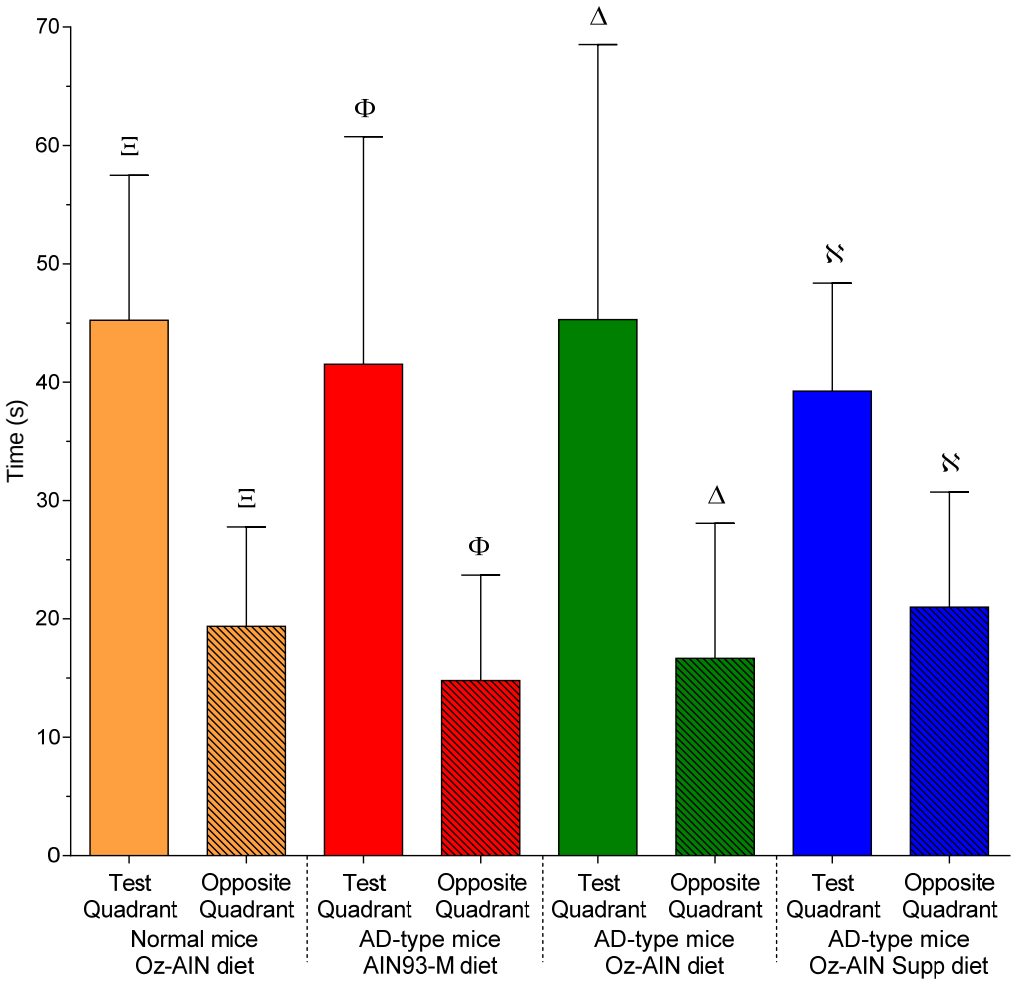


Figure 6A. Comparison of time (s) that 12 month old normal and Amy mice spent in the Test Quadrant (solid bars) and Opposite Quadrant (striped bars) during the Test trial of the Morris Water Maze. Normal mice fed the Oz-AIN diet (orange bars, n=12), Amy mice fed the AIN93-M diet (red bars, n=11), Amy mice fed the Oz-AIN diet (green bars, n=15), and Amy mice fed the Oz-AIN Supp diet (blue bars, n=16). Bars are mean ±SEM. Bars with matching symbols are significantly different with Student's *t*-tests. (Ξ) p=0.0001. (χ) p=0.0003. (Δ) p=0.004. (Φ) p=0.005.

Figure 6B. Distance travelled (m) by 12 month old normal and Amy mice in the Test and Opposite Quadrant during the Test Trial of the Morris Water Maze.

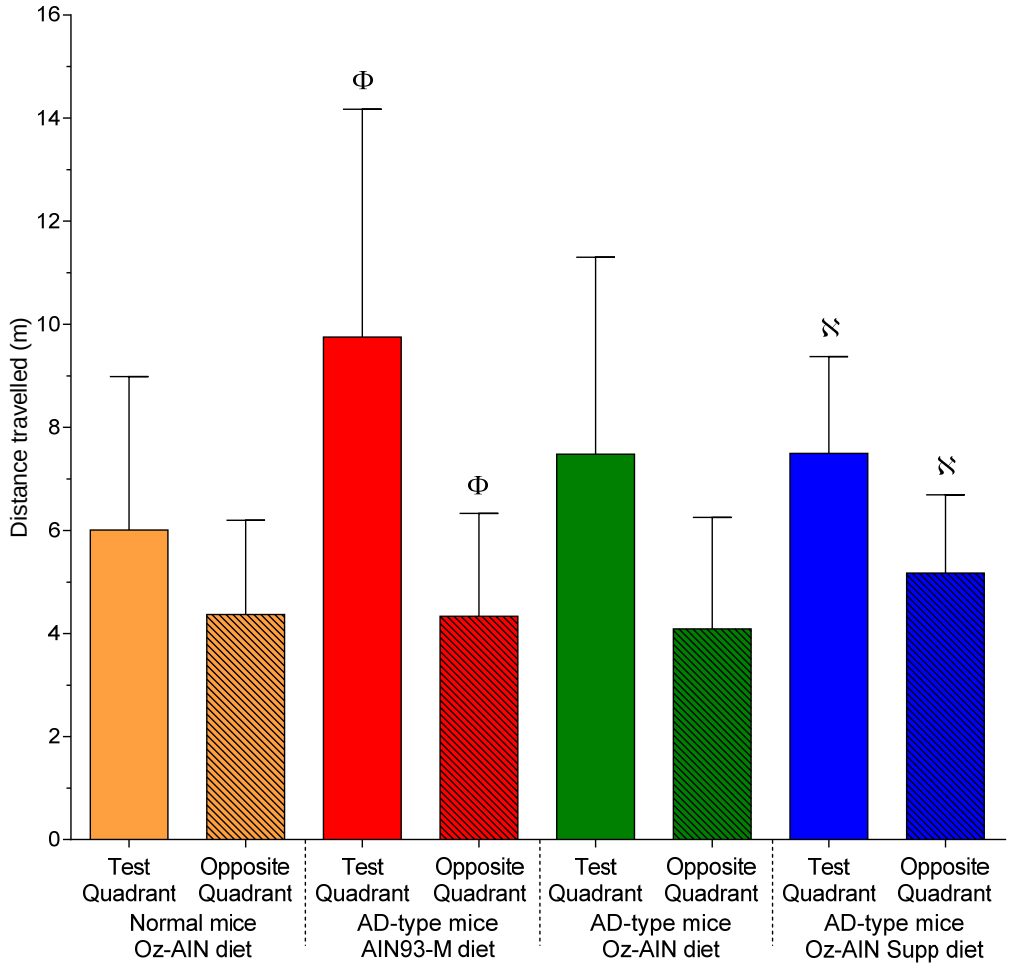


Figure 6B. Comparison of the distance travelled (m) by normal and Amy mice in the Test Quadrant (solid bars) and Opposite Quadrant (striped bars) during the Test trial of the Morris Water Maze. Normal mice fed the Oz-AIN diet (orange bars, n=12), Amy mice fed the AIN93-M diet (red bars, n=11), Amy mice fed the Oz-AIN diet (green bars, n=15), and Amy mice fed the Oz-AIN Supp diet (blue bars, n=16). Bars are mean ±SEM. Bars with matching symbols are significantly different with Student's *t*-tests. (ϰ) p=0.02. (Φ) p=0.032.

Between groups comparisons: Nutrient supplements against GENOTYPE EFFECTS on spatial memory abilities of 12 month old Amy mice.

One-way ANOVA's did not detect genotype effects on the percentage of time spent in the Test Quadrant (p=0.53, Table 21) or Opposite Quadrant (p=0.50, Table 21) by normal mice that were fed the Oz-AIN diet, Amy mice that were fed the Oz-AIN diet or Amy mice that were fed the Oz-AIN Supp diet. Similarly, one-way ANOVA's did not detect significant differences in the percentage distance travelled in the Test Quadrant (p=0.60, Table 22) or Opposite Quadrant (p=0.51, Table 22).

Furthermore, a one-way ANOVA did not detect significant differences in the number of times that normal mice or Amy mice that were fed the Oz-AIN diet, or Amy mice that were fed the Oz-AIN Supp diet, passed over the platform position (p=0.34, Table 23).

Collectively, this suggests that there were no genotype effects on spatial memory, and also that that nutrient supplements do not affect spatial memory abilities of 12 month old Amy mice.

Table 21. Percentage of time that 12 month old normal and Amy mice spent in the Test Quadrant and Opposite Quadrant of the Morris Water Maze during a 120 s test trial.

	Normal mice Oz-AIN diet	Amy mice Oz-AIN diet	Amy mice Oz-AIN Supp diet
Test Quadrant (%)	37.69 ±3.08	37.37 ±5.00	32.71 ±1.96
Opposite Quadrant (%)	16.14 ±2.21	17.08 ±2.23	17.49 ±2.09

All values are reported as mean ±SEM.

Table 22. Percentage of the total distance travelled by 12 month old normal and Amy mice in the Test Quadrant and Opposite Quadrant of the Morris Water Maze during a 120 s test trial.

	Normal mice Oz-AIN diet	Amy mice Oz-AIN diet	Amy mice Oz-AIN Supp diet
Test Quadrant (%)	29.98 ±2.41	35.73 ±5.60	32.06 ±2.21
Opposite Quadrant (%)	22.56 ±1.83	19.16 ±2.81	22.06 ±1.83

All values are reported as mean ±SEM.

Table 23. Number of times that 12 month old normal and Amy mice passed over the platform location during a 120 s test trial.

	Normal mice Oz-AIN diet	Amy mice Oz-AIN diet	Amy mice Oz-AIN Supp diet
Platform crossings	3.70 ±0.73	5.13 ±1.08	3.60 ±0.50

All values are reported as mean ±SEM.

Between groups comparisons: Nutrient supplements against DIET-TYPE EFFECTS on spatial memory abilities of 12 month old Amy mice.

There was no diet-type effect on spatial memory in 12 month old mice. The percent time spent in the Test Quadrant was the same for Amy mice that were fed the AIN93-M diet, the Oz-AIN diet or the Oz-AIN Supp diet ($p=0.66$, Table 24). Similarly, the percentage of time that mice spent searching in the Opposite Quadrant was the same for all dietary groups ($p=0.28$, Table 24). This suggests that all mice were equally disinterested in spending much time in the Opposite Quadrant to search for the submerged platform once it had been removed from the pool.

The percentage distance travelled in the Test Quadrant by Amy mice that were fed the AIN93-M diet, the Oz-AIN diet or the Oz-AIN Supp diet also indicates that all mice tried equally hard to locate the platform in the correct quadrant (Table 25). There were no significant differences detected for the percentage of overall distance that was spent in the Test Quadrant by the Amy mice that were fed the AIN93-M diet, the

Oz-AIN diet or the Oz-AIN Supp diet (p=0.77, Table 25). Similarly, there was no difference between treatment groups for the percent distance travelled in the Opposite Quadrant (p=0.31, Table 25).

There were no significant diet-type effects on the number of passes over the platform location (p=0.35, Table 26). However, Amy mice that were fed the Oz-AIN diet passed over the platform more times than Amy mice that were fed either the AIN93-M diet or the Oz-AIN Supp diet, suggesting that they may have searched the Test Quadrant with greater accuracy (Table 26).

These data demonstrate that diet-type does not affect spatial memory in 12 month old Amy mice. Amy mice that are fed the Oz-AIN Supp diet may have searched the Test Quadrant with greater accuracy, but this was not significant.

Table 24. Percentage of time that 12 month old Amy mice spent in the Platform Quadrant and Opposite Quadrant of the Morris Water Maze during a 120 s test trial.

	Amy mice AIN93-M diet	**Amy mice Oz-AIN diet**	**Amy mice Oz-AIN Supp diet**
Test Quadrant (%)	34.61 ±4.83	37.37 ±5.00	32.71 ±1.96
Opposite Quadrant (%)	12.33 ±2.24	17.08 ±2.23	17.49 ±2.09

All values are reported as mean ±SEM.

Table 25. Percentage of the total distance travelled by 12 month old Amy mice in the Test Quadrant and Opposite Quadrant of the Morris Water Maze during a 120 s test trial.

	Amy mice AIN93-M diet	**Amy mice Oz-AIN diet**	**Amy mice Oz-AIN Supp diet**
Test Quadrant (%)	36.42 ±5.54	35.73 ±5.60	32.06 ±2.21
Opposite Quadrant (%)	16.56 ±2.81	19.16 ±2.81	22.06 ±1.83

All values are reported as mean ±SEM.

Table 26. Number of times that 12 month old Amy mice passed over the platform location during a 120 s test trial.

	Amy mice AIN93-M diet	Amy mice Oz-AIN diet	Amy mice Oz-AIN Supp diet
Platform crossings	5.00 ±0.80	5.13 ±1.08	3.60 ±0.50

All values are reported as mean ±SEM.

6.2.4.2. Performance of 15 month old normal and Amy mice during the test trial in the Morris Water Maze.

Normal mice fed the Oz-AIN diet.

The 15 month old normal mice that were fed the Oz-AIN diet significantly more time in the Test Quadrant than in the Opposite Quadrant (p=0.04, Figure 7A). Mice also travelled further in the Test Quadrant than the Opposite Quadrant while searching for the platform (p=0.04, Figure 7B). This indicates that at 15 months of age, normal mice that were fed the Oz-AIN diet had intact spatial memory.

Amy mice fed the AIN93-M diet.

The 15 month old Amy mice that were fed the AIN93-M diet failed to demonstrated spatial memory. There was no significant difference in the time spent in the Test Quadrant or the Opposite Quadrant while searching for the removed platform (p=0.09, Figure 7A). There were also no differences in distance travelled in either quadrant (p=0.30, Figure 7B). This indicates that the 15 month old Amy mice that had been fed the AIN93-M diet did not recall where the platform was.

Amy mice fed the Oz-AIN diet.

The 15 month old Amy mice that were fed the Oz-AIN diet spent significantly more time in the Test Quadrant than in the Opposite Quadrant (p=0.002, Figure 7A).

Furthermore, mice travelled significantly further in the Test Quadrant while searching for the platform ($p=0.0003$, Figure 7B). This indicates that at 15 months, Amy mice that were fed the Oz-AIN diet had intact spatial memory.

Amy mice fed the Oz-AIN Supp diet.

The 15 month old Amy mice that were fed the Oz-AIN Supp diet spent significantly more time in the Test Quadrant than in the Opposite Quadrant ($p=0.0004$, Figure 7A). These mice also travelled significantly further in the Test Quadrant then the Opposite Quadrant while searching for the platform, indicating that they did have intact spatial memory.

Figure 7A. Time spent (s) in the Test and Opposite Quadrant during the Test Trial of the Morris Water Maze by 15 month old normal and Amy mice.

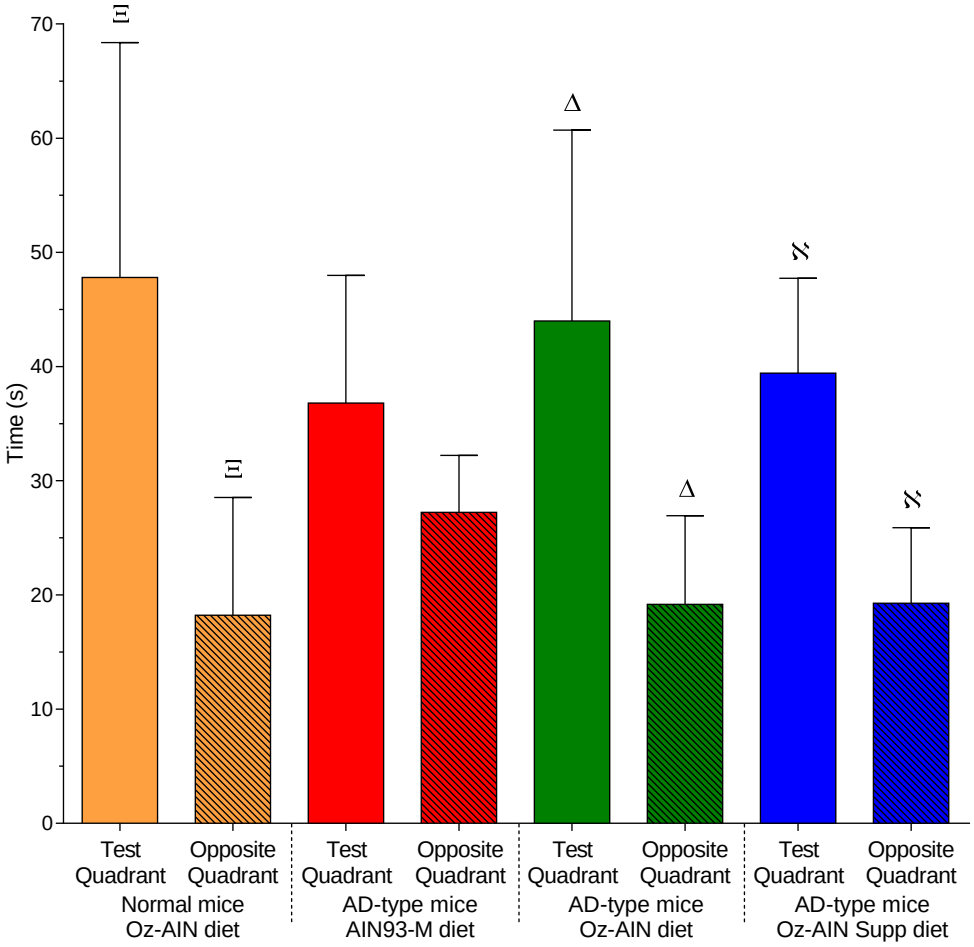


Figure 7A. Comparison of time spent (s) in the Test (solid bars) and Opposite (striped bars) quadrants during the Test trial of the Morris Water Maze for 15 month old mice. Normal mice fed the Oz-AIN diet (orange, n=8), Amy mice fed the AIN93-M diet (red, n=11), Amy mice fed the Oz-AIN diet (green, n=13), and Amy mice fed the Oz-AIN Supp diet (blue, n=12). Bars are mean ±SEM. Bars with matching symbols are significantly different with Student's *t*-tests. (ϰ) p=0.0004.
(Δ) p=0.002. (Ξ) p=0.04.

Figure 7B. Distance travelled (m) in the Test and Opposite Quadrant during the Test Trial of the Morris Water Maze by 15 month old normal and Amy mice.

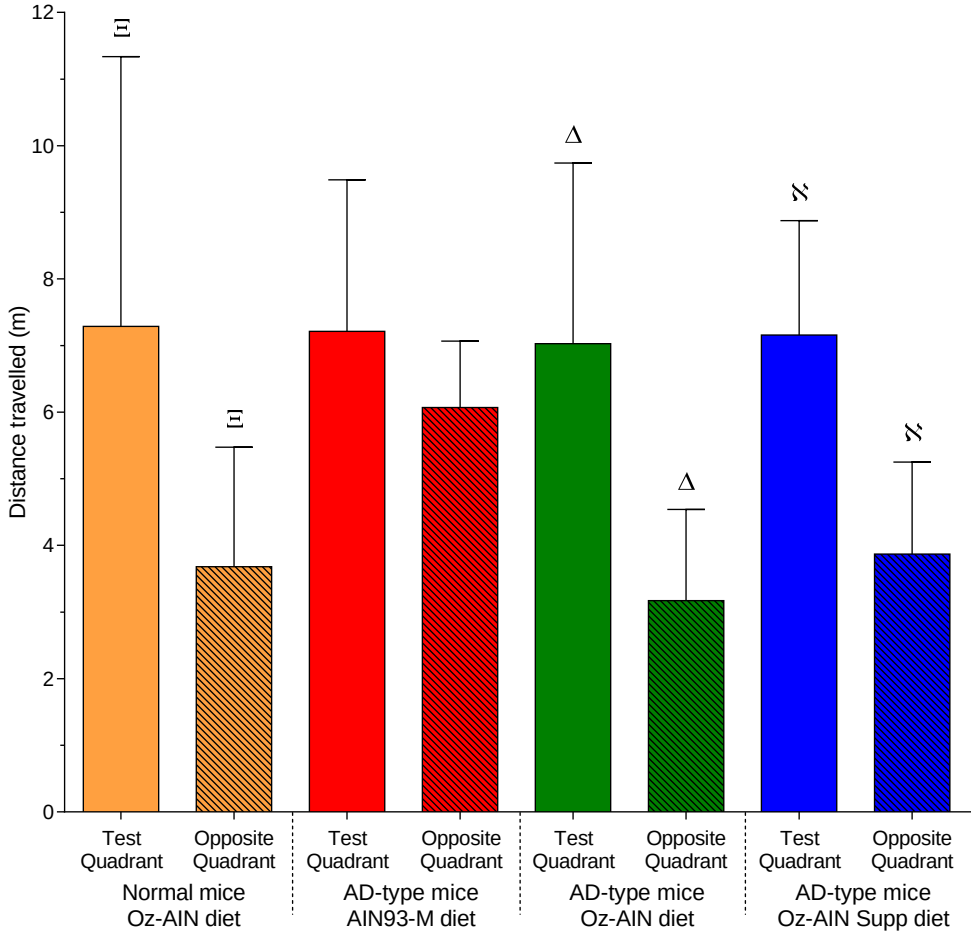


Figure 7B. Comparison of the distance travelled in the Test (solid bars) and Opposite (striped bars) quadrants during the Test trial of the Morris Water Maze for 15 month old mice. Normal mice fed the Oz-AIN diet (orange, n=8), Amy mice fed the AIN93-M diet (red, n=11), Amy mice fed the Oz-AIN diet (green, n=13), and Amy mice fed the Oz-AIN Supp diet (blue, n=12). Bars are mean ±SEM. Bars with matching symbols are significantly different with Student's *t*-tests. (ϰ) p=0.001. (Δ) p=0.003. (Ξ) p=0.04.

Between groups comparisons: Nutrient supplements against GENOTYPE EFFECTS on spatial memory abilities of 15 month old Amy mice.

Genotype did not affect spatial memory of 15 month old Amy mice. A one-way ANOVA did not detect significant differences in the percent time spent in the Test Quadrant while looking for the platform by the normal mice that were fed the Oz-AIN diet, Amy mice that were fed the Oz-AIN diet and Amy mice that were fed the Oz-AIN Supp diet ($p=0.81$, Table 27). Similarly, there were no significant differences in percentage of distance travelled in the Test Quadrant ($p=0.56$, Table 28). Further, there were no significant differences between the number of times that normal mice that were fed the Oz-AIN diet, Amy mice that were fed the Oz-AIN diet or Amy mice that were fed the Oz-AIN Supp diet passed over the platform ($p=0.65$, Table 29). Collectively, these data suggest that genotype did not affect spatial memory of 15 month old Amy mice.

Table 27. Percentage of time that 15 month old normal and Amy mice spent in the Test Quadrant and Opposite Quadrant of the Morris Water Maze during a 120 s test trial.

	Normal mice Oz-AIN diet	Amy mice Oz-AIN diet	Amy mice Oz-AIN Supp diet
Test Quadrant (%)	34.86 ±7.50	36.66 ±4.02	32.86 ±2.00
Opposite Quadrant (%)	13.30 ±3.39	15.99 ±1.87	16.07 ±1.59

All values are reported as mean ±SEM.

Table 28. Percentage of the total distance travelled by 15 month old normal and Amy mice in the Test Quadrant and Opposite Quadrant of the Morris Water Maze during a 120 s test trial.

	Normal mice Oz-AIN diet	Amy mice Oz-AIN diet	Amy mice Oz-AIN Supp diet
Test Quadrant (%)	36.93 ±5.84	37.59 ±3.54	32.62 ±2.09
Opposite Quadrant (%)	14.81 ±3.32	16.88 ±1.80	17.60 ±1.67

All values are reported as mean ±SEM.

Table 29. Number of times that 15 month old normal and Amy mice passed over the platform location during a 120 s test trial.

	Normal mice Oz-AIN diet	Amy mice Oz-AIN diet	Amy mice Oz-AIN Supp diet
Platform crossings	5.13 ±1.80	5.42 ±0.87	4.17 ±0.61

All values are reported as mean ±SEM.

Between groups comparisons: Nutrient supplements against DIET-TYPE EFFECTS on spatial memory abilities of 15 month old Amy mice.

Diet-type had an effect on spatial memory in 15 month old Amy mice. However, while there was no significant difference in the percent time that Amy mice that were fed the AIN93-M diet, the Oz-AIN diet or the Oz-AIN Supp diet spent in the Test Quadrant ($p=0.43$, Table 30), a one-way ANOVA detected significant differences in the percent time that mice spent in the Opposite Quadrant ($p=0.02$, Table 30). Amy mice that were fed the AIN93-M diet spent significantly more time in the Opposite Quadrant then Amy mice that were fed either the Oz-AIN diet ($p=0.03$, Table 30) or the Oz-AIN Supp diet ($p=0.02$, Table 30). Similar differences were also detected for the distance travelled in the Opposite Quadrant ($p=0.02$, table 31). Amy mice that were fed the AIN93-M diet travelled significantly further in the Opposite Quadrant than mice that were fed either the Oz-AIN diet ($p=0.02$, table 31) or the Oz-AIN Supp diet ($p=0.03$, Table 31). This suggests that Amy mice that were fed the AIN93-M diet were more inclined to search longer in the incorrect quadrants for the missing platform.

Despite spending a higher percentage of time and distance travelled in the wrong quadrant, Amy mice that were fed the AIN93-M diet made a similar number of passes over the platform position as Amy mice that were fed either the Oz-AIN diet ($p>0.99$, Table 32) or the Oz-AIN Supp diet ($p>0.99$, Table 32).

Collectively these data indicate that diet-type has an effect on spatial memory in 15 month old mice. However, while there was no difference in accuracy whilst in the Test Quadrant, the Amy mice that were fed the AIN93-M diet spent a greater percentage of time and overall distance in the wrong quadrant. This suggests that either the Oz-AIN diet is protective of spatial memory in 15 month old mice or that the mice that were fed the AIN93-M diet quickly learned that the platform was not where it was meant to be, and gave up searching.

Table 30. Percentage of time that 15 month old Amy mice spent in the Platform Quadrant and Opposite Quadrant of the Morris Water Maze during a 120 s test trial.

	Amy mice AIN93-M diet	Amy mice Oz-AIN diet	Amy mice Oz-AIN Supp diet
Test Quadrant (%)	30.67 ±3.11	36.66 ±4.02	32.86 ±2.00
Opposite Quadrant (%)	22.07 ±1.39	15.99 ±1.87$^{\#}$	16.07 ±1.59$^{\#}$

Diet-type effects were observed. All values are reported as mean ±SEM.
(#) p=<0.05 compared to Amy mice that were fed the AIN93-M diet.

Table 31. Percentage of the total distance travelled by 15 month old Amy mice in the Test Quadrant and Opposite Quadrant of the Morris Water Maze during a 120 s test trial.

	Amy mice AIN93-M diet	Amy mice Oz-AIN diet	Amy mice Oz-AIN Supp diet
Test Quadrant (%)	28.77 ±2.79	37.59 ±3.54	32.62 ±2.09
Opposite Quadrant (%)	24.44 ±1.52	16.88 ±1.80$^{\#}$	17.60 ±1.67$^{\#}$

Diet-type effects were observed. All values are reported as mean ±SEM.
(#) p=<0.05 compared to Amy mice that were fed the AIN93-M diet.

Table 32. Number of times that 15 month old Amy mice passed over the platform location during a 120 s test trial.

	Amy mice AIN93-M diet	Amy mice Oz-AIN diet	Amy mice Oz-AIN Supp diet
Platform crossings	4.89 ±0.96	5.42 ±0.87	4.17 ±0.61

All values are reported as mean ±SEM.

6.2.5. Summary of results.

The following tables provide a summary of the results from the MWM, and are arranged in order of the hypotheses that they address. The discussion section corresponds with the tables below.

Table 33. Aging effects on spatial learning and spatial memory abilities of normal and Amy mice at 6, 12 and 15 months.

Spatial Learning	*Normal mice that were fed the Oz-AIN diet:* Spatial learning abilities declined with age (ns). *Amy mice that were fed the AIN93-M diet:* Spatial learning abilities declined with age (improved latency, p=0.03, improved distance, ns). *Amy mice that were fed the Oz-AIN diet:* Spatial learning abilities declined with age (improved latency, p=0.001; improved distance, p=0.008). *Amy mice that were fed the Oz-AIN Supp diet:* Spatial learning abilities declined with age (improved latency, ns; improved distance, p=0.003).
Spatial Memory	*Normal mice that were fed the Oz-AIN diet:* Spatial memory improved with age. While aging mice consistently spent a similar percentage of time in the Test Quadrant (ns), they reduced the percentage of time spent in the Opposite Quadrant (p=0.04). *Amy mice that were fed the AIN93-M diet:* No effect of aging in Test Quadrant (ns). Percent time and distance in the Opposite Quadrant decreased over time (p=0.02), suggesting that as mice aged they had reduced preference to search the quadrant furthest from the platform. *Amy mice that were fed the Oz-AIN diet:* Spatial memory improved with age. As they aged, mice spent less time (p=0.008) and travelled shorter distances (ns) in the Opposite Quadrant. *Amy mice that were fed the Oz-AIN Supp diet:* Spatial memory improved with age. Mice travelled a further distance in the Test Quadrant as they aged (p=.04) and a lesser distance in the Opposite Quadrant as they aged (p=0.04).

Table 34. The potentially beneficial effects of nutrient supplements against GENOTYPE effects on spatial learning and memory abilities of 12 and 15 month old Amy mice.

Spatial learning	*12 months:* Genotype *did not* have an effect on spatial learning. Significant differences were observed in improved latencies, but not for improved distances travelled to the platform. *15 months:* Genotype *had* an effect on spatial learning abilities of Amy mice. Amy mice that were fed the Oz-AIN diet failed to learn the location of the platform. The genotype-effect was prevented with nutrient supplements.
Spatial memory	*12 months:* Genotype had no effect on spatial memory in 12 month old mice. This was not affected by nutrient supplements. *15 months:* Genotype had no effect on spatial memory in 15 month old mice. This was not affected by nutrient supplements.

Table 35. The potentially beneficial effects of nutrient supplements against DIET-TYPE effects on spatial learning and spatial memory abilities of 12 and 15 month old Amy mice.

Spatial learning	*12 months:* Diet-type *had* an effect on spatial learning abilities of Amy mice. Amy mice that were fed the Oz-AIN diet made the largest improvements after five training days. This was prevented with nutrient supplements. *15 months:* Diet-type *had* an effect on spatial learning abilities of Amy mice. Amy mice that were fed the Oz-AIN diet failed to learn the location of the platform. This diet-type effect was prevented with nutrient supplements.
Spatial memory	*12 months:* Diet-type *did not* have a significant effect on spatial memory, but Amy mice that were fed the Oz-AIN diet may have searched for the platform with greater accuracy. *15 months:* Diet-type *had* an effect on spatial memory in 15 month old Amy mice. Amy mice that were fed AIN93-M diet failed to demonstrate spatial memory. This diet-type effect was not affected by nutrient supplements.

6.3. Discussion.

The present study investigated the potential protective effects of nutrient supplements against spatial learning and spatial memory deficits associated with aging, genotype or diet-type. The spatial learning and spatial memory abilities of normal and Amy mice were assessed in the MWM at 6, 12 and 15 months.

In order to control for the effects of any age-related health problems that have been identified in other studies, such as cataracts or reduced mobility [487], mice were tested for their ability to respond to visual cues to locate the submerged platform in the MWM. The normal and Amy mice were able to locate a visible platform in the MWM when they were 6, 12 and 15 months old. This confirmed that aging did not affect ability to respond to visual cues and that all mice were equally able to locate the submerged platform.

Aging effects on spatial learning and spatial memory abilities of normal and Amy mice at 6, 12 and 15 months.

Consistent with the first aim of this chapter the effect of aging on spatial learning and spatial memory was investigated in normal and Amy mice.

Spatial Learning.

The normal mice that were fed the Oz-AIN diet made similar improvements in distance travelled to the platform at 6 and 12 months old. However, the improved distance at 15 months was much smaller than that at 12 months. The Amy mice that were fed the Oz-AIN diet made significantly smaller improvements in latency and distance travelled to the platform at 15 months compared to when they were 6 and 12 months old. This suggests that age-related spatial learning deficits in mice that are fed

a high-fat diet are most evident late in life. This is consistent with reports of other studies that report that a high-fat diet accelerates spatial learning deficits in normal and AD-type mice [186, 469, 480, 481, 483].

In contrast to this, the age-related decline in spatial learning abilities of Amy mice that were fed the AIN93-M diet or the Oz-AIN Supp diet appeared to be gradual and consistent. This implies that the decline in spatial learning abilities of mice that are fed either an optimal diet or nutrient supplements may be slower or more gradual than for mice that are fed a high-fat diet. Although some of these findings were marginal, they suggest that diet, and diet supplements, can have a positive effect on spatial learning abilities over time. This is consistent with reports from others, that dietary supplement combinations of fatty acids and polyphenolic compounds have beneficial effects on spatial learning abilities of mice [202, 488, 489]. .

Spatial Memory.

Unlike age-related decline in spatial learning ability, there was no clear age-related decline in spatial memory in normal or Amy mice. The amount of time that mice spent in the Opposite Quadrant decreased with age, indicating that mice had a reduced preference for the quadrant on the opposite side of the pool while they were searching for the platform. This may reflect an age-related improvement in searching strategies.

The different types of searching strategies used by mice can explain the behaviours underlying differences in latencies and distances travelled to the platform. Mice with intact spatial memory, for example, will swim straight to the platform because they remember where it is in relation to where they are in the maze [96, 139, 462]. Other mice may remember that the platform was a particular distance from the pool wall,

and spend search time swimming broad laps around the MWM arena. This search strategy is called 'chaining' [488]. Although chaining is more efficient than the random search strategies employed by mice with impaired spatial learning abilities, it may result in what appears to be poorer performances in the MWM, despite mice having intact, *albeit* limited spatial learning and spatial memory abilities [139, 488, 490]. Janus reports that normal mice may rely on chaining strategies in the early stages of learning in the MWM, however in the latter stages and in the test trial, mice use more efficient spatial strategies to locate the submerged platform [490]. On the other hand, AD-type mice don't appear to demonstrate progression from non-spatial to spatial search strategies [490]. This is to be expected, as spatial learning and memory are hippocampal dependent tasks, and the hippocampus is one of the primary regions of the brain affected in AD [82, 83, 86, 87].

Parachikova *et al.* report that feeding mice a nutrient supplement combination rich in polyphenols and antioxidants conserved hippocampal abilities of AD-type mice, making them indistinguishable from normal mice [204]. This suggests that the Amy mice that were fed the Oz-AIN Supp diet, which also contained polyphenolic compounds and antioxidants, may have had intact hippocampal function. Therefore, it is possible that the decreased time spent in the Opposite Quadrants as mice aged reflect improved search strategies, whereby mice may not have recalled exactly where the platform *was* but remembered where the platform *was not*. The consequence of this is that mice spent less time in the quadrant least likely to contain the missing platform.

Amy mice that were fed the Oz-AIN diet made the greatest improvements in spatial memory and search strategy as they aged. Although this relationship was non-significant, it point to a disparity with research concerning diet and cognitive ability.

Specifically, saturated fat has been demonstrated to have a detrimental effect on spatial memory [472, 479, 484] as has B-vitamin deficiency [330, 465], and both of these are characteristics of the Oz-AIN diet. It may therefore be expected that the Oz-AIN diet would have a detrimental effect on cognitive abilities of Amy mice, which was not that case in the current study. This suggests that, although the Oz-AIN diet may have elements that impair spatial memory, the Oz-AIN diet may have somehow had an optimal effect on spatial strategies in aging Amy mice. It is possible that some other feature of the Oz-AIN diet reduced these otherwise well documented characteristics.

Alternately, these results may have been a consequence of over training mice. Mice were assessed in the MWM at 6, 12 and 15 months of age. Mice may have become more familiar with the task at each age, as assessments were only 3 months apart. This may have had several effects on performance in the MWM as mice aged. In support of this, Mielke *et al.* used a longitudinal study to demonstrate the effects of a high-fat diet on weight gain, hippocampal insulin signalling and cognitive abilities of aging normal mice. They report that whilst the high-fat diet impaired glucose metabolism and insulin signalling in the hippocampus, there were no differences in performance in the MWM after 5 or 10 months of feeding. However, they did observe impaired performance in a bar pressing task. Meilke *et al.* initially suggest that the reason they did not observe differences in performance in the MWM may be that the test is that the strain of mice they used is particularly good at performing tasks such as the MWM. However, rats are more suited to water maze tasks [96], and have also been used to demonstrate the detrimental effects of high-fat diets on spatial memory [491]. The other alternative to explain why Mielke *et al.* and the current study both have not observed spatial learning deficits in mice that have been fed high-

fat diets may be that mice were over-trained and recalled the platform location on subsequent assessments.

In order to control for any effects of familiarity at 12 months and 15 months the platform was placed in a new position at each age. When mice were 6 months old, they were trained to find a submerged platform in the South West quadrant of the pool; when mice were 12 months old the platform was in the North West quadrant of the pool; and when mice were 15 months old the platform was in the South East quadrant of the pool. Therefore, it is unlikely that they retained memory for the platform location over the three months between tests. Nonetheless, this possibility can not be completely ruled out.

Although the location of the platform was moved, the familiarity with the task itself may have contributed to improvement over time, in terms of reducing levels of anxiety. Certainly, anxiety has been reported to impair performance of mice in other behavioural tests [468, 492], and AD-type mice have been reported to be more prone to anxiety than normal mice [493]. The increased familiarity and reduced anxiety may have enabled mice to search for the platform more effectively. This does not necessarily mean that mice had better memory for the platform location at 15 compared to 6 months, or that their better performance was solely due to reduction of anxiety. Rather, it means that unlike other studies in which anxiety was a confounding factor, anxiety may not have been an issue for mice in this study because they became more familiar with the task. It does, however, emphasise the importance of including other measures such as distance travelled or passes over the platform location to compare spatial memory between *aging* mice, as these factors are less likely to be confounded by the effects of repetitive testing.

Summary of the effects of aging.

Collectively, studies concerning the effect of aging on spatial learning and spatial memory indicate that spatial learning abilities decline with age in normal and Amy mice and that the rate of decline may be affected by diet-type. Age related deficits in spatial learning were not apparent in normal and Amy mice that had been fed the Oz-AIN diet until they were 15 months old. On the other hand, Amy mice that received an optimal diet or nutrient supplements appeared to have a slower and gradual decline in spatial learning skills. These results also suggest that aging does not have a detrimental effect on spatial memory in normal or Amy mice. It is proposed here that this may have been a consequence of improved search strategies as mice aged, or a result of familiarity with the task.

The potentially beneficial effects of nutrient supplements against GENOTYPE effects on spatial learning and memory abilities of 12 and 15 month old Amy mice.

Consistent with the second aim of this chapter the potential for nutrient supplements to prevent *genotype effects* on spatial learning and spatial memory in 12 and 15 month old mice were investigated using the MWM.

Spatial Learning

In Chapter 5 genotype did not have an effect on spatial learning of 12 month old normal or Amy mice that were fed the Oz-AIN diet. The findings of the present study confirm those findings, specifically, that genotype did not have an effect on spatial learning abilities of 12 month old Amy mice.

The absence of differences between genotypes for their improvements of latency to the platform after five training days amongst normal mice could potentially reflect a

'ceiling' effect. The Amy mice that were fed the Oz-AIN diet took longer than normal mice to locate the platform on Day 1 of the acquisition period, but all mice reached the platform with similar latencies on Day 5. It is possible that the latency on Day 5 reflects the optimal time that it will take 12 month old mice to reach the submerged platform. In this scenario, the normal mice that were fed the Oz-AIN diet had less room for improvement before reaching optimal latencies than Amy mice that were fed the Oz-AIN diet. Therefore, other measures of spatial memory that are not time dependent can provide further insight into spatial learning abilities of 12 month old AD-type mice. These may include tests such as a multiple T maze, whereby the primary indicators of learning are the number of correct choices a mouse makes while it guides its way through a maze to receive a reward [468].

Because there were no genotype effects observed at 12 months, any beneficial effects of nutrient supplements on spatial learning abilities of 12 month old Amy mice can not be attributed to preventing genotype effects. Therefore, the effects of nutrient supplements at 12 months of age will be discussed further in the context of diet-type effects on spatial learning (pp. 412-414).

Genotype effects have been demonstrated at in young Ad-type mice that carry similar genetic mutations as the Amy mouse (i.e. they carry both the APP mutation and the PSEN1 mutation, see p.23 for explanation of genotype of Amy mice) [156, 158, 159]. Chen *et al.* report that 8 month old APP/PSEN1 mice take longer to locate the submerged platform after six training days, compared to age-matched controls [159]. Similarly, Nagakura *et al.* report that PS1/APP mice take longer to reach the platform after four training days, when they are only 4 months old [158]. Weismann *et al.* report that olfactory deficits in Amy mice were not detected until they were older than

ages observed in other models, and that at 11 months Amy mice take longer to locate the submerged platform than control mice after four training days [488].

Similar to Wiesmann *et al.* genotype effects were observed amongst old Amy mice that were fed the Oz-AIN diet. The 15 month old Amy mice that were fed the Oz-AIN diet did not make improvements of latency or distance travelled to the platform after five training days. In contrast to this, the 15 month old normal mice that were fed the Oz-AIN diet made significant improvements by Day 2, and then improved again on Day 5. There was a great deal of variance of distance travelled on each training day within groups, which may be why significant differences were no observed for changes in distance travelled.

At 15 months of age, nutrient supplements prevented the genotype effects on spatial learning abilities in Amy mice that were fed the Oz-AIN diet. Nutrient supplements such as fish oil have been demonstrated to have a beneficial effect on cognitive functioning in AD-type and aging rodents [86, 329, 481, 494]. Docosahexaenoic acid supplementation enhances brain levels of docosahexaenoic acid and enhances synaptic transmission in mouse hippocampus [494]. Furthermore, dietary intervention with fish oil decreases brain ω-6:ω-3 ratios, which also increases spatial learning in the MWM [489]. In other mouse models of AD, dietary docosahexaenoic acid supplements improved spatial learning abilities in AD-type mice [86, 329]. In the context of these reports, the findings of the current study suggest that the beneficial effect of the Oz-AIN Supp diet in preventing spatial learning deficits in Amy mice may be attributed to the beneficial effects of the docosahexaenoic acid that had been added to the Oz-AIN Supp diet.

Spatial Memory.

Consistent with the studies of the detrimental effect of the Oz-AIN diet that are presented in Chapter 5, genotype did not have an effect on spatial memory in 12 month old Amy mice that are fed the Oz-AIN diet. However, the current study is not consistent with Chapter 5 in regards to the spatial memory abilities of 15 month old mice. Chapter 5 reported that the 15 month old Amy mice that were fed the Oz-AIN diet did not demonstrate spatial memory in the MWM, because they spent equal percentages of time in the Test and Opposite Quadrant. This led to the conclusion that at 15 months, genotype does have an effect on spatial memory. However, in the current study, 15 month old Amy mice that were fed the Oz-AIN diet did demonstrate spatial memory, and hence no genotype effects are reported. It is proposed here that there were large variations within groups, which may have masked any differences that did exist.

Without any significant genotype effects on the spatial memory abilities of 12 and 15 months old Amy mice, it is difficult to discuss whether or not nutrient supplements may alter genotype effects on spatial memory in Amy mice. The spatial memory abilities of 12 and 15 month old Amy mice that were fed the Oz-AIN Supp diet are discussed in relation to diet-type effects on spatial memory.

Summary of GENOTYPE effects.

There were no genotype effects on spatial learning or spatial memory for 12 month old mice or for 15 month old mice. There were, however, descriptively clear genotype effects on spatial learning abilities when Amy mice were 15 months old, specifically, Amy mice's spatial learning declined with age, whereas normal mice's spatial learning did not. These genotype effects were prevented with nutrient supplements,

because the performance of Amy mice on the supplementary diet was better than Amy mice on the Oz-AIN diet. Collectively, these results do not support the hypothesis that genotype will have an effect on spatial learning and spatial memory in 12 and 15 month old Amy mice, and that this can be prevented with nutrient supplements. However, genotype-effects on spatial learning abilities of 15 month old Amy mice were clearly present, and they were prevented with nutrient supplements.

The potentially beneficial effects of nutrient supplements against DIET-TYPE effects on spatial learning and spatial memory abilities of 12 and 15 month old Amy mice.

Consistent with the third aim of this chapter the potential for nutrient supplements to prevent diet-type effects on spatial learning and spatial memory in 12 and 15 month old mice were carried out using the MWM.

Spatial Learning.

The studies that focused on the potentially detrimental effects of the Oz-AIN diet on spatial learning in Chapter 5 reported that the Oz-AIN diet may have had a beneficial effect on spatial learning amongst 12 month old Amy mice. Consistent with this, the current study also found that the Oz-AIN diet improved spatial learning abilities of 12 month old Amy mice. This contradicts a lot of the current literature that states that a high-fat diet has detrimental effects on spatial learning abilities of mice [186, 469, 480, 481, 483]. However, the beneficial effects of the Oz-AIN diet may not necessarily be attributed to the macronutrient content of the diet, but may instead be related to other diet-related behaviours such as increased environmental enrichment and activity associated with high-fat foods [167, 369, 482, 495].

In contrast to Chapter 5, the current study also reports significant effect of diet-type on spatial learning abilities of 15 month old Amy mice. However, unlike the apparently protective effect of the Oz-AIN diet at 12 months of age, the 15 month old Amy mice that were fed the Oz-AIN diet made no improvement in latency to platform after five training days. On the other hand, Amy mice that were fed the AIN93-M diet made significant improvements in latency and distance travelled to the platform after five training days. The beneficial effect of the AIN93-M diet on spatial learning abilities of Amy mice was so great that the improvements in distance and latency to the MWM platform were greater than that of normal mice. This suggests that at 15 months of age, the effect of diet-type is stronger than that of genotype on spatial learning abilities of Amy mice.

As already described, there is a great wealth of literature that describes the detrimental effect of a high-fat diet on cognitive abilities such as spatial learning [186, 469, 480, 481, 483]. However, other features of the Oz-AIN diet may have also impaired spatial learning in Amy mice. The Oz-AIN diet is low in folate, containing 43% less folate than recommended levels. Homocysteinemia, caused by folate and vitamin B12 deficiencies, leads to cognitive impairment in rodent models [334, 496]. Similar to the present study, Bernardo et al. tested the effect of dietary vitamin B deficiency on spatial learning in aged mice. They report that aged AD-type transgenic mice that were fed a folate deficient, homocysteinemia inducing diet had impaired spatial learning abilities in the MWM [496]. This suggests that the spatial learning deficits in the 15 month old Amy mice in the current study may be attributed to folate deficiency coupled with the high-fat content of the Oz-AIN diet.

The nutrient supplements had an effect on the diet-type induced changes in spatial learning abilities at 12 and 15 months of age. However, owing to the finding that the Oz-AIN diet had potentially beneficial effects on spatial memory, the ability for nutrient supplements to prevent Oz-AIN diet-induced changes in behaviour may not always be a benefit to performance.

It was tentatively suggested in Chapter 5 that one of the benefits of the Oz-AIN diet at 12 months of age may have been associated with food-related behaviour such as increased activity and its reported effects on cognition [167, 369, 482, 495]. While the Oz-AIN Supp diet shared many characteristics with the Oz-AIN diet, such as being high in fat, the Oz-AIN Supp diet had also been supplemented with dietary supplements that included curcumin, alpha-lipoic acid and fish oil as a source of docosahexaenoic acid. It is possible that either of these components, or a combination of the three reduced the palatability or pleasure derived from consumption of the Oz-AIN Supp diet. Therefore, mice were less likely to interact with their food, and the beneficial effects of food-associated behaviours that were proposed in Chapter 5, would have been reduced. However, there is very little evidence to support reduced palatability associated with any of the nutrient supplements used in this study, and even if the Amy mice that were fed the Oz-AIN Supp diet interacted with their food less, this is not likely to have played a large role in spatial learning and spatial memory abilities.

The apparent detrimental effect of the nutrient supplements on spatial learning at 12 months was not present among 15 month old mice. The 15 month old Amy mice that were fed the Oz-AIN diet performed as well, if not better than, Amy mice that were fed the AIN93-M diet. It is suggested here that the discrepancies in spatial learning abilities of 12 and 15 month old Amy mice that were fed the Oz-AIN Supp diet may

be due to the different effects of the Oz-AIN diet on spatial learning and spatial memory at these two ages. Chapter 5 reported that the Oz-AIN diet may have been beneficial for spatial learning and spatial memory at 12 and eighteen months but not 15 months. Similarly, in the current study, the 12 month old Amy mice that were fed the Oz-AIN diet had better spatial learning skills than when they were 15 months old.

While the pathological changes behind the differing effects of the Oz-AIN Supp diet on spatial learning abilities of 15 month old Amy mice have not been determined by the present study, it is clear that, irrespective of age, the effects of the Oz-AIN diet were prevented by the Oz-AIN Supp diet. This suggests that features of the Oz-AIN diet such as the ω-3 fatty acids or polyphenolic compounds, which have previously been demonstrated to prevent high-fat diet induced changes in AD-type mice [217, 225], may interfere with the beneficial effects of the Oz-AIN diet in 12 month old age.

As an example of this, the ω-3 fatty acids found in fish oil have been are promoted as having powerful anti-inflammatory capabilities, and have therapeutic benefits in maintaining cardiovascular health, neuronal development, mental health and obesity [497-500]. However, using the data from the Selenium and Vitamin E Cancer Prevention Trial (SELECT), Brasky *et al.* report that high plasma phospholipid long-chain ω-3 fatty acid content was associated with significantly higher risk for prostate cancer [501]. Men in the highest quartile had a 71% increased risk for high-grade prostate cancer [501]. Similar findings have been found in two other studies [502, 503]. This suggests that nutrient supplements that are considered to be beneficial may actually be dangerous in the context of different life stage or diet.

Spatial Memory.

Similar to Chapter 5, the Amy mice in the current studies that were fed the AIN93-M diet or the Oz-AIN diet demonstrated intact spatial memory. However, the current chapter does not report a beneficial diet-type effect on spatial memory in 12 month old Amy mice. While there are other studies that have demonstrated that diet-type did not affect spatial memory in adult mice, they have reported that irrespective of diet-type, mice fail to demonstrate spatial learning after 10 months of age [308]. As discussed earlier in this chapter, it is possible that mice demonstrated spatial memory as a consequence of over-training, familiarity or repeated testing. However, even the effects of over-training or repeated testing demonstrate the rodents' ability to learn and remember complex tasks. In order to control for the effects of repeated testing behavioural tests could be conducted using separate groups of mice at 6, 12, and 15 months.

In the current study the only group of 15 month old mice that did not demonstrate spatial memory was the Amy mice that were fed the AIN93-M diet, which is a low-fat diet. This implies that a diet that is higher in fats may be beneficial for spatial memory in aged mice. Consistent with this, the Amy mice that were fed the Oz-AIN Supp diet, which had the highest total fat content of all diets used, also demonstrated intact spatial memory at 15 months of age.

While the mechanism for the beneficial effects of the Oz-AIN diet was not determined in the current study, potential candidates for dietary components may be discovered by exploring the characteristics of the Oz-AIN diet that were unique relative to the Oz-AIN Supp diet and the AIN93-M diet. This includes being low in anti-oxidants such as folate, and being high in saturated fat. The detrimental effects of both of these characteristics on cognitive function are well established [171, 183, 186,

187, 208, 210, 296, 326], and any potential mechanism is purely speculative at this point. However, it would be beneficial to continue studies of specific effect of various fats on cognitive abilities of Amy mice, with and without essential anti-oxidants such as folate.

An alternate explanation for the findings of the benefit of the O-AIN diet in the current study is that in the Test Trial, the Amy mice that were fed the AIN93-M diet quickly learned that the platform was not in the expected location in the pool and looked elsewhere in the pool for a new escape path. The consequence of this is may be that they spent less time in the correct quadrant, even though they had intact spatial memory. Decreasing the length of the Test Trial could potentially prevent these effects. Comparison of latencies to first reaching the location of the missing platform during the Test Trial may indicate how accurately mice searched for the platform [468]. This would also provide further information about searching strategies of normal and Amy mice that are fed different diets.

Summary of DIET-TYPE effects.

Collectively, this study has indicated that diet-type has different effects on spatial learning abilities of 12 and 15 month old Amy mice. Consistent with reports from others about the detrimental effects of a high-fat diet, 15 month old Amy mice that were fed the Oz-AIN diet did not demonstrate spatial learning. However, the sub-optimal Oz-AIN diet appeared to prevent spatial learning deficits in 12 month old Amy mice. Under these circumstances, nutritional supplementation was not always beneficial for the spatial learning abilities of Amy mice that were fed a high-fat diet. The prevention of Oz-AIN diet effects on spatial learning meant that while 15 month old Amy mice that were fed the Oz-AIN Supp diet had improved spatial learning

abilities, they had poorer spatial learning abilities at 12 months of age, relative to other mice.

There were diet-type effects on spatial memory amongst 15 month old Amy mice. The current findings suggest that high total fat content may prevent spatial memory deficits in aged Amy mice. Amy mice that were fed the Oz-AIN diet or the Oz-AIN Supp diet, but not those fed the AIN93-M diet, demonstrated that they had intact spatial memory by spending a significantly greater proportion of the test trial in the correct quadrant (Figure 7). However, the majority of research reports that a high-fat diet is detrimental to spatial learning and spatial memory abilities. It is possible that the mice that were fed the AIN93-M diet had better spatial learning and memory than mice that were fed the Oz-AIN or the Oz-AIN Supp diets, and quickly learned that the platform was not in the correct quadrant. The consequence of this would be that they spent a greater proportion of the test searching in other quadrants.

6.4. Conclusion

The principal findings of this study were that age has an effect on spatial learning and spatial memory abilities in normal and Amy mice. While spatial learning abilities decreased with age, spatial memory abilities of normal and Amy mice appeared to improve. This may have been a consequence of the repetitive testing in the MWM, and that spatial memory at 12 and 15 months may have been re-enforced by training 3 months prior. Genotype and diet-type impaired spatial learning of Amy mice at 15 months of age, and this was prevented with nutrient supplements. However, despite failing to demonstrate spatial learning at 15 months, Amy mice that were fed the Oz-AIN diet demonstrated intact spatial memory. In contrast to this, Amy mice that were fed the AIN93-M diet did not demonstrate spatial memory but demonstrated intact spatial learning abilities. This suggests that at 15 months of age spatial learning and

spatial memory deficits are independent processes, which is similar to proposals from others [155]. Collectively, these results suggest that spatial learning and spatial memory are separate processes in mice that are independently affected by age, genotype, and diet.

Chapter 7: The effects of genotype and the Oz-AIN diet on ability to smell.

7. Background.

The olfactory bulb and tract are two regions of the brain that are important for the sense of smell. These regions degenerate in a β-amyloid dependent manner in the early stages of AD, and olfactory dysfunction is more severe in AD patients than normal healthy controls [504]. In light of this, olfactory ability has the potential to be a diagnostic marker for AD [505, 506]. However, before this can take place, a clearer understanding of the relationship between olfactory abilities and AD needs to be determined.

While olfactory decline has been observed in *some* AD mouse models, it is not well characterised in Amy mice [122, 505, 507, 508]. Owing to the fact that Amy mice are a robust model for other behavioural features of AD, such as cognitive decline [132, 509-511], it would be useful to establish whether or not olfactory dysfunction also occurs in Amy mice.

Olfactory functioning can also be regulated by food intake and nutritional status [512, 513]. For example, undernourishment can alter receptor populations in the nasal epithelium of rats [513], and hunger increases olfactory sensitivity in frogs, which enables identification of food odors at lower concentrations [514]. Satiety, on the other hand has been demonstrated to reduce olfactory neuronal activity in rats [512, 515]. Such research suggests that nutritional status and food intake can modulate olfactory abilities across a wide range of species.

The aims of the study described in this chapter are to:

1. **Demonstrate the effects of genotype on global olfactory function of normal and Amy mice (Figure 1A).**

   This was achieved by making two comparisons:

   (i) Olfactory abilities of normal and Amy mice that were fed the AIN93-M diet were compared.

   (ii) Olfactory abilities of normal mice and Amy mice that were fed the Oz-AIN diet were compared.

2. **Determine the effects of the Oz-AIN diet-type on global olfactory function in normal and Amy mice (Figure 1B).**

   This was achieved by comparing genotype matched mice that were fed one of two diets.

   (i) Normal mice that were fed either the AIN93-M diet or the Oz-AIN diet.

   (ii) Amy mice that were fed the AIN93-M diet or the Oz-AIN diet.

3. **Characterise olfactory function of normal and Amy mice at different stages of adult life.**

There were three hypotheses for the studies in this chapter. First, it was hypothesised that genotype has an effect on olfactory function and that Amy mice have worse olfactory abilities than diet-matched normal mice. Secondly, it was hypothesised that diet-type has an effect on olfactory function independently of genotype, and that mice that are fed the Oz-AIN diet have poorer olfactory ability than mice that are fed the AIN93-M diet. Third, it was hypothesised that olfactory decline occurs with aging, and this is the most pronounced in Amy mice that are fed the Oz-AIN diet.

This study has made use of a modified version of the Buried Food Pellet Test [114], which is called the Buried Chocolate Test (BCT), to assess global olfactory function. The BCT is a simple test, where the primary measures are the latency and distance travelled before a mouse uncovers a buried piece of chocolate. A mouse that has intact olfactory ability will be able to locate the chocolate quickly and within a short distance [114, 115]. The procedures used to conduct the BCT are described in detail in the methods section of this chapter (pp. 427-428).

The main findings from the BCT were that the genotype effects on olfactory ability are dependent on diet. Amy mice that have been fed a sub-optimal diet (the Oz-AIN diet) have poorer olfactory abilities than normal mice throughout life. However, Amy mice that have been fed an optimal diet (the AIN93-M diet) have similar, if not better olfactory abilities than normal mice. While the Oz-AIN diet was associated with age-related olfactory decline, the AIN93-M diet appeared to enhance olfactory abilities throughout life. These changes were more pronounced in Amy mice than normal mice, suggesting that Amy mice are more susceptible to diet-type effects on global olfactory function.

7.1. Methods.

7.1.1. Animals.

All experiments were approved by the Commonwealth Scientific and Industrial Research Organisation (CSIRO) Animal Welfare Committee, Australia in accordance with National Health and Medical Research Council guidelines.

Female Amy (APPswe/PSEN1dE9) mice and their female normal (C57bl/6) littermates were bred at and provided by Flinders University Animal Facility,

Bedford Park, South Australia. Genotype was confirmed by PCR and agarose gel electrophoresis, as described in Appendix I. Amy mice and normal mice were each fed either the Oz-AIN diet or the AIN93-M diet from weaning until the end of the study. Mice were housed (n<6) in cages lined with sawdust and had free access to food and water.

Mice were tested in the BCT at 6 and 12 months (Figure 1). Group sizes are reported in Table 1. Some mice were excluded from analyses due to either: changes in apparatus while the BCT was being established at CSIRO; or due to software not recording trials correctly. As a consequence of this, the group sizes used in the current study are between n=6-8, which is smaller than originally anticipated. However, group sizes between six and ten have been successfully used by others to demonstrate genotype and treatment effects on olfactory dysfunction in mice, and were therefore deemed acceptable for use in the current study [116, 118, 516].

Table 1. The number of mice assessed in the Buried Chocolate Test at 6 and 12 months of age.

	Normal mice AIN93-M diet	**Normal mice Oz-AIN diet**	**Amy mice AIN93-M diet**	**Amy mice Oz-AIN diet**
Treatment group size	n = 7	n = 8	n = 6	n = 6

7.1.2. Study design.

This study made use of a two-factorial design, where one factorial was genotype (normal versus Amy mice) and the other factorial was diet-type (AIN93-M diet versus Oz-AIN diet).

Aim 1: To determine GENOTYPE EFFECTS on olfactory ability of normal and Amy mice.

Consistent with the first aim of this study, the effect of genotype on olfactory ability at either 6 or 12 months of age was assessed through comparisons between normal mice and Amy mice that were fed the AIN93-M diet (Figure 1A). A second set of comparisons was made between 12 month old normal mice and Amy mice that were fed the Oz-AIN diet (Figure 1A).

Aim 2: To determine DIET-TYPE EFFECTS on olfactory ability of normal and Amy mice.

Consistent with the second aim of this study, the effect of the Oz-AIN diet on olfactory ability at 6 and 12 months was determined by comparison of normal mice that were fed either the AIN93-M diet or the Oz-AIN diet (Figure 1B). A second comparison was made between 6 and 12 month old Amy mice that were fed either the AIN93-M diet or the Oz-AIN diet (Figure 1B).

Aim 3. To determine the effect of AGING on olfactory ability of Amy mice.

Consistent with the third aim of this study, olfactory ability of 6 month old and 12 month old mice were compared. The effect of aging on olfactory ability of normal and Amy mice that were fed the AIN93-M diet or the Oz-AIN diet were compared using *within groups* comparisons of olfactory abilities at 6 and 12 months. Diet-type and genotype effects on age-related changes in olfactory abilities were investigated using the same two-factorial analysis described above.

Figure 1A. The study design used to investigate genotype effects on olfactory abilities.

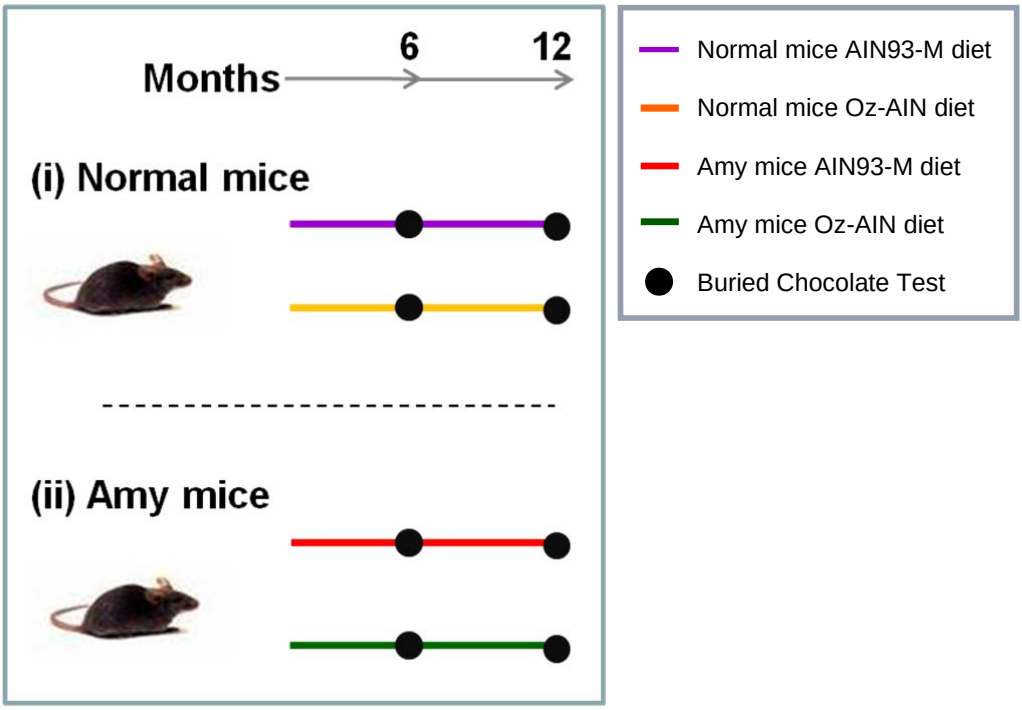


Figure 1A. Genotype effects on olfactory function.

The Buried Chocolate Test (BCT) was used to assess olfaction ability. Mice were assessed at 6 and 12 months. Genotype effects on olfactory ability were assessed by comparing diet-matched normal mice and Amy mice.

(i) Comparisons between normal mice (orange line) and Amy mice (red line) that were fed the AIN93-M diet were made to demonstrate genotype effects when mice are fed an optimal diet.

(ii) Comparisons between normal mice (yellow line) and Amy mice (green line) that were fed the Oz-AIN diet were compared to demonstrate genotype effects in when mice are challenged with a sub-optimal diet.

Figure 1B. The study design used to investigate diet-type effects on olfactory abilities.

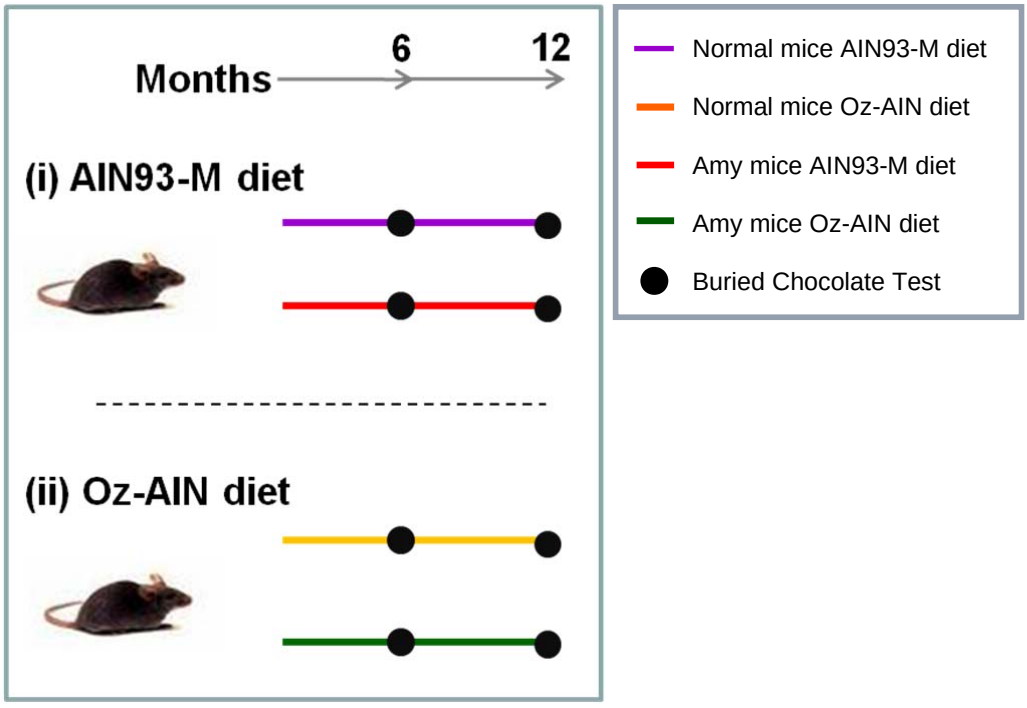


Figure 1B. Diet-type effects on olfactory function.

The Buried Chocolate Test (BCT) was used to assess olfaction ability. Mice were assessed at 6 and 12 months. Diet-type effects on olfactory ability were assessed by comparing genotype-matched mice that were fed either the AIN93-M diet or the Oz-AIN diet.

(i) Comparisons between normal mice were fed either the AIN93-M diet (orange line) or the Oz-AIN diet (yellow line) were made to demonstrate the effects of a sub-optimal diet.

(ii) Comparisons between Amy mice that were fed either the AIN93-M diet (red line) or the Oz-AIN diet (green line) were made to demonstrate the effects of a sub-optimal diet on olfactory abilities of Amy mice.

Figure 1C. The study design used to investigate the effect of aging from 6 to 12 months on olfactory abilities of normal and Amy mice.

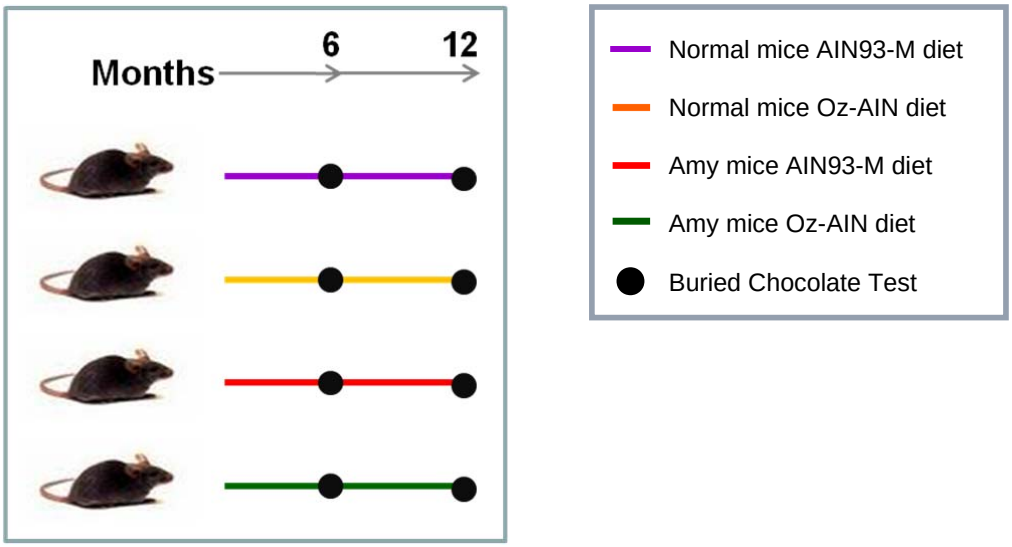


Figure 1C: Aging effects on olfactory function.

The Buried Chocolate Test (BCT) was used to assess olfaction ability. Mice were assessed at 6 and 12 months.

Within groups comparisons of the performance of mice in the BCT were made to demonstrate the change in olfactory abilities with aging.

Between groups comparisons of the change in olfactory abilities with aging were made to establish whether genotype or diet-type had an effect on age-associated changes in olfactory ability.

7.1.3. Apparatus.

The BCT Arena was a large plastic mouse cage (45 cm x 24 cm x 20 cm) lined with sawdust. A digital camera that was connected to a laptop running Stoelting ANYmaze software (Stoelting Co., Wood Dale, USA) was positioned 80 cm above the area (See Appendix III for details on configuration of ANYmaze software for the BCT). A piece of milk chocolate (0.8–1.0 g) (Cadbury Dairy Milk Chocolate©, Kraft Foods Australia Pty Ltd, (formerly Cadbury Pty Ltd)) was buried 0.5 cm below the surface of the saw dust, approximately 5 cm from the end of the arena (Figure 2).

Figure 2. Buried Chocolate Test arena.

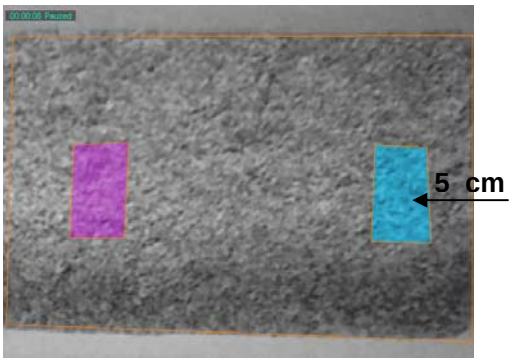


Figure 2. The Buried Chocolate Test arena is a 45 cm x 24 cm x 20 cm cage lined with sawdust. A piece of chocolate (0.8 – 1.0 g) is buried approximately 5 cm from either the left or right end of the arena.

7.1.4. Protocol.

The day prior to the test mice received a piece of chocolate (0.8 – 1.0 g) for 2 hours and then fasted overnight (6:00 PM – 7:00 AM).

All tests were conducted between 7:00 AM and 9:00 AM to restrict factors that may vary motivation such as different fasting length or circadian effects [113, 517].

Mice were placed in the centre of the arena, and latency (s) to uncover a buried piece of chocolate was recorded. Mice who failed to uncover the chocolate after 3 minutes were removed from the arena and latency was recorded as 180 s (maximum time). If mice accidentally uncovered the buried chocolate (kicked up the sawdust as they ran over it), testing was terminated and results excluded from analysis.

Whether mice found the chocolate or failed, all mice were presented with the chocolate and returned to their cage with free access to food and water. After each test, the sawdust in the arena was discarded and replaced with fresh sawdust. This was done to remove any confounding effects of scent of previous mice tested.

7.1.5. Data collection and storage.

Latency to uncover the chocolate (s), total distance travelled before reaching the chocolate (m) and average speed travelled during each trial (m/s) were recorded by Stoelting ANYmaze software (Stoelting Co.).

All data was stored in excel files and analysed using GraphPadPrism^{R} Software (Prism 5 for Windows, version 5.04, GraphPad Software inc.).

7.1.6. Data analysis.

To confirm that the latencies, distances, and average speed were accurately recorded by ANYmaze software, the latencies to uncovering the buried chocolate were also recorded manually from video footage by an experimenter that was blinded to the treatment condition of each mouse. Any trials where the manual and digitally recorded latencies differed by >2 s were considered not to have been detected accurately by ANYmaze software. These data were excluded from analyses.

Latency (s) and distance travelled (m) to the buried chocolate were the primary measures of olfactory function. At 6 and 12 months of age, genotype and diet-type effects on olfactory function were determined using two-way ANOVA and Bonferroni post tests. With low numbers of mice in some treatment groups, the interaction, diet-type or genotype effects may have been hard to detect with a two-way ANOVA. Therefore, analysis of diet-type-matched or genotype-matched mice was also carried out using Student's *t*-tests. The effect of aging *within* groups was established by comparing the performance of mice at 6 and 12 months of age with Students *t*-tests. The effect of diet-type and genotype on age-induced changes in olfactory ability was carried out with 2-way ANOVA. All data is reported as mean ±SEM. For all comparisons, statistical significance was set at $p<0.05$.

7.2. Results.

7.2.1. Performance of 6 month old normal and Amy mice in the Buried Chocolate Test.

Genotype effects on latency (s) to uncover the buried chocolate.

A two-way ANOVA indicated that genotype accounted for 9.90% of the variation between groups for latency (s) to uncover the buried chocolate ($p=0.16$, Figure 3A). Bonferroni post tests revealed that there were differences in the latencies of normal mice and Amy mice that were fed the Oz-AIN diet ($p>0.99$, Figure 3A). This suggests that there were no genotype effects on latency to uncover a chocolate at 6 months between mice that are fed a sub-optimal diet. Bonferroni post tests also did not detect significant differences between normal and Amy mice that were fed the AIN93-M diet ($p=0.20$, Figure 3A). However, with such small numbers in these comparisons, significant differences may have been hard to detect with a two-way ANOVA. Therefore, the latencies of 6 month old normal and Amy mice that were fed the AIN93-M diet were also compared using Students *t*-tests. Students *t*- tests revealed that the latency of 6 month old normal mice that were fed the AIN93-M diet to uncover the buried chocolate was significantly different to that of Amy mice that were fed the AIN93-M diet ($p=0.02$, Figure 3A). This suggests that there are genotype effects between normal and Amy mice that are fed the AIN93-M diet.

These results indicate that genotype-effects on the latency for 6 month old mice to locate a buried chocolate may be dependent on diet-type. While there were no genotype effects between mice that had been fed a suboptimal diet, there were genotype effects between mice that had been fed the optimal diet (AIN93-M diet). While this is suggestive of an interaction, a two-way ANOVA did not detect a

genotype-diet-type interaction on latency to the buried chocolate (5.97 % of variance, p=0.20, Figure 3A).

Genotype effects on distance (m) before uncovering the buried chocolate.

A two-way ANOVA revealed that genotype accounted for 18.20% of the variance of distance travelled by 6 month old mice before uncovering the pellet (p=0.06, Figure 3B). While this was not significant at $p<0.05$, it is significant at $p<0.10$. This suggests that there may have been trends for genotype to effect the distance travelled by 6 month old mice before uncovering the chocolate.

Bonferroni post tests did not detect significant differences between treatment groups. As previously suggested, the treatment group sizes may not have been large enough for a two-way ANOVA to detect significant differences. Therefore, Student *t*-tests were also carried out. Students *t*-tests revealed that Amy mice that were fed the Oz-AIN diet travelled significantly further before uncovering the chocolate than normal mice that were fed the Oz-AIN diet (p=0.04, Figure 3B). This suggests that there were genotype effects on olfactory ability between normal and Amy mice that were fed the Oz-AIN diet.

The normal mice and Amy mice that were fed the AIN93-M diet travelled similar distances before uncovering the chocolate (p=0.74, Figure 3B), indicating that genotype does not affect distance travelled by mice that are fed an ideal diet. This contrasts the findings of the effect of genotype on latency to uncover the pellet, which reported that genotype effects exist between mice fed the AIN93-M diet, but not the Oz-AIN diet.

Comparisons of speeds travelled during the test may explain how these discrepancies have occurred (Figure 3C). A two-way ANOVA revealed that there was a significant

diet-type-genotype interaction on speed travelled (42.23% of overall variance, p=0.001, Figure 3C), and non-significant trends to indicate genotype effects on speed travelled (8.15% of variance, p=0.10, Figure 3C). Bonferroni post tests determined that these variances could be attributed to the speeds travelled by normal mice that were fed the AIN93-M diet, that travelled significantly faster than Amy mice that were fed the AIN93-M diet while searching for the chocolate (p=0.02, Figure 3C). The implications of this are that it makes it hard to interpret data describing latency to locate the buried chocolate. However, the normal mice and the Amy mice that were fed the AIN93-M diet reached the pellet within similar distances, confirming that they had similar olfactory abilities, even though the normal mice that were fed the AIN93-M diet travelled faster. Student *t*-tests indicated that there were non-significant trends for Amy mice that were fed the Oz-AIN diet to travel faster than normal mice that were fed the same diet, suggesting that genotype des have an effect on speed travelled (p=0.08, Figure 3C).

Collectively, these data suggest that there were no genotype effects on olfactory abilities of mice that were fed the AIN93-M diet as they travelled similar distances before locating the pellet. However, genotype may affect olfactory abilities of normal mice and Amy mice that were fed the Oz-AIN diet. The Amy mice that were fed the Oz-AIN diet travelled further before locating the chocolate than normal mice that were fed the Oz-AIN diet, at slightly faster speeds.

Figure 3A. Latency (s) for 6 month old normal and Amy mice to uncover a buried piece of chocolate.

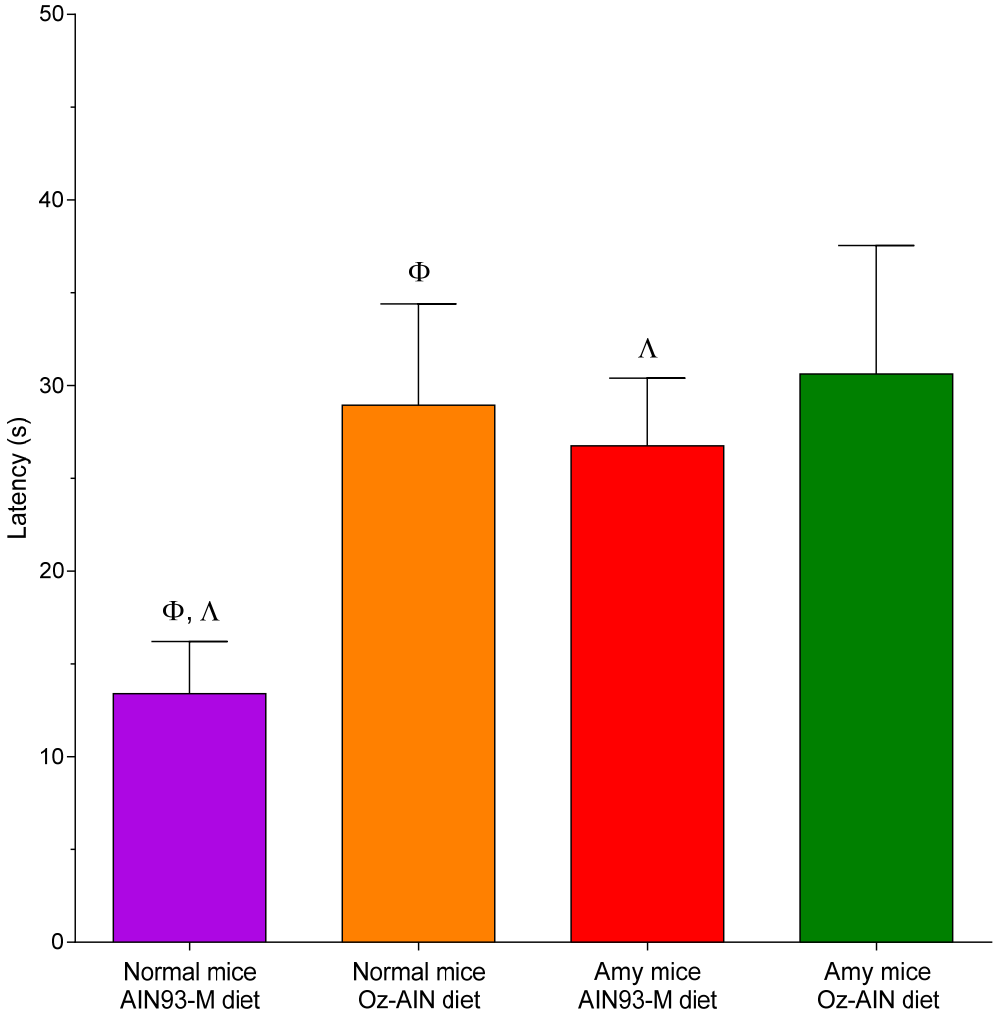


Figure 3A. 6 month old normal mice fed the AIN93-M diet (orange bar, n=7), normal mice fed the Oz-AIN diet (yellow bar, n=8), Amy mice fed the AIN93-M diet (red bar, n=6), Amy mice fed the Oz-AIN diet (green bar, n=6). Bars represent mean ±SEM. Bars with matching symbols were significantly different with Students *t*-test. (Φ) p=0.04. (Λ) p=0.02.

Figure 3B. Distance travelled (m) by 6 month old normal and Amy mice before uncovering a buried piece of chocolate.

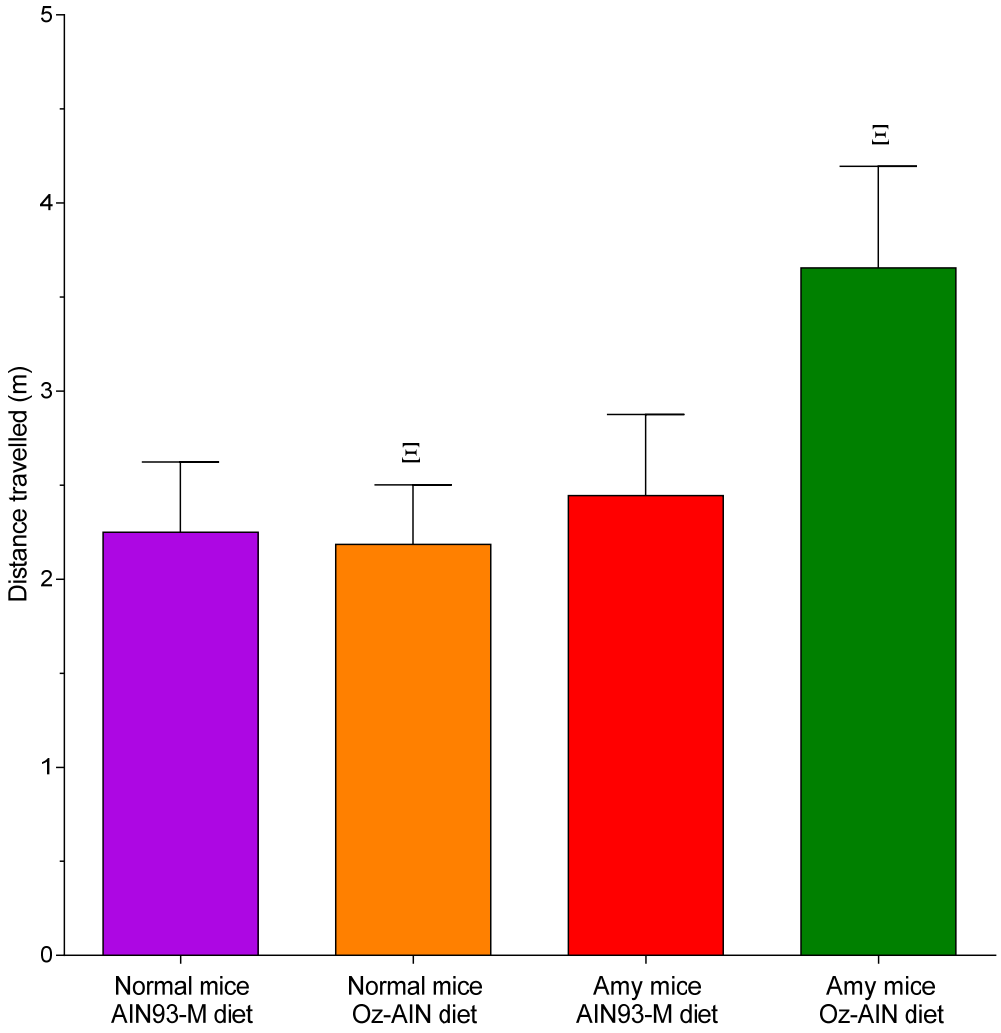


Figure 3B. 6 month old normal mice fed the AIN93-M diet (orange bar, n=7), normal mice fed the Oz-AIN diet (yellow bar, n=8), Amy mice fed the AIN93-M diet (red bar, n=6), Amy mice fed the Oz-AIN diet (green bar, n=6). Bars represent mean ±SEM. Bars with matching symbols are significantly different with Students *t*-test. (Ξ) p=0.04.

Figure 3C. Speed travelled (m/s) by 6 month old normal and Amy mice whilst looking for a buried piece of chocolate.

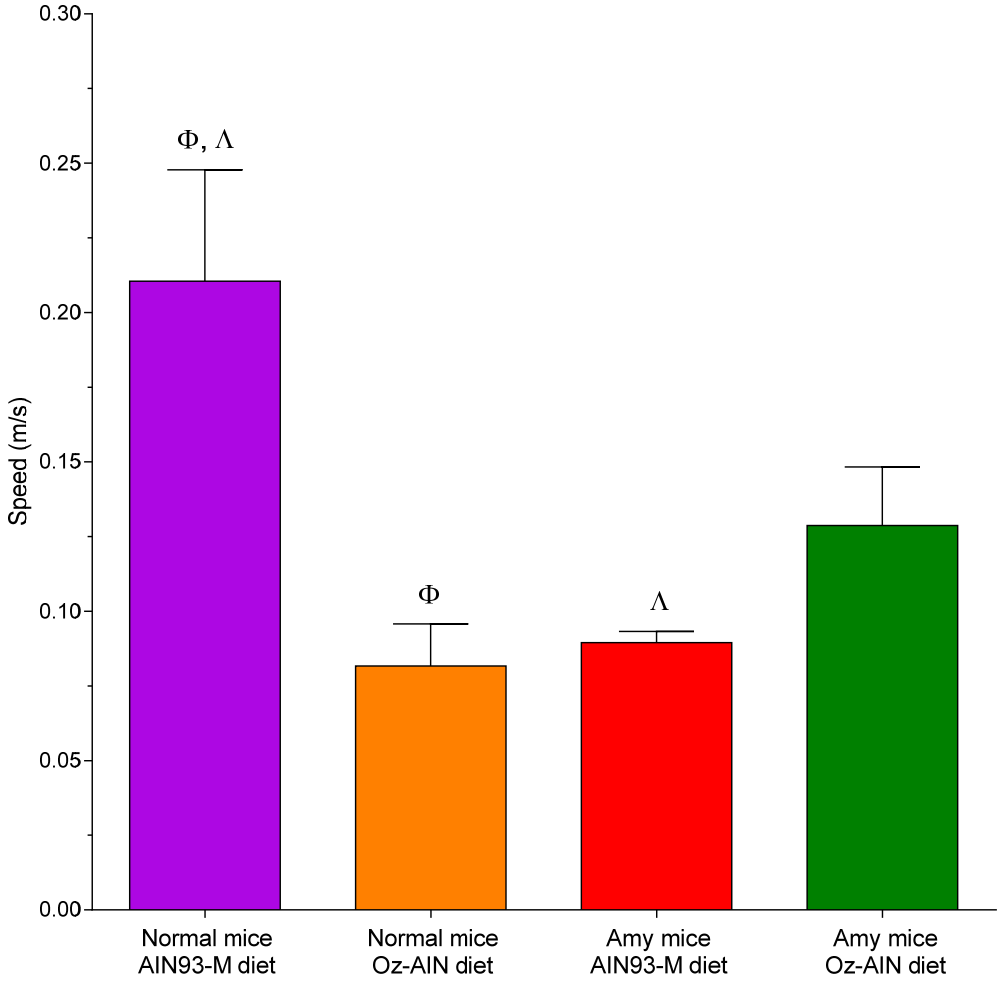


Figure 3C. 6 month old normal mice fed the AIN93-M diet (orange bar, n=7), normal mice fed the Oz-AIN diet (yellow bar, n=8), Amy mice fed the AIN93-M diet (red bar, n=6), Amy mice fed the Oz-AIN diet (green bar, n=6). Bars represent mean ±SEM. Bars with matching symbols are significantly different with Students *t*-test. (Ξ) p=0.04.

Diet-type effects on latency (s) to uncover the buried chocolate.

A two-way ANOVA detected that diet-type accounted for 6.50% of the variation between groups for the latency (s) for 6 month old mice to uncover the buried chocolate ($p=0.08$, Figure 3A). Bonferroni post tests did not detect significant differences between groups. However, Students *t*-tests indicated that the normal mice that were fed the AIN93-M diet located the chocolate faster than the normal mice that were fed the Oz-AIN diet ($p=0.04$, Figure 3A). Similarly, the 6 month old Amy mice that were fed the AIN93-M diet also found the buried chocolate faster than Amy mice that were fed the Oz-AIN diet (Figure 3A). However, this failed to achieve significance with either Bonferroni post tests ($p>0.99$) or Students *t*-tests ($p=0.64$). These data suggest that diet-type may have an effect on latency to uncover the buried chocolate for normal mice but not Amy mice (Figure 3A).

Diet-type effects on distance (m) before uncovering the buried chocolate.

A two-way ANOVA detected that diet-type accounted for 8.61% of the overall variation between groups for the distance travelled before uncovering the buried chocolate ($p=0.19$, Figure 3B). Bonferroni post tests did not detect significant diet-type effects between normal mice ($p>0.99$, Figure 3B) or Amy mice ($p=0.40$, Figure 3B). Student *t*-tests confirmed that diet does not affect the distance travelled by Amy mice that are fed either the AIN93-M diet or the Oz-AIN diet ($p=0.13$, Figure 3B). Similarly, diet-type did not affect distance travelled by normal mice that were fed the AIN93-M diet or the Oz-AIN diet whilst searching for the buried chocolate ($p=0.90$, Figure 3C).

As stated on pages 431-432, the normal mice that were fed the AIN93-M diet travelled faster than Amy mice that were fed the AIN93-M diet whilst searching for

the chocolate (Figure 3C). Similarly, the normal mice that were fed the AIN93-M diet travelled significantly faster than normal mice that were fed the Oz-AIN diet ($p=0.006$, Figure 3C). The consequence of moving faster was that the normal mice that were fed the AIN93-M diet located the chocolate before normal mice that were fed the Oz-AIN diet. Therefore, the latencies to the chocolate may not necessarily reflect better olfactory skills. The finding that there were no differences in the distances travelled by 6 month normal mice that were fed either the AIN93-M diet or the Oz-AIN diet suggests that there were no differences in their olfactory abilities.

Summary of performance of normal and Amy mice in the Buried Chocolate Test at 6 months.

Taken together, the results from the BCT indicate that there genotype affects the olfactory abilities of 6 month old Amy mice. Genotype impaired olfactory abilities of mice that were fed the Oz-AIN diet. The 6 month old Amy mice that are fed the Oz-AIN diet travelled further before uncovering the chocolate compared to normal mice that are fed the Oz-AIN diet. However, genotype did not affect the olfactory abilities of Amy mice that were fed an optimal diet (AIN93-M diet). This indicates that the genotype effects on olfactory abilities of 6 month old Amy mice are diet-type dependent.

The 6 month old normal mice that were fed the AIN93-M diet found the chocolate with shorter latencies than those fed the Oz-AIN diet, suggesting diet-type may affect olfactory abilities of 6 month old normal mice.

7.2.2. Performance of 12 month old normal and Amy mice in the Buried Chocolate Test.

Genotype effects on latency (s) to uncover the buried chocolate.

A two-way ANOVA detected that at 12 months of age, genotype accounted for 19.49% of variation between groups for the latency (s) to uncover the buried chocolate ($p=0.0005$, Figure 4A). Bonferroni post tests did not detect significant genotype effects between normal and Amy mice that were fed the AIN93-M diet ($p>0.99$, Figure 4A). However, Bonferroni post tests revealed that the significant genotype effects could be attributed to differences in latencies of normal and Amy mice that were fed the Oz-AIN diet. The 12 month old Amy mice that were fed the Oz-AIN diet took significantly longer to locate the buried chocolate than normal mice that were fed the Oz-AIN diet ($p=0.004$, Figure 4A). This was also significant with a Student's *t*-test ($p=0.01$, Figure 4A).

These data suggest that while genotype does not affect the latency to uncover a buried chocolate for 12 month old mice that are fed an optimal diet (AIN93-M), genotype does affect latency to uncover a chocolate for 12 month old mice that are fed a sub-optimal diet (Oz-AIN diet).

Genotype effects on distance (m) before uncovering the buried chocolate.

A two-way ANOVA detected that genotype accounted for 2.04% of the variation between groups for the distance travelled (m) before 12 month old mice uncovered the buried chocolate ($p=0.49$, Figure 4B). Bonferroni post tests did not detect significant genotype effects on the distance travelled by normal and Amy mice that were fed the AIN93-M diet ($p>0.99$) or the Oz-AIN diet ($p=0.57$). Students *t*-tests indicated that there were non-significant trends for normal mice that were fed the

AIN93-M diet to travel further than Amy mice that were fed the AIN93-M diet before locating the chocolate (p=0.08). Students *t*-tests did not detect significant genotype effects between normal and Amy mice that were fed the Oz-AIN diet, despite there being a large difference in distance travelled whilst searching for the chocolate (p=0.21, Figure 4B). The failure for this to achieve significance is probably due to the large variation in distances travelled by Amy mice that were fed the Oz-AIN diet.

Normal mice that were fed the AIN93-M diet travelled faster than Amy mice that were fed the AIN93-M diet (0.175 ±0.023 m/s and 0.057 ±0.20 m/s respectively, p=0.007, Figure 4C). This suggests that the Amy mice that were fed the AIN93-M diet may have had better olfactory abilities than normal mice that were fed the AIN93-M diet at 12 months of age, as they located the chocolate within shorter distances (Figure 4B). The Amy mice that were fed the Oz-AIN diet had poorer olfactory abilities than normal mice that were fed the Oz-AIN diet when they were 12 months old. They took significantly longer, and travelled further distances than normal mice before they located the chocolate.

Collectively, these data suggest that genotype has opposing effects on the olfactory abilities of 12 month old mice, depending on the diet that mice received. Amy mice that have been fed an ideal diet (AIN93-M diet) may have better olfactory abilities than normal mice that were fed the AIN93-M diet. The Amy mice that were fed the sub-optimal diet (the Oz-AIN diet) have poorer olfactory abilities than normal mice that were fed the Oz-AIN diet.

Figure 4A. Latency (s) for 12 month old normal and Amy mice to uncover a buried piece of chocolate.

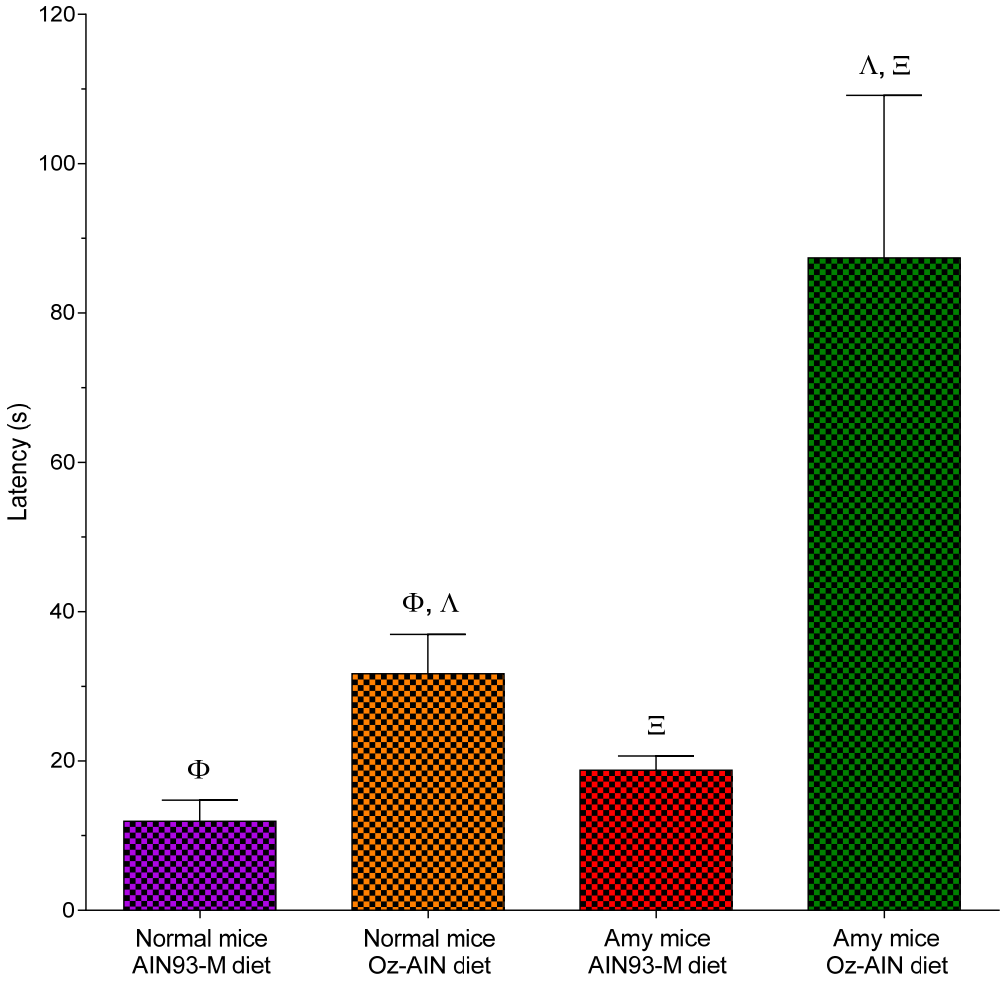


Figure 4A. 12 month old normal mice fed the AIN93-M diet (orange bars, n=7), normal mice fed the Oz-AIN diet (yellow bars, n=8), Amy mice fed the AIN93-M diet (red bar, n=6), Amy mice fed the Oz-AIN diet (green bar, n=6). Bars represent mean ±SEM. Bars with matching symbols are significantly different with Students *t*-tests. (Φ) p=0.02. (Λ) p=0.01. (Ξ) p=0.02.

Figure 4B. Distance travelled (m) by 12 month old normal and Amy mice before uncovering a buried piece of chocolate.

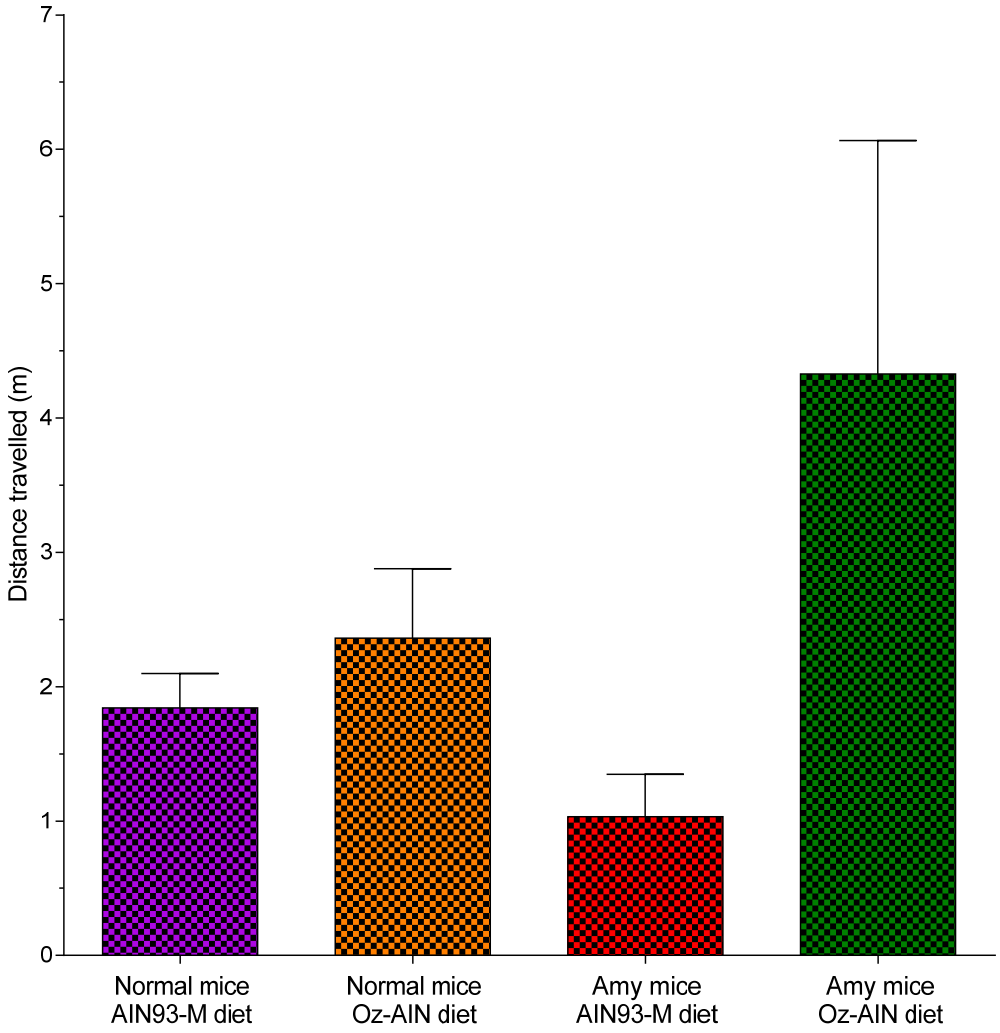


Figure 4B. 12 month old normal mice fed the AIN93-M diet (orange bars, n=7), normal mice fed the Oz-AIN diet (yellow bars, n=8), Amy mice fed the AIN93-M diet (red bar, n=6), Amy mice fed the Oz-AIN diet (green bar, n=6). Bars represent mean ±SEM.

Figure 4C. Speed travelled (m/s) by 12 month old normal and Amy mice whilst looking for a buried piece of chocolate.

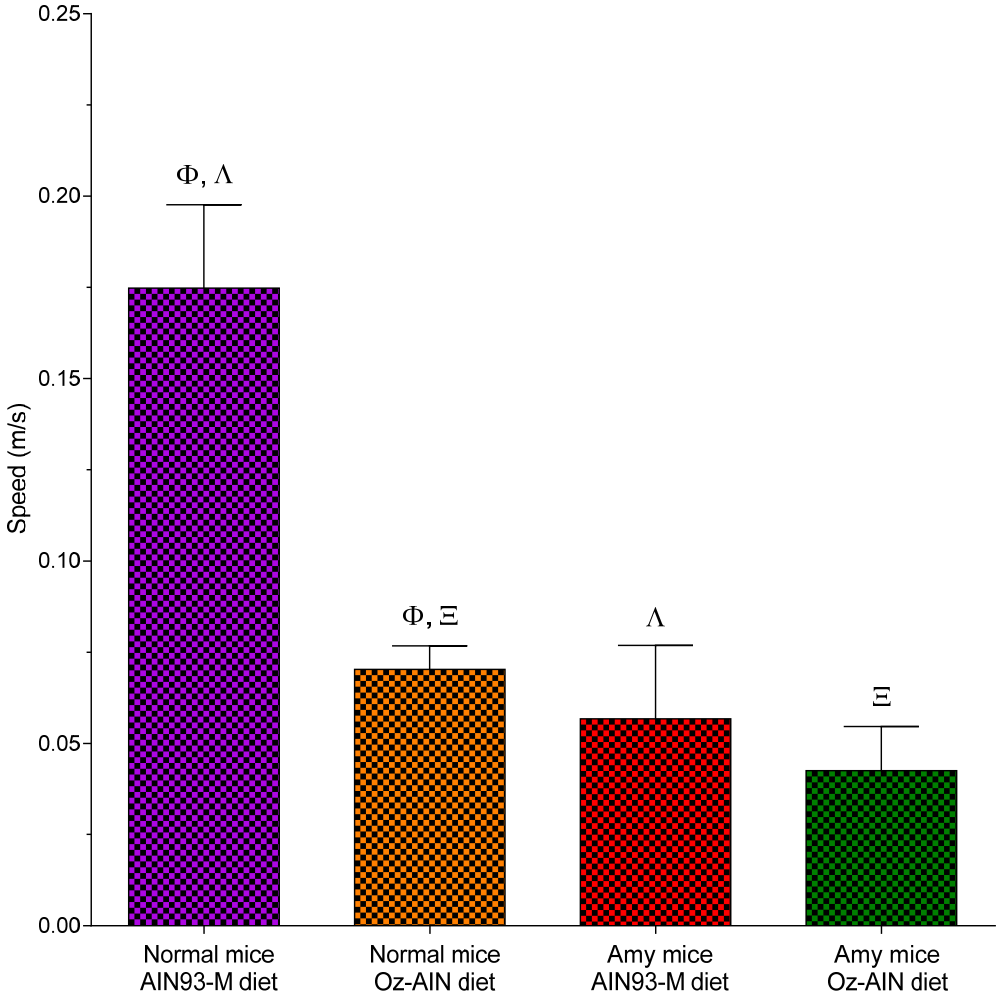


Figure 4C. 12 month old normal mice fed the AIN93-M diet (orange bars, n=7), normal mice fed the Oz-AIN diet (yellow bars, n=8), Amy mice fed the AIN93-M diet (red bar, n=6), Amy mice fed the Oz-AIN diet (green bar, n=6). Bars represent mean ±SEM. Bars with matching symbols are significantly different with Students *t*-tests. (Φ) p=0.0005. (Λ) p=0.007. (Ξ) p=0.05.

Diet-type effects on latency (s) to uncover the buried chocolate.

A one-way ANOVA revealed that diet-type accounted for 38.94 % of the overall variation of latency to uncover the buried chocolate (p=0.0003, Figure 4A). There was also a significant diet-type-genotype interaction on latency to uncover the chocolate (11.91 %, p=0.02, Figure 4A). Bonferroni post tests revealed latencies to uncover the chocolate were not different between normal mice that were fed either the AIN93-M diet or the Oz-AIN diet (p=0.77, Figure 4A).Suggesting that diet-type does not affect olfactory abilities of normal mice.

However, the Amy mice that were fed the Oz-AIN diet took significantly longer to locate the chocolate than Amy mice that were fed the AIN93-M diet (p=0.02, Figure 4A). This indicates that diet-type effects on olfactory abilities of 12 month old mice are dependent on genotype, and that the Oz-AIN diet impairs olfactory functioning in Amy but not normal mice.

Diet-type effects on distance (m) before uncovering the buried chocolate.

A one-way ANOVA revealed that diet-type accounted for 22.14% of the variation of distance travelled before locating the buried chocolate (p=0.03, Figure 4B). Bonferroni post tests did not detect significant differences between normal mice that were fed the AIN93-M diet or the Oz-AIN diet (p=0.45, Figure 4B). There were non-significant trends that indicated that Amy mice that were fed the Oz-AIN diet travelled further than Amy mice that were fed the AIN93-M diet before locating the buried chocolate (p=0.11, Figure 4B). The failure of this to achieve significance may have been due to the large variation in distances travelled by Amy mice that were fed the Oz-AIN diet.

Summary of performance of normal and Amy mice in the Buried Chocolate Test at 12 months.

The data from the BCT indicate that 12 month old Amy mice that have been fed the Oz-AIN diet from weaning have impaired olfactory function, and this can be attributed to genotype effects, diet-type effects and a genotype-diet-type interaction. Amy mice that were fed the Oz-AIN diet took longer (s) and travelled further (m) to uncover the buried chocolate than Amy mice that were fed the AIN93-M diet or normal mice that were fed the Oz-AIN diet.

In contrast to this, the Amy mice that were fed the AIN93-M diet located the chocolate within shorter distances than normal mice that were fed the same diet. Whilst they took marginally longer to locate the chocolate, this is likely to be due to the finding that they travelled significantly slower than normal mice that were fed the AIN93-M diet. This suggests that at 12 months of age, the Amy mice that were fed the AIN93-M diet may have had better olfactory skills than normal mice that were fed the same diet.

7.2.3. Comparison of the changes in latency (s), distance travelled (m) and average speed (m/s) to determine age-related changes in olfactory abilities of mice at 6 and 12 months of age.

Section 7.2.1 described the genotype and diet-type effects on olfactory function in 6 month old mice. Section 7.2.2 described the genotype and diet-type effects on olfactory function in 12 month old mice. The current section investigates the changes that occur with aging. Comparisons of latency (s), distance travelled (m) and average speed (m/s) are made *within groups* to determine whether or not aging from 6 to 12 months does have an effect on olfactory abilities. The age-associated changes in latency (s) distance (m) and average speed travelled are also compared *between groups* to determine whether diet-type or genotype influence age-associated change in olfactory abilities.

The effect of aging from 6 to 12 months on the latency (s) to uncover the buried chocolate.

Aging from 6 to 12 months had different effects on the time taken to uncover the buried chocolate for each group of mice. The 12 month old Amy mice that were fed the Oz-AIN diet took significantly longer to locate the buried chocolate compared to when they were 6 months ($p=0.03$, Figure 5). The 12 month old normal mice that were fed the Oz-AIN diet also took longer to locate the buried chocolate at 12 months compared to 15 months, however this was not significant ($p=0.08$, Figure 5). In contrast to this, the normal mice that were fed the AIN93-M diet found the chocolate faster at 12 months compared to 6 months ($p=0.03$ Figure 5). While the Amy mice that were fed the AIN93-M diet also reduced latency to uncover the chocolate, this was not significant ($p=0.17$, Figure 5).This failed to achieve significance, however

this is likely to be due to the large variation within groups. This suggests that aging had a beneficial effect on latency to locate a buried chocolate for mice that were fed the AIN93-M diet, and a detrimental effect for mice that were fed the Oz-AIN diet.

Figure 5. A comparison of the latency (s) to uncover a buried chocolate by 6 and 12 month old normal and Amy mice.

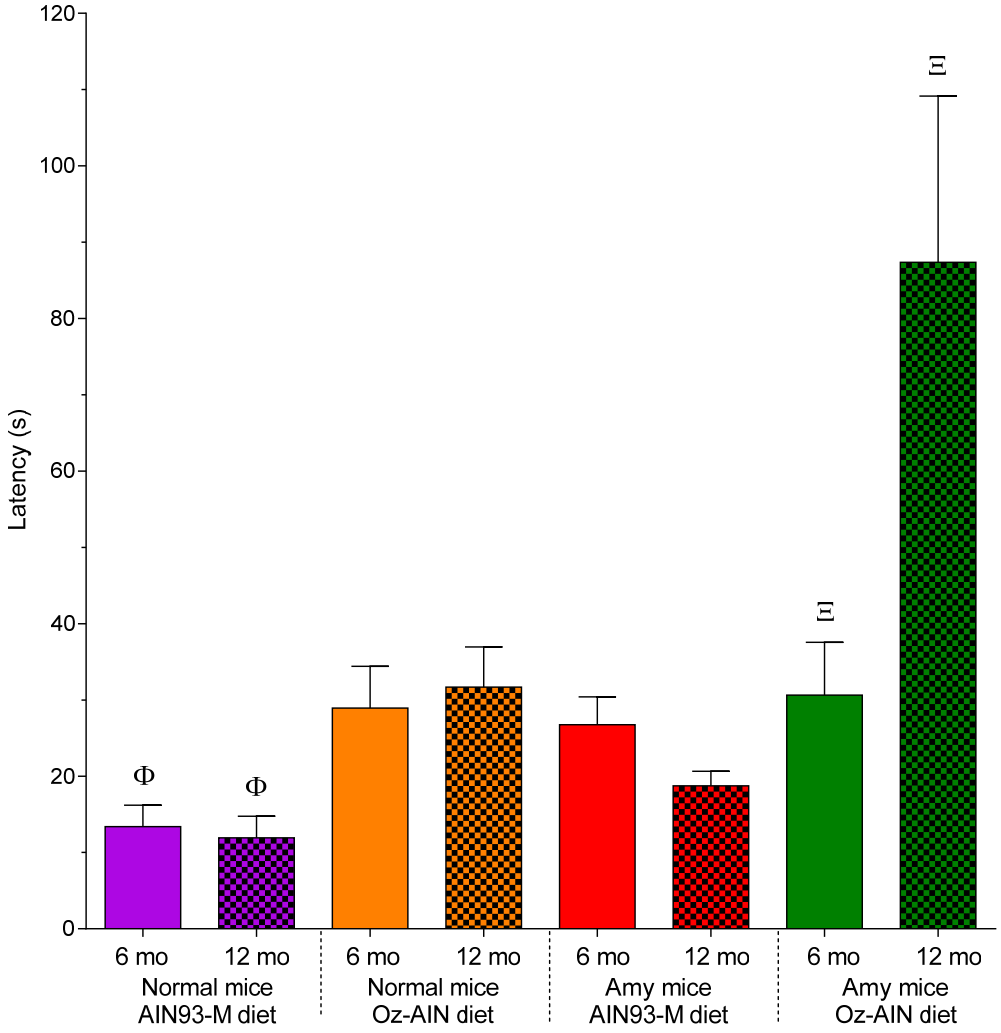


Figure 5. A comparison of the latency (s) to uncover a buried piece of chocolate between 6 month (solid bars) and 12 month old (checked bars) mice. Normal mice fed the AIN93-M diet (yellow solid and checked bars, n=7), Normal mice fed the Oz-AIN diet (orange solid and checked bars, n=8), Amy mice fed the AIN93-M diet (red solid and checked bars, n=6), Amy mice that were fed the Oz-AIN diet (green solid and checked bars, n=6). Bars are mean ±SEM. Bars with matching symbols were significantly different with paired Students *t*-test. p=0.03.

A two way ANOVA of the overall changes in latency to uncover the chocolate revealed a significant diet-type-genotype interaction that accounted for 24.64% of the overall variance ($p=0.0008$). Diet-type and genotype also had significant effects on the change in latencies with aging, and accounted for 24.64% and 14.65% of the overall variance, respectively ($p=0.008$ and $p=0.006$ respectively, Table 2). Bonferroni post tests revealed that there were significant diet-type effects between Amy mice that were fed either the AIN93-M or the Oz-AIN diet ($p<0.0001$, Table 2). This may be attributed to the fact that Amy mice that were fed the Oz-AIN diet took significantly longer to locate the chocolate at 12 months compared to 6 months, whilst the Amy mice that were fed the AIN93-M diet took less time as they got older (Figure 5, Table 2). However, there were no significant diet-type effects on the overall changes of latency of normal mice that were fed either the AIN93-M diet or the Oz-AIN diet ($p>0.99$, Table 2). This indicates that Amy mice are more susceptible to the effects of aging on age-associated olfactory dysfunction than normal mice.

Bonferroni post tests revealed that the genotype effects on the overall change in latency to uncover the buried chocolate were between mice that were fed the Oz-AIN diet, but not the AIN93-M diet ($p<0.0001$ and $p>0.99$ respectively, Table 2). These results indicate that the Amy mice that were fed the Oz-AIN diet had the greatest overall increase in latency to uncover the buried chocolate, and that this can be attributed to both diet-type and genotype effects.

The ratios of the change of latency to uncover the buried chocolate were also compared (Table 2). A two-way ANOVA revealed that diet-type had the strongest effect on ratios of age-associated change, and accounted for 50.43% of the overall variance ($p<0.0001$, Table 2). Genotype and the diet-type-genotype interaction

accounted for 16.51% and 23.18% of the overall difference in ratios ($p<0.0001$ and $p<0.0001$ respectively, Table 2). Bonferroni post tests revealed diet-type affected the ratio for the change in latency between Amy mice that were fed either the AIN93-M diet or the Oz-AIN diet ($p<0.0001$, Table 2), but not normal mice that were fed the AIN93-M or the Oz-AIN diet ($p>0.99$, Table 2). The change in latency for Amy mice that were fed the Oz-AIN diet was 2.79 ±0.25 s which was a significantly greater change than that for normal mice that were fed the Oz-AIN diet (1.27 ±0.10, $p<0.0001$, Table 2). These comparisons confirm the findings in the paragraph above, that Amy mice that are fed the Oz-AIN diet are more susceptible to age-related increase in latency to uncover the buried chocolate, and that this is attributed to both diet-type and genotype effects.

Table 2. Genotype and diet-type effects on the difference and ratios of the change in the latency (s) for normal and Amy mice to uncover the buried chocolate at 6 and 12 months.

	Normal mice AIN93-M diet	**Normal mice Oz-AIN diet**	**Amy mice AIN93-M diet**	**Amy mice Oz-AIN diet**
6 months (mean latency)	13.39 ±2.81	28.94 ±5.45	26.75 ±3.65	30.62 ±6.93
12 months (mean latency)	11.92 ±2.82	31.67 ±5.26	18.75 ±1.92	87.38 ±21.78
Difference (s)	**-1.47 ±0.43**	**6.19 ±2.80**	**-8.01 ±4.45**	**56.75 ±15.11**
Ratio (15 : 6)	**0.88 ±0.05**	**1.27 ±0.10**	**0.75 ±0.14**	**2.79 ±0.25**

All numbers are mean ±SEM. Ratios are a comparison of latency (s) at 12 months relative to 6 months. Differences and ratios were calculated individually for each mouse and averaged to determine mean ±SEM. Genotype effects were determined by comparisons between mice that were fed he same diet. Diet-type effects were determined by comparisons between mice that were the same genotype.

The effect of aging from 6 to 12 months on the distance travelled (m) before uncovering the buried chocolate.

Consistent with the data describing change in latency with aging, age had different effects on the change in distance travelled before uncovering the chocolate at 6 and 12 months for each treatment group (Figure 5). Amy mice that were fed the Oz-AIN diet travelled further at 12 months compared to 6 months, however this was not significant ($p=0.63$, Figure 5). Normal mice that were fed the Oz-AIN diet travelled similar distance at 12 months compared to when they were 6 months old ($p=0.31$, Figure 5).

Consistent with their change in latency as they aged, the Amy mice that were fed the AIN93-M diet travelled shorter distances before uncovering the chocolate at 12 months compared to when they were 6 months (1.03 ±0.31 m and 2.45 ±0.43 m respectively, $p=0.02$, Figure 5). The normal mice that were fed the AIN93-M diet also travelled shorter distances when they were 15 months old, however this was not significant (1.84 ±0.26 m and 2.25 ±0.37 m respectively, $p=0.15$, Figure 5).

Collectively, these data indicate that the Oz-AIN diet has a detrimental effect on the change in distance travelled in the BCT as mice age from 6 to 12 months, and that the AIN93-M diet has a beneficial effect on the change in distance travelled. These diet-type effects on age-associated change in distance are explored further in *between groups* comparisons on page 452.

Figure 6. A comparison of the distance travelled before uncovering a buried chocolate between 6 and 12 month old normal and Amy mice.

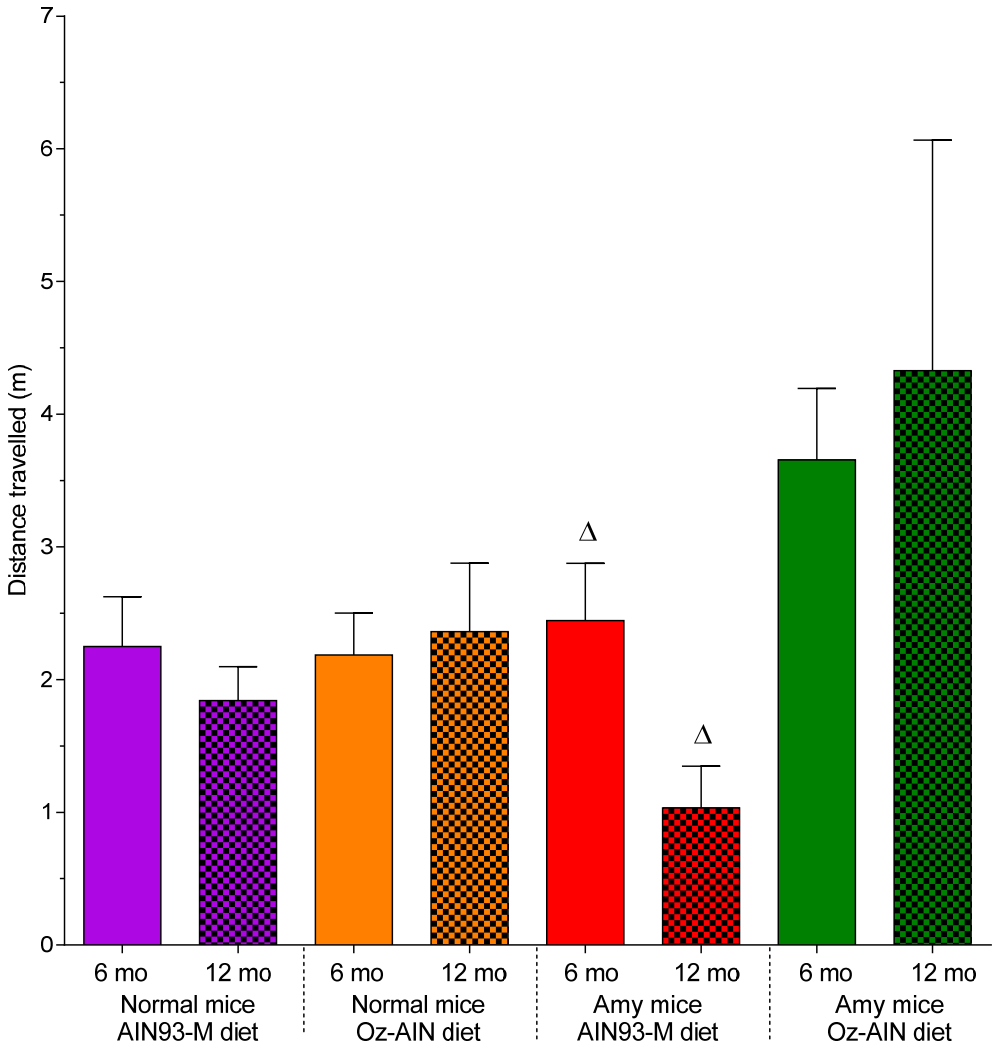


Figure 6. A comparison of the distance travelled (m) before uncovering a buried piece of chocolate between 6 month (solid bars) and 12 month old (checked bars) mice. Normal mice fed the AIN93-M diet (yellow solid and checked bars, n=7), Normal mice fed the Oz-AIN diet (orange solid and checked bars, n=8), Amy mice fed the AIN93-M diet (red solid and checked bars, n=6), Amy mice that were fed the Oz-AIN diet (green solid and checked bars, n=6). Bars are mean ±SEM. Bars with matching symbols were significantly different with paired Students *t*-test. p=0.02.

As stated on page 450, comparisons of the distance that mice travelled before uncovering the buried chocolate at 6 and 12 months suggested that there may be diet-type effects on change in distance travelled with aging. However, a two-way ANOVA indicated that diet-type only accounted for 15.44% of the overall variance of age-associated change in distance (p=0.10, Table 3). Bonferroni post tests and Students *t*-tests also did not detect significant differences in overall changes between groups (Table 3). Furthermore, neither genotype effects nor a genotype-diet-type interaction were detected by a two-way ANOVA, as they only accounted for 3.91% or 6.34% of the overall variance (p=0.39 and p=0.28 respectively, Table 3).

A two-way ANOVA of the ratios of distance travelled at 12 months compared to 6 revealed that diet-type had a significant effect on the degree that performance changed, and accounted for 32.67% of the overall variance (p=0.01, Table 3). While Bonferroni post tests and Students *t*-tests did not significant differences between groups, the Oz-AIN diet was associated with an increase in distance travelled and the AIN93-M diet was associated with a decrease in distance travelled (Table 3). There were no genotype effects or a diet-type-genotype interaction on the ratio of change in distance travelled (p=0.17 and p=0.76 respectively, Table 3).

Collectively, these results indicate that neither genotype nor diet-type affect the change in distance travelled as mice age from 6 to 12 months. However, the degree to which changes occur, and whether changes reflect improvement or detriment in distance travelled is affected by diet-type.

Table 3. Genotype and diet-type effects on the difference and ratios of the change in the distance travelled (m) before normal and Amy mice uncover the buried chocolate at 6 and 12 months.

	Normal mice AIN93-M diet	Normal mice Oz-AIN diet	Amy mice AIN93-M diet	Amy mice Oz-AIN diet
6 months (mean distance)	2.25 ±0.37	2.19 ±0.32	2.45 ±0.43	3.66 ±0.54
12 months (mean distance)	1.84 ±0.26	2.36 ±0.52	1.03 ±0.31	4.33 ±1.74
Difference (s)	**0.04 ±0.72**	**0.50 ±0.44**	**-1.41 ±0.30**	**0.67 ±1.24**
Ratio (15 : 6)	**0.78 ±0.12**	**1.29 ±0.17**	**0.41 ±0.13**	**1.06 ±0.34**

All numbers are mean ±SEM. Ratios are a comparison of distance (m) at 12 months relative to 6 months. Differences and ratios were calculated individually for each mouse and averaged to determine mean ±SEM. Genotype effects were determined by comparisons between mice that were fed he same diet. Diet-type effects were determined by comparisons between mice that were the same genotype.

The effect of aging from 6 to 12 months on average speed travelled (m/s) before uncovering the buried chocolate.

While speed travelled in the BCT decreased with age for all mice, this was only significant for the Amy mice that were fed the Oz-AIN diet ($p=0.03$, Figure 7). This indicates that the increased latencies with aging for the Amy mice that were fed the Oz-AIN diet may have been due to mice moving slower, and may therefore not be a true reflection of changes in olfactory ability. However, the distance travelled while searching for the chocolate also increased with age, supporting the conclusion that aging had a detrimental effect on olfactory abilities of Amy mice that were fed the Oz-AIN diet.

Figure 7. A comparison of the average speed travelled (m/s) by normal and Amy mice at 6 and 12 months whilst searching for a buried chocolate.

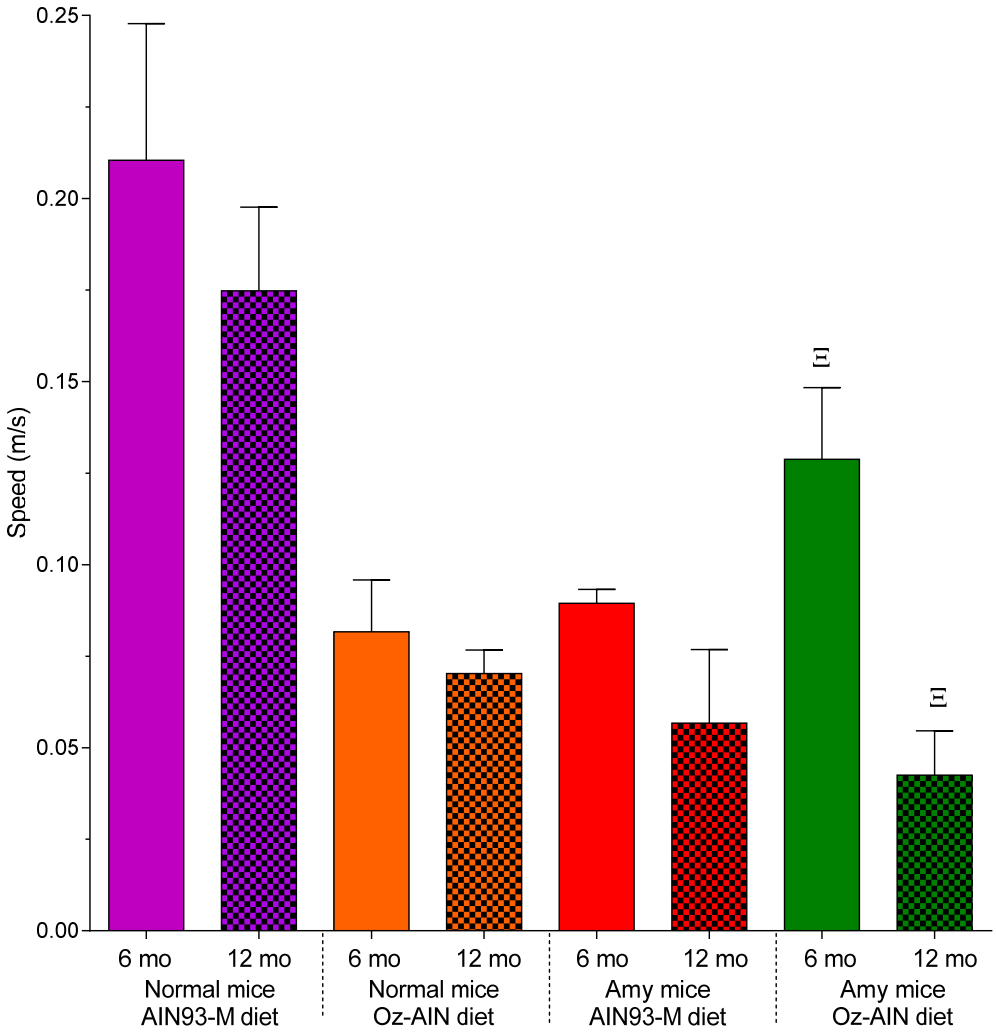


Figure 7. A comparison of the average speed travelled (m/s) whilst searching for a buried piece of chocolate between 6 month (solid bars) and 12 month old (checked bars) mice. Normal mice fed the AIN93-M diet (yellow solid and checked bars, n=7), Normal mice fed the Oz-AIN diet (orange solid and checked bars, n=8), Amy mice fed the AIN93-M diet (red solid and checked bars, n=6), Amy mice that were fed the Oz-AIN diet (green solid and checked bars, n=6). Bars are mean ±SEM. Bars with matching symbols were significantly different with paired Students *t*-test. p=0.03.

A two-way ANOVA revealed that genotype accounted for 21.56% of the overall change in speed travelled as mice aged from 6 to 12 months ($p=0.03$, Table 4). However, diet-type and a diet-type-genotype interaction only accounted for 8.80% and 3.74% respectively ($p=0.16$ and $p=0.35$ respectively, Table 4). While Bonferroni post tests did not detect differences between groups, Students *t*-test revealed that the age-related decline of speed travelled Amy mice that were fed the Oz-AIN diet was significantly greater than that of normal mice that were fed the Oz-AIN diet ($p=0.02$, Table 4). This suggests that the genotype effects that were detected by the two-way ANOVA may be between normal and Amy mice that have been fed the Oz-AIN diet, and that Amy mice that are fed the Oz-AIN diet have a greater decline in speed travelled between 6 and 12 months.

A two-way ANOVA of the ratio of change also detected significant genotype effects, and reported that while genotype accounted for 35.27% of the overall variance ($p=0.01$), diet-type and the diet-type-genotype interaction only accounted for 0.36% and 6.40% respectively ($p=0.77$ and $p=0.23$ respectively, Table 4). Bonferroni post tests revealed that while the ratios of age-associated change in speed were significantly different between normal and Amy mice that had been fed the Oz-AIN diet ($p=0.05$) there were no differences between normal and Amy mice that were fed the AIN93-M diet ($p>0.99$, Table 4). This may be attributed to the finding reported here that Amy mice that were fed the Oz-AIN diet made the largest change in speed, and were more affected by aging than other groups of mice (Figure 7, Table 4).

Collectively, these results suggest that genotype has a significant effect on age-associated changes in speed travelled, and that this is the most apparent mice that have been fed the Oz-AIN diet. The age-associated decline in speed travelled is

greater for Amy mice that are fed the Oz-AIN diet than for normal mice that are fed the Oz-AIN diet.

Table 3. Genotype and diet-type effects on the difference and ratios of the change in the average speed travelled (m/s) by normal and Amy mice whilst searching for the buried chocolate at 6 and 12 months.

	Normal mice AIN93-M diet	**Normal mice Oz-AIN diet**	**Amy mice AIN93-M diet**	**Amy mice Oz-AIN diet**
6 months (mean speed)	0.211 ±0.037	0.082 ±0.014	0.090 ±0.004	0.130 ±0.020
12 months (mean speed)	0.175 ±0.023	0.070 ±0.006	0.057 ±0.020	0.043 ±0.012
Difference (s)	**0.006 ±0.038**	**-0.006 ±0.016**	**-0.027 ±0.015**	**-0.086 ±0.023**
Ratio (15 : 6)	**0.89 ±0.10**	**1.04 ±0.17**	**0.61 ±0.21**	**0.36 ±0.11**

All numbers are mean ±SEM. Ratios are a comparison of average speed travelled (m/s) at 12 months relative to 6 months. Differences and ratios were calculated individually for each mouse and averaged to determine mean ±SEM. Genotype effects were determined by comparisons between mice that were fed he same diet. Diet-type effects were determined by comparisons between mice that were the same genotype.

7.3. Discussion.

The current study has reported genotype and diet-type effects on the olfactory abilities of 6 and 12 month old Amy mice.

Genotype affects olfactory function in the Buried Chocolate Test.

Consistent with the first aim of this study, genotype effects on olfactory function were investigated in normal and Amy mice at 6 and 12 months of age.

The comparison of the olfactory abilities of the 6 month old normal and Amy mice that were fed the AIN93-M diet suggest that genotype does not affect olfactory functions of Amy mice that are fed an optimal diet. This is consistent with reports

from others that AD-type mice that over express amyloid-precursor protein do not have impaired olfactory abilities [123, 508]. However, to make conclusions based on comparisons between mice that are fed the AIN93-M diet would be to state that "*if a mouse is fed an ideal diet that meets all their nutritional requirements, genotype does not have an effect on olfactory abilities*". In this context, such a conclusion hardly adds to the understanding of the relationship between anosmia and AD in humans. The main reason for this, apart from the obvious fact that these are rodents and not primates, is that mice received an optimal diet. Unfortunately, most Western diets do not meet nutritional requirements. It may be more valuable to gain an understanding of genotype effects on olfactory function when mice are fed a sub-optimal diet that is similar to a Western diet, such as a typical Australian-type diet.

Comparison of the olfactory abilities of normal and Amy mice that were fed the Oz-AIN diet at 6 and 12 months suggest that genotype *does* affect the olfactory abilities of Amy mice that are fed a sub-optimal diet.

Wesson *et al*. report that in the Tg2576 mouse model of AD, that β-amyloid dependent neuronal hyperactivity leads to neurodegeneration in brain regions associated with olfaction, resulting in olfactory dysfunction [505]. Wu *et al.* recently demonstrated that 3 month old Amy mice develop β-amyloid deposits in central olfactory cortices such as the anterior olfactory nucleus, which are accompanied by neurodegeneration of olfactory neurons [518]. This is consistent with reports from others that β-amyloid deposition in the olfactory tract precedes β-amyloid deposition in the hippocampus [122].

Chapter 4 proposed that the high fat content of the Oz-AIN diet potentially led to increased β-amyloid deposits throughout the brains of Amy mice, which supports findings from others [472, 519]. This suggests a potential mechanism for the impaired

olfactory abilities of Amy mice that were fed the Oz-AIN diet. Potentially, β-amyloid deposition in Amy mice begins in the olfactory bulbs, and that this was accelerated in Amy mice that were fed the Oz-AIN diet compared to those fed the AIN93-M diet. This may have caused accelerated neuronal loss in olfactory pathways, resulting in the olfactory dysfunction that has been observed in the current chapter. However, without measurements of β-amyloid in the 6 month old Amy mouse brains and without neuronal counts in the olfactory bulbs and tract, this is hard to conclude.

An alternate explanation for these findings may be that the Amy mice that were fed the Oz-AIN diet were simply less motivated to find the chocolate than normal mice that were fed the same diet. There were trends for the Amy mice to travel slower whilst looking for the chocolate, which may be interpreted as there being less "urgency" for the mice to uncover the food reward. However, the Amy mice and normal mice had fasted for the same period of time, suggesting that there should have been no differences in motivation of they could both smell the chocolate. Therefore, these results are interpreted as reflecting olfactory dysfunction in Amy mice that were fed the Oz-AIN diet.

It is hard to determine whether or not there were differences in the olfactory abilities of the 12 month old normal and Amy mice that were fed the AIN93-M diet. The Amy mice that were fed the AIN93-M diet located the chocolate within significantly shorter distances, which suggests that they had better olfactory abilities than normal mice. However, the majority of the literature reports that AD-type mice have poorer olfactory abilities than normal mice [122, 520, 521].

It is possible that the normal and Amy mice that were fed the AIN93-M diet could both smell the chocolate, but that the Amy mice were able to search for the chocolate with greater efficiency than normal mice. However, Chapter 5 reported that at 12

months of age normal and Amy mice that were fed the AIN93-M diet could equally figure out and learn the location of a submerged platform in the Morris Water Maze, suggesting that they had similar problem solving abilities. Therefore, it is unlikely that the Amy mice that were fed the AIN93-M diet were more efficient in their search strategy, as this would have been reflected in the Morris Water Maze tests as well.

Alternately, the differences in performance of normal and Amy mice in the BCT may be a reflection of different anxiety levels of normal and Amy mice. This suggestion is based on the observations that during BCT trials that the normal mice would often dart across the arena, resulting in normal mice having higher average speeds than Amy mice. This type of activity was initially interpreted as being indicative of anxiety. However, anxiety is more likely to have occurred in the AD-type mice than the normal mice, as anxiety is a behavioural characteristic of AD [7, 10] and has been reported in several different AD mouse models [492, 509]. Others have reported that there are no differences in the anxiety levels of normal mice and Amy mice [522, 523]. This indicates that anxiety is not likely to be the cause of the increased speeds of the normal mice, and therefore not likely to have effected performance in the BCT. The erratic behaviour of the normal mice (darting across the BCT arena) have certainly had an impact on their latencies and distances travelled. However, it also indicates that they either (i) could not smell the buried chocolate, or (ii) they could smell the chocolate but they were not interested in it. As discussed above, mice should have been equally motivated to locate the chocolate, as they were all fasted overnight. However, this possibility cannot be ruled out completely. Therefore, these results are interpreted as suggesting that while normal and Amy mice that were fed the AIN93-M diet may have both been able to smell the chocolate, the olfactory abilities of Amy mice may have been better.

This study supports the hypothesis that genotype has an effect on olfactory abilities of Amy mice. However, whether this is a beneficial or a detrimental effect appeared to be dependent on diet. Amy mice that were fed a sub-optimal diet had poorer olfactory abilities than normal mice that were fed the same diet. It is likely that this is due to enhanced β-amyloid deposition and neuronal degeneration throughout the olfactory tracts. However this study does not have the neuropathological data to support this conclusion.

The Amy mice that were fed the AIN93-M diet located the chocolate within shorter distances than normal mice that were fed the AIN93-M diet, suggesting that they may have better olfactory abilities. While this contrasts the current literature, other possible explanations for the differences between normal and Amy mice that were fed the AIN93-M diet such as differing search strategies or anxiety levels are less likely. It is therefore concluded that at 12 months of age, the Amy mice that were fed the AIN93-M diet that have been used in the current study had better olfactory skills than normal mice that were fed the same diet.

Diet-type affects olfactory function in the Buried Chocolate Test.

Consistent with the second aim of this study, diet-type effects on olfactory function were investigated in mice that were fed either the AIN93-M diet or the Oz-AIN diet. At 6 months, diet-type did not have an effect on the olfactory abilities of Amy mice, but may have affected olfactory abilities of normal mice.

As reported in Chapter 2, by 6 months of age, the normal mice that were fed the Oz-AIN diet weighed significantly more than mice that were fed the AIN93-M diet. Similarly, while food intake was the same between dietary groups, energy intake was higher for 6 month old mice that were fed the Oz-AIN diet. This may be relevant to

the findings of the current study, as diet induced obesity and nutritional status can alter olfactory functioning [121, 512, 513, 515]. Insulin and leptin, both of which play a role in obesity and food metabolism, can alter olfactory processing [513]. For example, undernourished mice (food access for 2 hours each day) have lower plasma levels of insulin, fewer insulin receptors in the olfactory mucosa and were able to locate a buried chocolate faster than mice that had prolonged access to food (8 hours each day) [513]. This suggests that undernourishment may enhance olfactory abilities or food-odor driven behaviours in mice.

In an olfactory test that was similar to the one used in the current study, Tucker *et al.* demonstrated that mice that had been fed a high-fat diet were unable to locate a fatty-scented cookie, but rapidly located a chocolate candy [121]. They suggest that this is due to sensory specific satiety, whereby eating a specific food type to the point of satiety decreases pleasantness or enjoyment of that food [121]. Tucker *et al.* reported that despite being unable to locate the fatty-scented cookie, the normal mice were able to discriminate between two very similar fats that were in their high-fat diet [121]. It is possible that the normal mice that were fed a high-fat diet could smell the buried fatty pellet but were not motivated to find it as it was not novel.

Table 4. Comparison of the macronutrient content of the AIN93-M diet and the Oz-AIN diet with the macronutrient content of the chocolate pieces* that were used in the Buried Chocolate Test.

	Carbohydrate (% energy)	Protein (% energy)	Fat (% energy)
AIN93-M diet	75.9%	14.1%	10.0%
Oz-AIN diet	46.7%	16.8%	33.0%
Chocolate pieces	44.06%	6.04%	49.82%

*Cadbury chocolate pieces (Dairy Milk Chocolate©, Kraft Foods Australia Pty Ltd, (formerly Cadbury Pty Ltd)).

The buried chocolate pellet that was used in the current study was novel for mice that were fed either the AIN93-M diet or the Oz-AIN diet. However, the macronutrient profile of the chocolate pellet was more similar to that of the Oz-AIN diet than that of the AIN93-M diet (Table 4), suggesting that it was less novel for mice that were fed the Oz-AIN diet. The total fat content of the chocolate pellet was higher than that of the Oz-AIN diet, but both diets had substantially more fat than the AIN93-M diet. Similarly, both diets had fewer carbohydrates than the AIN93-M diet. The similarity of the Oz-AIN diet to the chocolate pellet suggests that the reward for finding the chocolate was less novel for the 6 month old normal mice that were fed the Oz-AIN diet. This may partially explain the longer latencies but similar distances to locate the chocolate. The normal mice that were fed the Oz-AIN diet may have been just as accurate in searching for the chocolate, hence travelling similar distances as mice that were fed the AIN93-M diet. However, the chocolate was less novel, and therefore there may have been less urgency to locate the food.

At 12 months of age, normal mice that were fed the AIN93-M diet travelled similar distances to locate the buried chocolate as mice that were fed the Oz-AIN diet, suggesting that they no longer considered the food reward as being novel. This also suggests that diet does not affect the olfactory ability of 12 month old normal mice. This is similar to findings of others that report that while aspects of diet such as calorific intake may alter some odour guided behaviours, they do not completely impair performance in olfactory tests such as the BCT [513].

The 12 month old Amy mice that were fed the Oz-AIN diet took significantly longer and travelled further before uncovering the chocolate than Amy mice that were fed the AIN93-M diet. This demonstrates that diet does affect olfactory ability of Amy mice, and that feeding Amy mice the Oz-AIN diet impairs olfactory function. As discussed above, in the early stages of AD, β-amyloid accumulates within the olfactory bulbs and tract leading to hyperactivity, neuronal loss, and eventual anosmia [505] and β-amyloid deposition is enhanced by high-fat diet feeding [198, 472]. Taken together, this suggests that the high-fat nature of the Oz-AIN diet accelerated β-amyloid accumulation in the olfactory bulbs and tracts, leading to enhanced olfactory loss at 12 months of age. While this could be further supported with immunohistochemical and immunofluorescent techniques to visualise β-amyloid and neuronal populations within the olfactory bulbs and tracts of Amy mice, this was not part of the design of this study, as there were no mice killed at 12 months of age. However, comparisons of the β-amyloid deposit counts at 15 months of age indicated that Amy mice that were fed the Oz-AIN diet had more amyloid throughout their brains (Chapter 4). This suggests that it is possible that the 12 month old Amy mice that were fed the Oz-AIN diet may have had more amyloid than age-matched Amy mice that were fed the AIN93-M diet.

These results indicate that the high-fat, sub optimal Oz-AIN diet accelerates olfactory dysfunction in Amy mice. This is consistent with previous studies that have demonstrated that high-fat diets have also effect other behavioural features of AD, such as anxiety and exploratory behaviour [524].

Olfactory dysfunction with aging, in the Buried Chocolate Test.

Consistent with the third aim of this study, the effect of aging on olfactory function was investigated in normal and Amy mice that were fed either the AIN93-M diet or the Oz-AIN diet. These comparisons were made between mice when they were aged 6 and 12 months of age, to reflect olfactory ability during early and late adulthood.

Behavioural features such as speed and exploratory behaviour decline with aging in normal female mice [525], and these confounding factors must be taken into account when comparing latency data in aging animals. The average speeds of mice decreased with age. However, this was only significant for the Amy mice that were fed the Oz-AIN diet. This suggests that, apart from Amy mice that were fed the Oz-AIN diet, change in latency with age is not likely to be confounded by changes in mobility or speed. This provides strength to the interpretation that that the change in latency to uncover the pellet at 6 and 12 months does reflect olfactory decline for normal mice that were fed either the AIN93-M diet or the Oz-AIN diet, and for Amy mice that were fed the AIN93-M diet. This is consistent with reports from others who report that olfactory sensitivity and acuity decline with age in normal mice [526]. However, changes in the latency to uncover the chocolate may reflect changes in mobility for the Amy mice that were fed the Oz-AIN diet. Coupled with the fact that the distances travelled at 6 and 12 months were not significant for the Amy mice that were fed the Oz-AIN diet, it is possible that age-associated changes of latency to uncover the chocolate were more likely to be due to mobility, and not olfactory ability for Amy

mice that were fed the Oz-AIN diet. This implies that Amy mice that were fed the Oz-AIN diet may have been the least susceptible to age-related changes in olfactory abilities.

The implication that the Amy mice that were fed the Oz-AIN diet were the least susceptible to age-related olfactory dysfunction than other mice was unexpected. As briefly mentioned on page 457, Wu *et al.* report that Amy mice develop β-amyloid deposition in central olfactory cortices by the time they are 3 months old [518]. This is accompanied by significant olfactory decline and degeneration of olfactory sensory neurons [518]. It is possible that the behavioural tests used by Wu *et al.* and the current study account for the discrepancies in results. Wu *et al*. used a habituation / dishabituation test to evaluate olfactory abilities of Amy mice [518], whilst the current study has used a modified version of the Buried Food Pellet Test. The habituation / dishabituation test requires some degree of learning and recognition of a scent, and therefore may be confounded by cognitive abilities of mice. However, Wu *et al.* also demonstrated that Amy mice did not develop cognitive deficits until 9 months, and performed as well as normal mice in the Morris Water Maze at 3 months, when olfactory dysfunction first started to appear [518]. Other research groups that have also used modified versions of the Buried Food Pellet Test, and have reported that AD-type mice do not demonstrate olfactory decline with aging [527]. However, these studies have also did not demonstrate olfactory decline with aging in normal mice, suggesting that, the Buried Food Test and tests similar to it, may not actually be ideal for evaluating olfactory abilities in aging mice. It may be worthwhile to evaluate olfactory changes in aging mice using tests such as odor identification tests, or odor aversion tests, to determine whether or not the Amy mice that were fed the Oz-AIN diet were resilient to age-related olfactory abilities.

It must be emphasised that the suggestion that Amy mice that were fed the Oz-AIN diet were resilient to age-related olfactory decline is based on the finding that their increased latencies at 12 months may have been confounded by mice moving slower. It is likely that, although they did move slower with age, they may have lost olfactory function.

As discussed above, it is possible that the Amy that were fed the Oz-AIN diet had amyloid deposits and neuronal loss throughout the olfactory bulbs and tract, and this may have been accelerated by the Oz-AIN diet [472, 518, 519]. If this was indeed the case, it is possible that olfactory dysfunction starts much earlier in the Amy mice, and that by 6 months of age, the rate of decline for Amy mice that were fed the Oz-AIN diet had started to slow. As a result of this, the change observed from 6 to 12 months would not be as apparent as it was for other mice.

Interestingly, diet-type had opposing effects on olfactory abilities of mice. While the Oz-AIN diet appeared to have a detrimental effect on olfactory abilities of normal mice, the mice that were fed the AIN93-M diet appeared to improved olfactory function with age. Furthermore, this age-relate improvement was greater amongst Amy mice that were fed the AIN93-M diet was greater than that of normal mice that were fed the normal mice that were fed the AIN93-M diet. This suggests that feeding Amy mice and optimal diet enhances olfactory abilities with aging. These findings are surprising as others have reported a detrimental effect of age on olfactory function rather than a protective effect [526].

Mice that received the Oz-AIN diet irrespective of genotype, the mice that were fed the AIN93-M diet improved olfactory abilities with aging. It is possible that this suggests that AIN93-M diet may have improved olfactory functioning with aging in both normal and Amy mice. Irrespective of diet While there were slight increases in

the amount of time it took to uncover the chocolate (Figure 5A) the Amy mice that were fed the AIN93-M diet travelled a significantly shorter distance before uncovering the buried chocolate at 15 months of age compared to when they were 6 months old (1.360.29 m compared to 2.44 ±0.43 m, Figure 5B).

It is possible that mice stumbled across the buried chocolate by accident, which enabled them to locate the chocolate faster than other mice, however this is unlikely. It is more likely that the numbers of mice that were used in the current study were not large enough and have resulted in a false positive. In the comparisons of the distance travelled by Amy mice that were fed the AIN93-M diet, only the data from three mice were compared. It may therefore be beneficial to repeat this study with larger numbers of mice in order to confirm these findings.

7.4. Conclusion.

Taken together, assessment of the performance of normal and Amy mice in the BCT indicate that there are genotype effects on the olfactory abilities of Amy mice, and that these genotype effects are diet-type dependent.

The Oz-AIN diet may have accelerated olfactory dysfunction in Amy mice, and as a result Amy mice that were fed the Oz-AIN diet had poorer olfactory abilities than either normal mice that were fed the Oz-AIN diet or the Amy mice that were fed the AIN93-M diet. A likely mechanism for the olfactory dysfunction in Amy mice that were fed the Oz-AIN diet is by high-fat diet induced increase in β-amyloid deposition and subsequent neuronal loss throughout the olfactory bulbs and tract. However, the current study does not have the neuropathological data to support this.

The AIN93-M diet was associated with an improvement in olfactory abilities as mice aged, and this was more apparent in Amy mice than normal mice. While this contradicts much of the current literature that AD-type mice have poorer olfactory abilities than normal mice, it is proposed that improvement may be associated with age-related hormonal changes in Amy mice. This needs be investigated in further detail.

Chapter 8: The effect of nutrient supplements on genotype and diet-type induced olfactory dysfunction in Amy mice.

8. Background.

Olfactory dysfunction has been reported to precede memory dysfunction in AD patients and in AD-mouse models [505, 506]. However, olfactory dysfunction is not as well characterised in the Amy mouse as it is in other mouse models of AD [122, 508, 509].

Chapter 7 of this thesis characterised olfactory function in the Amy mouse model of AD. Genotype effects were reported, and these effects were dependent on diet-type. While the Oz-AIN diet may have had a detrimental effect on olfactory abilities of Amy mice, the AIN93-M diet enabled Amy mice to improve with age by degrees that were greater than normal mice. The role of the present chapter is to describe data that investigated abilities of nutrient supplements to prevent the genotype and diet-type effects on olfactory abilities of Amy mice.

Further to this, the study described in the current chapter also compares the olfactory abilities of Amy mice at 6 and 15 months. The benefit of these comparisons are that (i) extending the age gap between which mice are compared provides a greater window to observe changes; and (ii) results can be better related to the β-amyloid pathology in the 15 month old Amy mouse brains that were described in Chapter 4.

The aims of the study described in this chapter are to:

1. **Determine whether or not genotype induced olfactory dysfunction can be prevented with nutrient supplements (Figure 1A).**

   This was achieved by making two sets comparisons:

   (i) Olfactory abilities of normal and Amy mice that were fed the Oz-AIN diet were assessed in the Buried Chocolate Test (BCT) and compared at 6 and 15 months.

   (ii) Olfactory abilities of Amy mice that were fed the Oz-AIN Supp were assessed in the BCT at 6 and 15 months and compared with those of age matched normal or Amy mice that were fed the Oz-AIN diet.

2. **Determine whether or not diet-type induced olfactory dysfunction can be prevented with nutrient supplements (Figure 1B).**

   This was achieved by making two sets of comparisons:

   (i) Olfactory abilities of Amy mice that had been fed the AIN93-M diet or the Oz-AIN diet were assessed in the BCT and compared at 6 and 15 months.

   (ii) Olfactory abilities of Amy mice that were fed the Oz-AIN Supp diet were assessed in the BCT and compared with those of Amy mice that were fed either the AIN93-M diet or the Oz-AIN diet.

3. **Characterise olfactory function of normal and Amy mice at different stages of adult life.**

   This was achieved by making within group comparisons of olfactory abilities of normal mice that were fed the Oz-AIN diet, Amy mice that were fed the AIN93-M diet, Amy mice that were fed the Oz-AIN diet and Amy mice that were fed the Oz-AIN Supp diet at 6 and 15 months.

There were three sets of hypotheses for this chapter. First, it was hypothesised that Amy mice fed the Oz-AIN diet have poorer olfactory abilities than normal mice fed the Oz-AIN diet, and that this could be prevented by nutrient supplements, so that Amy mice fed the Oz-AIN Supp diet would perform similarly to normal mice that were fed the Oz-AIN diet. Second it was hypothesised that Amy mice fed the Oz-AIN diet would have poorer olfactory abilities than Amy mice fed the AIN93-M diet, and that this could be prevented by nutrient supplements, so that Amy mice fed the Oz-AIN Supp diet would perform as well as Amy mice fed the AIN93-M diet. Third, it was hypothesised that 6 month old mice would have better olfactory abilities than 15 month old mice.

This study has made use of a modified version of the Buried Food Pellet Test, which is called the Buried Chocolate Test (BCT). The apparatus and protocol that were used are the same as those used in Chapter 7 (sections 7.1.3. and 7.1.4. pp. 427-428). The study design and statistics used are described in the methods section of this chapter.

The main findings of the BCT were that diet-type, but not genotype, had a significant effect on olfactory abilities of 6 and 15 month old Amy mice. The nutrient supplements were able to prevent diet-type effects at 6 months of age, but not at 15 months. As a consequence of this, age-associated olfactory dysfunction was the greatest for Amy mice that were fed the Oz-AIN Supp diet. Furthermore, while all mice that were fed the high-fat diet had significant age-related olfactory decline, the Amy mice that were fed the AIN93-M diet had similar olfactory abilities at 6 and 15 months of age. This indicates that the high-fat diet accelerates age-related olfactory dysfunction.

8.1. Methods.

8.1.1. Animals.

All experiments were approved by the Commonwealth Scientific and Industrial Research Organisation (CSIRO) Animal Welfare Committee, Australia in accordance with National Health and Medical Research Council guidelines.

Female Amy (APPswe/PSEN1dE9) mice and their female normal (C57bl/6) littermates were bred at and provided by Flinders University Animal Facility, Bedford Park, South Australia. Genotype was confirmed by PCR and agarose gel electrophoresis, as described in Appendix I. Amy mice were divided into three dietary groups and were fed the AIN93-M diet, the Oz-AIN diet or the Oz-AIN Supp diet from weaning. Normal mice were fed the Oz-AIN diet from weaning and were used as a control. Mice were housed (n<6) in cages lined with sawdust, and had free access to food and water.

Mice underwent testing in the BCT at 6 and 15 months of age (Figure 1). Treatment group sizes are reported in Table 1. Group sizes between six and ten have been successfully used by others to demonstrate the genotype and treatment effects on olfactory dysfunction in mice, and were therefore deemed acceptable for use in the current study [116, 118, 516].

Table 1. The number of mice assessed in the Buried Chocolate Test at 6 and 15 months of age.

	Normal mice Oz-AIN diet	**Amy mice AIN93-M diet**	**Amy mice Oz-AIN diet**	**Amy mice Oz-AIN Supp diet**
Treatment group size	n = 6	n = 9	n = 9	n = 8

8.1.2. Study design.

Aim 1: To investigate the ability of nutrient supplements to PREVENT GENOTYPE EFFECTS on olfactory function of normal and Amy mice.

Consistent with the first aim of the study, the ability of nutrient supplements to prevent genotype effects on olfactory ability was assessed at 6 and 15 months of age (Figure 1A).

Normal mice and Amy mice that were fed the Oz-AIN diet were compared to demonstrate genotype effects of olfactory abilities (Figure 1A). The ability of nutrient supplements to prevent genotype effects on olfactory abilities was investigated by comparing Amy mice that had been fed the Oz-AIN Supp diet with either: normal mice that were fed the Oz-AIN diet or Amy mice that were fed the Oz-AIN diet (Figure 1A).

Aim 2: To investigate the ability of nutrient supplements to PREVENT DIET-TYPE EFFECTS on olfactory function of Amy mice.

Consistent with the second aim of the study, the ability of nutrient supplements to prevent genotype effect on olfactory ability was assessed at 6 and 15 months of age (Figure 1B). Amy mice that were fed the AIN93-M diet and Amy mice that were fed the Oz-AIN diet were compared to demonstrate the diet-type effects on olfactory function (Figure 1B).

The ability of nutrient supplements to prevent diet-type effects on olfactory abilities was investigated by comparing Amy mice that were fed the Oz-AIN Supp diet with either: Amy mice that were fed the AIN93-M diet or Amy mice that were fed the Oz-AIN diet (Figure 1B).

Aim 3: To investigate the effect of AGING on olfactory abilities of Amy mice.

Consistent with the third aim of this study, the effect of aging was investigated through comparisons of the olfactory abilities of mice at 6 and 15 months (Figure 1C).

Age-related olfactory dysfunction was investigated using *within* groups and *between* groups comparisons. The *within* groups comparisons were made by comparing performance in the BFPT at 6 and 15 months to demonstrate the change in olfactory with age. The normal mice that were fed the Oz-AIN diet, Amy mice that were fed the Oz-AIN diet, Amy mice that were fed the AIN93-M diet and Amy mice that were fed the Oz-AIN Supp diet were included in these comparisons (Figure 1C). The *between* groups comparisons of the change in olfactory ability with age, were made to determine genotype and diet-type effects on age-related olfactory dysfunction.

Figure 1A. The study design used to investigate the ability of nutrient supplements to prevent Genotype effects on olfactory abilities.

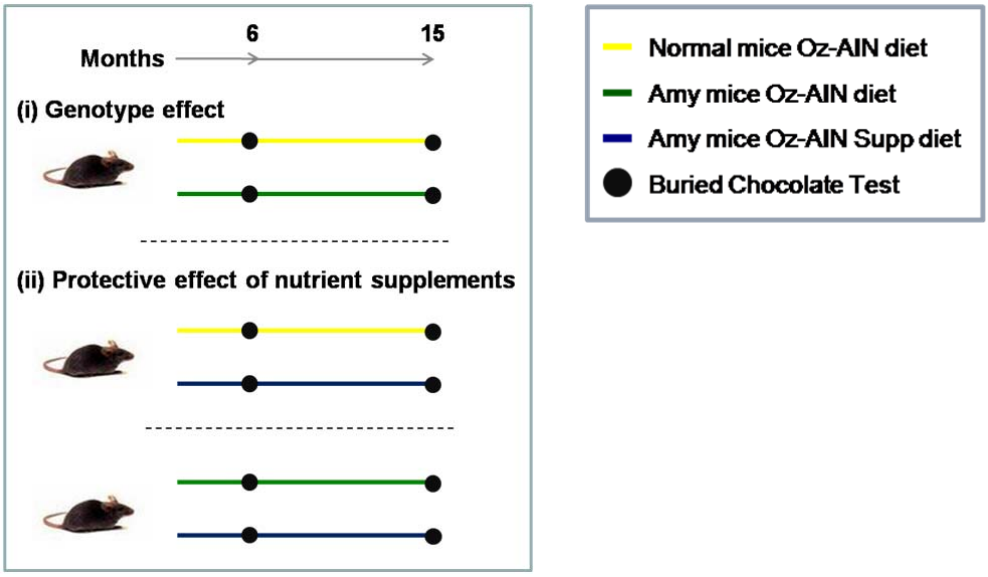


Figure 1A. The Buried Chocolate Test (BCT) was used to assess olfactory abilities in mice. Mice underwent assessments at 6 and 15 months old. The ability of nutrient supplements to prevent genotype effects were determined over two stages:

(i) Comparisons between normal mice (yellow line) and Amy mice (green line) that had been fed the Oz-AIN diet at 6 and 15 months old were made to demonstrate genotype effects when mice are challenged with a sub-optimal diet.

(ii) The Amy mice that were fed the Oz-AIN Supp diet (blue line) were compared with the normal mice and Amy mice that were fed the Oz-AIN diet to demonstrate whether or not nutrient supplements could prevent genotype induced olfactory function.

Figure 1B. The study design used to investigate the ability of nutrient supplements to prevent Diet-type effects on olfactory abilities.

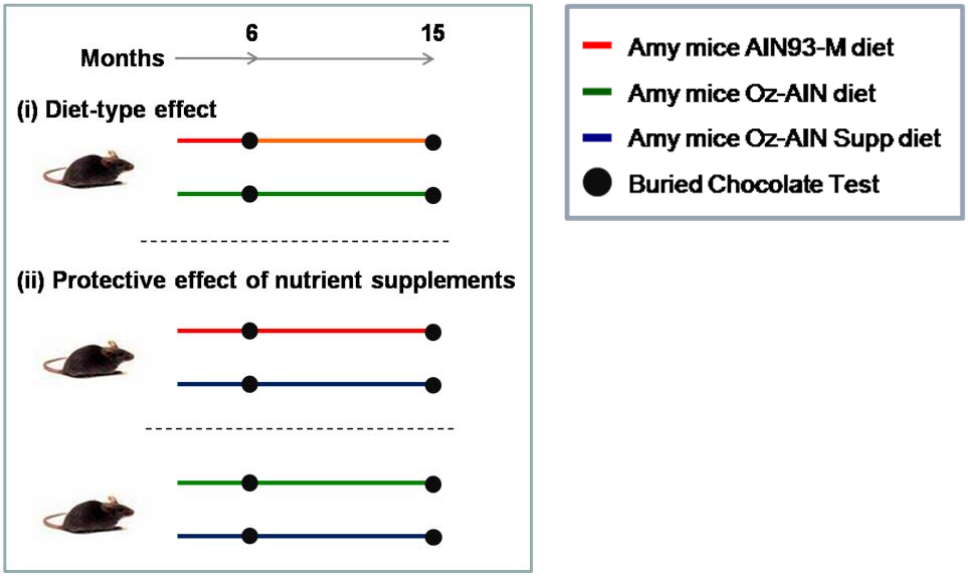


Figure 1B. The Buried Chocolate Test (BCT) was used to assess olfactory abilities in mice. Mice underwent assessments at 6 and 15 months old. Diet-type effects were demonstrated over two stages:

(i) Comparisons between Amy mice that were fed the AIN93-M diet (red line) and Amy mice that were fed the Oz-AIN diet (green line) that at 6 and 15 months old were made to demonstrate diet-type effects.

(ii) The Amy mice that were fed the Oz-AIN Supp diet (blue line) were compared with the Amy mice that had been fed either the AIN93-M diet or the Oz-AIN diet to determine whether or not nutrient supplements prevent diet-type induced olfactory function.

Figure 1C. The study design used to investigate the effect of aging on olfactory abilities of normal and Amy mice.

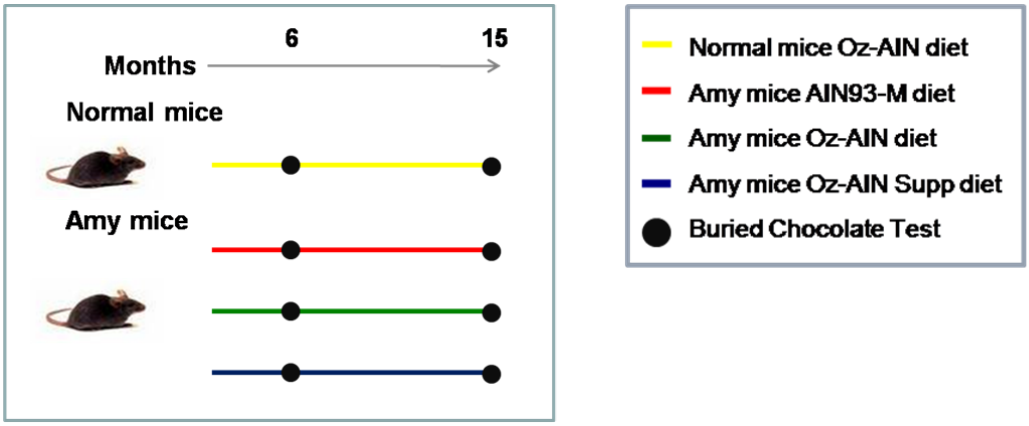


Figure 1C. The Buried Chocolate Test (BCT) was used to assess olfactory abilities in mice. Mice underwent assessments at 6 and 15 months of old.

Aging effects *within groups* were determined by comparing the olfactory abilities of normal mice that were fed the Oz-AIN diet (yellow line), Amy mice that were fed the Oz-AIN diet (green line), Amy mice that were fed the AIN93-M diet (red line), and Amy mice that were fed the Oz-AIN Supp diet (blue line) when they were 6 months old with their olfactory abilities at 15 months old.

Aging effects *between groups* were determined by comparing the change of olfactory abilities from 6 to 15 months between groups.

(i) Genotype effects on the change of olfactory abilities with aging were investigated through comparisons between normal mice that were fed the Oz-AIN diet (yellow line), Amy mice that were fed the Oz-AIN diet (green line), and Amy mice that were fed the Oz-AIN Supp diet (blue line).

(ii) Diet-type effects on the change of olfactory abilities with aging were investigated through comparisons between Amy mice that were fed the AIN93-M diet (red line), Amy mice that were fed the Oz-AIN diet (green line), and Amy mice that were fed the Oz-AIN Supp diet (blue line).

8.1.3. Apparatus.

The apparatus that was used to conduct the BCT and maze set up are the same as described in Chapter 7 (7.1.3., p. 434).

8.1.4. Protocol.

Mice underwent testing in the BCT at 6 and 15 months of age.

The protocols that were used to conduct the BCT are the same as those described in Chapter 7 (7.1.4., p. 435).

8.1.5. Data collection.

Latency (s), distance travelled (m) and average speed (m/s) travelled by mice before uncovering the buried piece of chocolate were recorded by Stoelting ANYmaze software (Stoelting Co., Wood Dale, USA).

All data was stored in excel files and analysed using GraphPad Prism^{R} Software (Prism 5 for Windows, version 5.04, GraphPad Software inc., CA, USA).

8.1.6. Data analysis.

Latency (s) and distance travelled (m) were the primary measures of olfactory function. To confirm that the latencies, distances, and average speed were accurately recorded by ANYmaze software, the latencies to uncover the buried chocolate were also recorded manually from video footage by an experimenter that was blinded to the treatment condition of each mouse. Any trials where the manual and digitally recorded latencies differed by >2 s were considered not to have been detected accurately by ANYmaze software. These trials were excluded from analyses.

To achieve Aim 1 and Aim 2, comparisons were made *between groups* using one-way ANOVA and Bonferroni post tests when mice were 6 and 15 months old. With low numbers of mice in some treatment groups, diet-type or genotype effects may have been hard to detect with a one-way ANOVA. Therefore, analysis of diet-type-matched or genotype-matched mice was also carried out using Student's *t*-tests. The ability of nutrient supplements to prevent genotype effects were established by comparisons between normal mice that were fed the Oz-AIN diet, Amy mice that were fed the Oz-AIN diet, and Amy mice that were fed the Oz-AIN Supp diet. The ability of nutrient supplements to prevent diet-type effects were established by comparing Amy mice that were fed the AIN93-M diet, the Oz-AIN diet or the Oz-AIN Supp diet using one-way ANOVA and Bonferroni post tests.

To achieve Aim 3, the effect of aging *within* groups was established by comparing the performance of mice at 6 and 15 months of age with Student's *t*-tests. The ability of nutrient supplements to prevent the genotype or diet-type effects on age-related olfactory dysfunction was also investigated. The ratio of olfactory abilities at 15 months : 6 months was calculated for each treatment group. These ratios were compared using one-way ANOVA and Bonferroni post tests. All data is reported as mean ±SEM. For all comparisons, statistical significance was set at $p<0.05$.

8.2. Results.

8.2.1. Mice at 6 months old.

8.2.1.1. The ability of nutrient supplements to prevent genotype effects on olfactory abilities of 6 month old mice in the Buried Chocolate Test.

The ability of nutrient supplements to prevent genotype effects on latency (s).

A one-way ANOVA did not detect significant differences in latency to locate the buried chocolate between the 6 month old normal mice that were fed the Oz-AIN diet, Amy mice that were fed the Oz-AIN diet and Amy mice that were fed the Oz-AIN Supp diet ($p=0.18$, Figure 2). Amy mice that were fed the Oz-AIN diet took longer to locate the buried chocolate (31.76 ±7.22 s and 25.27 ±5.36 s respectively, Figure 2). However, this failed to achieve significance with Bonferroni multiple comparisons tests ($p>0.99$). Students *t*-test also did not detect significance between normal and Amy mice that were fed the Oz-AIN diet ($p=0.59$, Figure 2). This confirms that at 6 months of age, there were no genotype effects on latency (s) to find the buried chocolate.

The Amy mice that were fed the Oz-AIN Supp diet found the chocolate faster than normal mice that were fed the Oz-AIN diet and Amy mice that were fed the Oz-AIN diet (16.20 ±3.90 s compared to 25.27 ±5.36 s and 31.76 ±7.22 s respectively, Figure 2). However, the purposes of these comparisons were to demonstrate the ability of nutrient supplements to prevent genotype effects on olfactory abilities. There were no genotype effects on latency (s) to uncover the buried chocolate. Therefore, the effects of nutrient supplements on latency to the buried chocolate will be discussed in the context of diet-type effects on olfactory abilities, on pages 486 and 525-528.

Figure 2. The ability of nutrient supplements to prevent potential genotype effects on latency (s) for 6 month old normal and Amy mice to locate a buried piece of chocolate.

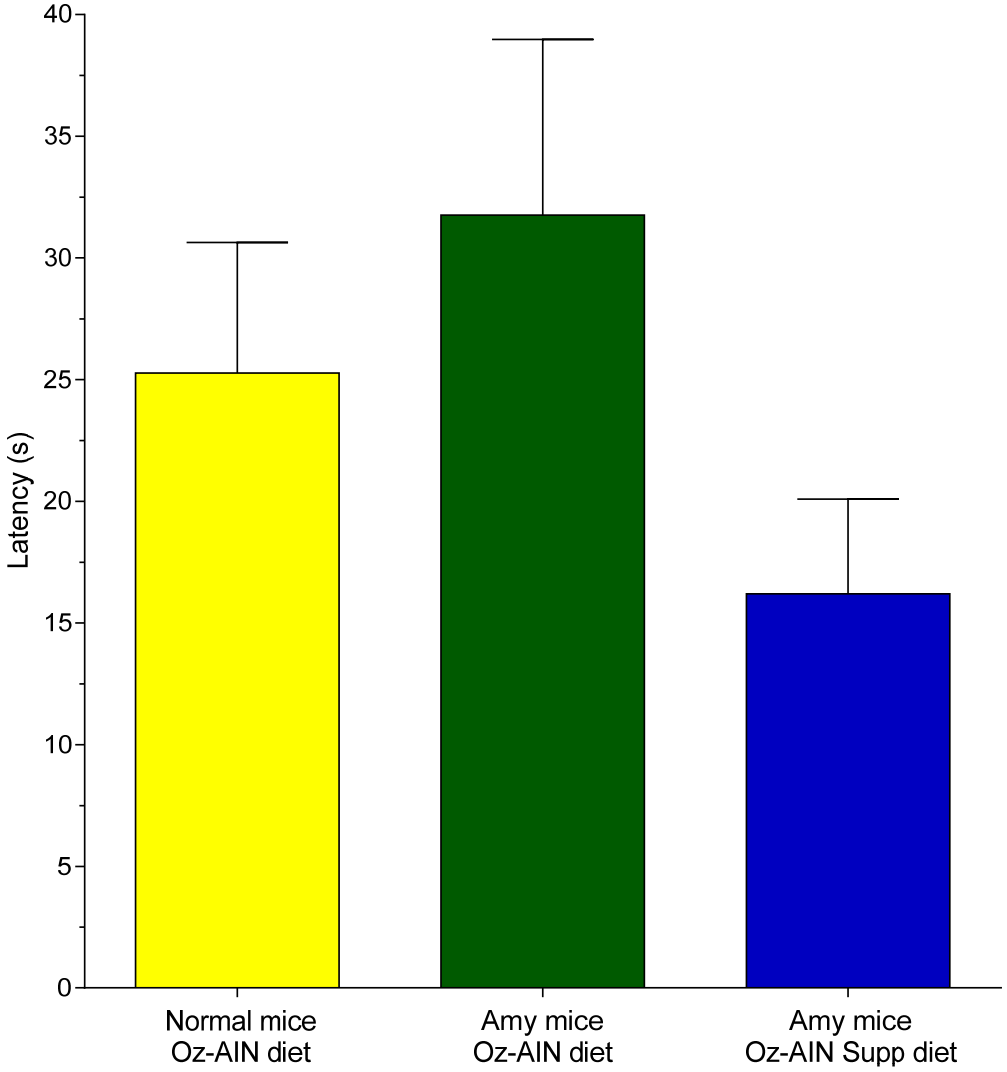


Figure 2. Normal mice fed the Oz-AIN diet (yellow bar, n=6), Amy mice fed the Oz-AIN diet (green bar, n=9), Amy mice fed the Oz-AIN Supp diet (blue bar, n=8). Bars represent mean ±SEM.

The ability of nutrient supplements to prevent genotype effects on distance (m).

A one-way ANOVA did not detect significant differences in distance travelled by 6 month old normal and Amy mice whilst looking for the buried chocolate ($p=0.07$, Figure 3). While these differences were not significant at $p<0.05$, they were significant at $p<0.10$. This indicates that there may have been trends for genotype to affect distance travelled by 6 month old mice before uncovering the buried chocolate. Amy mice that were fed the Oz-AIN diet travelled further than normal mice that were fed the Oz-AIN diet. This did not achieve significance with Bonferroni post tests (3.03 ±0.55 m and 2.71 ±0.53 m respectively, $p>0.99$, Figure 3). Students *t*-test also did not detect significance between normal and Amy mice that were fed the Oz-AIN diet ($p=0.73$, Figure 3). This suggests that at 6 months of age, there were no genotype effects on distance travelled (m) before normal or Amy mice that were fed the Oz-AIN diet located the buried chocolate.

The trends for difference between groups that was detected by the one-way ANOVA may have been due to the distances travelled by Amy mice that were fed the Oz-AIN Supp diet (Figure 3). Bonferroni post tests revealed trends to suggest that Amy mice that were fed the Oz-AIN Supp travelled shorter distances than Amy mice that were fed the Oz-AIN diet (1.29 ±0.49 m and 3.03 ±0.55 m respectively, $p=0.08$, Figure 3). This was confirmed with a Student's *t*-test ($p=0.03$). However, it may not be appropriate to make comparisons that investigate the ability of nutrient supplements to prevent genotype effects on distance to the chocolate at 6 months of age, as there were no genotype effects. Therefore, the distance to the buried chocolate of 6 month old Amy mice that were fed the Oz-AIN Supp diet will be discussed further in the context of the effect of diet on olfactory abilities (p. 488 and pp. 525-528).

Figure 3. The ability of nutrient supplements to prevent potential genotype effects on the distance travelled (m) by 6 month old normal and Amy mice before locating a buried piece of chocolate.

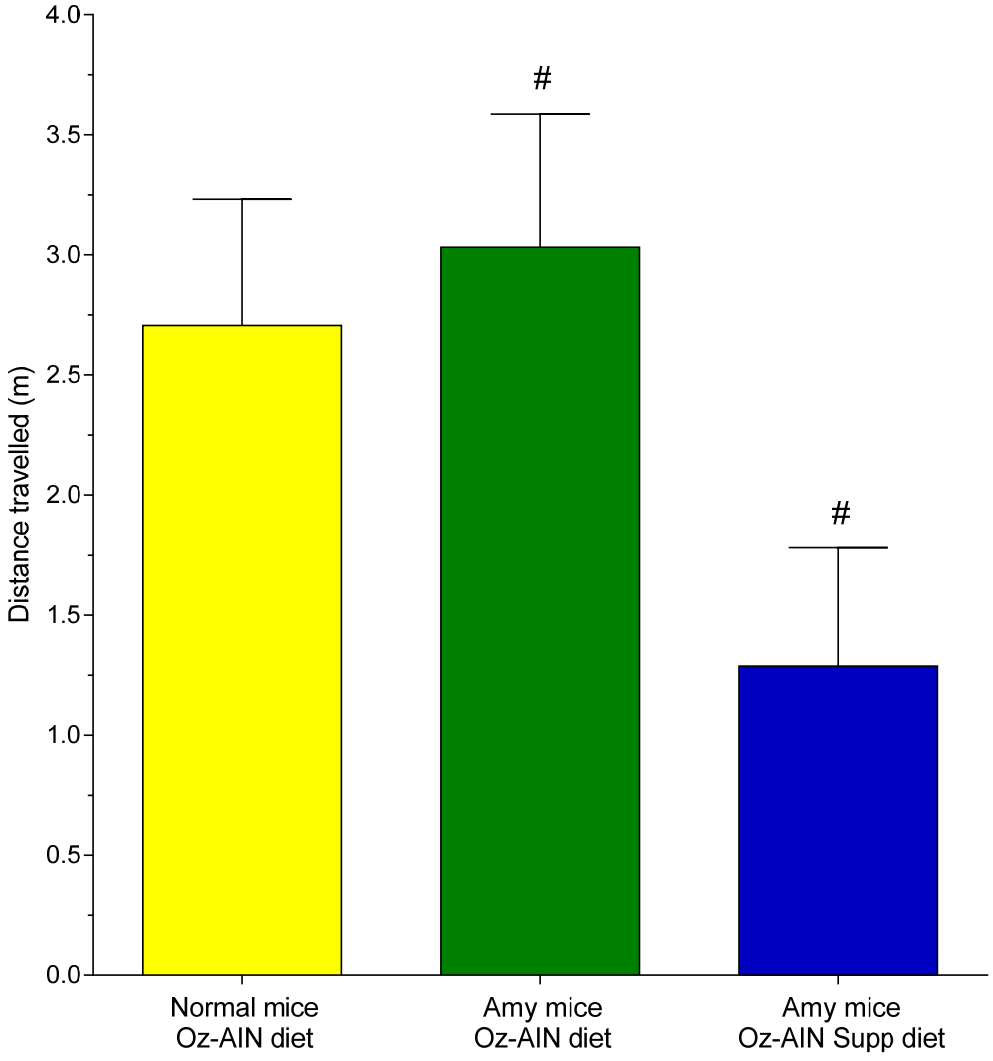


Figure 3. Normal mice fed the Oz-AIN diet (yellow bar, n=6), Amy mice fed the Oz-AIN diet (green bar, n=9), Amy mice fed the Oz-AIN Supp diet (blue bar, n=8). Bars represent mean ±SEM. Bars with matching symbols were significantly different with Students *t*-test. (#) p=0.03.

The ability of nutrient supplements to prevent genotype effects on speed (m/s).

A one-way ANOVA revealed that there were significant differences in the average speed (m/s) of mice whilst searching for the buried chocolate ($p=0.07$, Figure 4). This suggests that it may be hard to accurately determine whether or not genotype effects on latency (s) or distance travelled (m) by mice did exist. However, significant differences were not detected between normal or Amy mice that were fed the Oz-AIN diet by either Bonferroni post tests ($p>0.999$) or Students *t*-tests ($p=0.063$, Figure 4). The variances in average speed (m/s) of 6 month old mice that were that were detected by the one-way ANOVA are attributed to the speeds travelled by Amy mice that were fed the Oz-AIN Supp diet. The effect of the Oz-AIN Supp diet on speeds travelled by 6 month old mice is discussed further on pages 486 and 525-528.

Figure 4. The ability of nutrient supplements to prevent potential genotype effects on the average speed travelled (m/s) by 6 month old Amy mice whilst searching for a buried piece of chocolate.

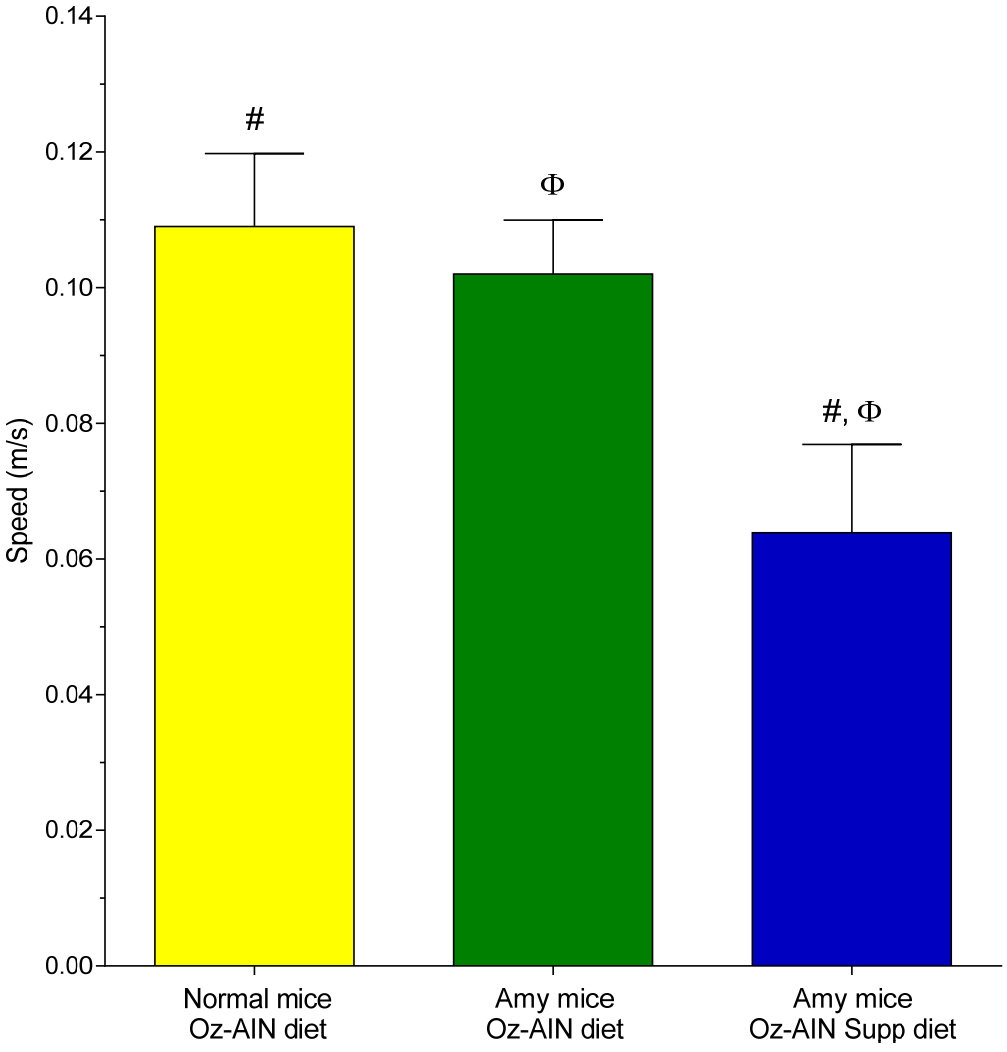


Figure 4. Normal mice fed the Oz-AIN diet (yellow bar, n=6), Amy mice fed the Oz-AIN diet (green bar, n=9), Amy mice fed the Oz-AIN Supp diet (blue bar, n=8). Bars represent mean ±SEM. Bars with matching symbols were significantly different with Students *t*-test. (#) p=0.05. (Φ) p=0.02.

8.2.1.2. The ability of nutrient supplements to prevent diet-type effects on olfactory abilities of 6 month old mice in the Buried Chocolate Test.

The ability of nutrient supplements to prevent diet-type effects on latency (s).

A one-way ANOVA did not detect significant diet-type effects on latency (s) to locate the buried chocolate by 6 month old Amy mice ($p=0.10$, Figure 5). While these differences were not significant at $p<0.05$, they were significant at $p<0.10$. This indicates that there may have been trends for diet-type effects on latency (s) for 6 month old Amy mice to uncover the buried chocolate (Figure 5).

Amy mice that were fed the Oz-AIN diet took longer to find the buried chocolate than Amy mice that were fed the AIN93-M diet (31.76 ±7.22 s and 20.64 ±2.69 s respectively, Figure 5). The Amy mice that were fed the Oz-AIN Supp diet found the buried chocolate in almost half the time that Amy mice that were fed the Oz-AIN diet (16.20 ±3.90 s and 31.76 ±7.22 s respectively, $p=0.09$, Figure 5). While not significant, the Amy mice that were fed the Oz-AIN Supp diet also found the buried chocolate with shorter latencies than Amy mice that were fed the AIN93-M diet (16.20 ±3.90 s and 20.64 ±2.69 s respectively, $p=0.35$, Figure 5).

These data suggest that diet may affect the latency (s) for Amy mice to locate a buried piece of chocolate. While the Oz-AIN diet may increase the time taken for mice to uncover the buried chocolate, the nutrient supplemented diet improved latencies to times that were faster than those of mice that were fed an ideal diet. This suggests that nutrient supplements are beneficial for olfactory functioning in 6 month old mice.

Figure 5. The ability of nutrient supplements to prevent potential diet-type effects on the latency (s) for 6 month old Amy mice to locate a buried piece of chocolate.

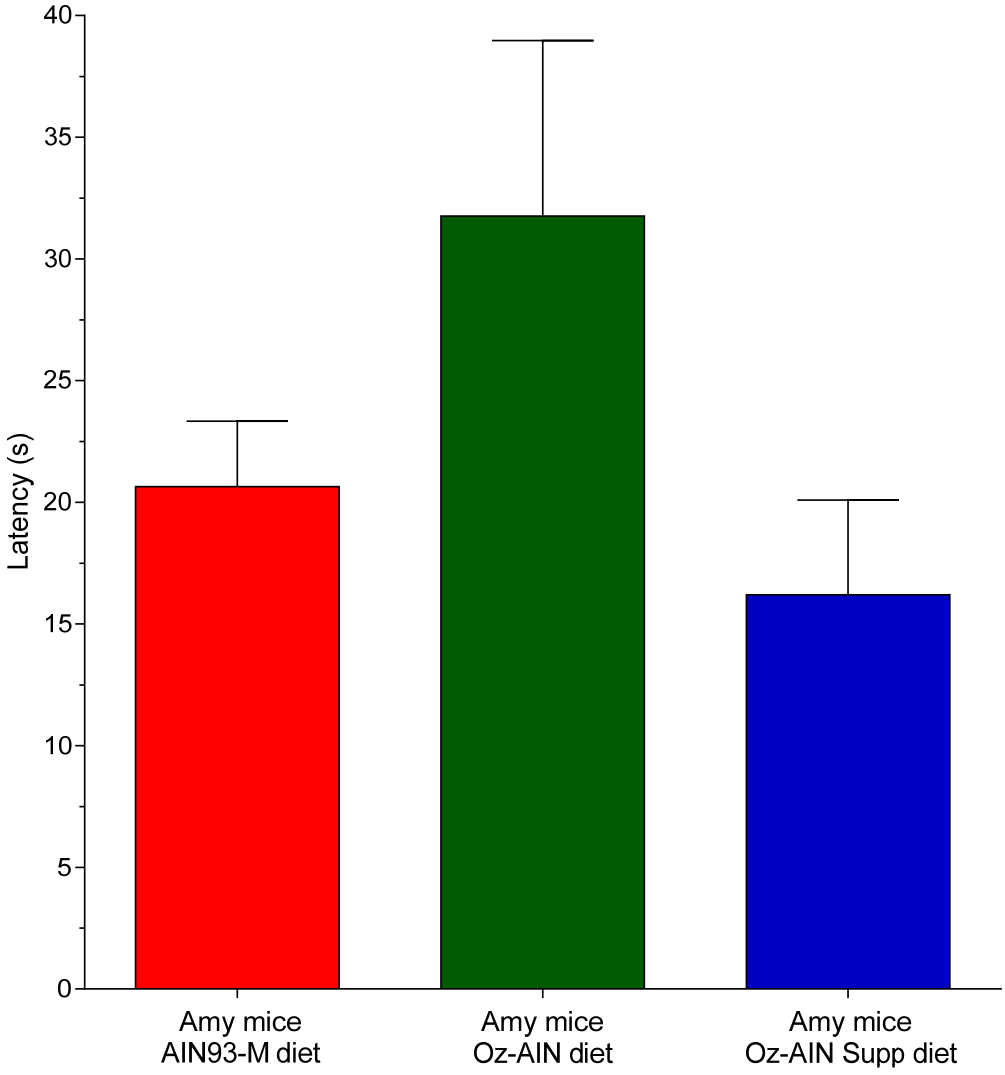


Figure 5A. Amy mice fed the AIN93-M diet (red bar, n=9), Amy mice fed the Oz-AIN diet (green bar, n=9), Amy mice fed the Oz-AIN Supp diet (blue bar, n=8). Bars represent mean ±SEM.

The ability of nutrient supplements to prevent diet-type effects on distance (m).

A one-way ANOVA revealed that there were significant differences in the distance travelled (m) by 6 month old mice before locating a buried chocolate ($p=0.03$, Figure 6). Bonferroni multiple comparisons tests revealed that Amy mice that were fed the Oz-AIN diet travelled significantly further than Amy mice that were fed the Oz-AIN Supp diet (3.03 ±0.55 m and 1.29 ±0.49 m respectively, $p=0.03$, Figure 6). This was confirmed with Students *t*-tests ($p=0.03$, Figure 6). There were also trends that indicated that Amy mice that were fed the Oz-AIN diet travelled further than Amy mice that were fed the AIN93-M diet before locating the buried chocolate (3.03 ±0.55 m and 1.99 ±0.21 m respectively, $p=0.10$, Students *t*-test, Figure 6).

Similar to the data that describe latency (s) to locate the buried chocolate, these data suggest that the sub-optimal diet impaired olfactory abilities in Amy mice. The nutrient supplements not only improved olfactory abilities at 6 months, but enabled mice to located the chocolate after travelling shorter distances than mice that were fed the AIN93-M diet.

Figure 6. The ability of nutrient supplements to prevent potential diet-type effects on the distance travelled (m) by 6 month old Amy mice before locating a buried piece of chocolate.

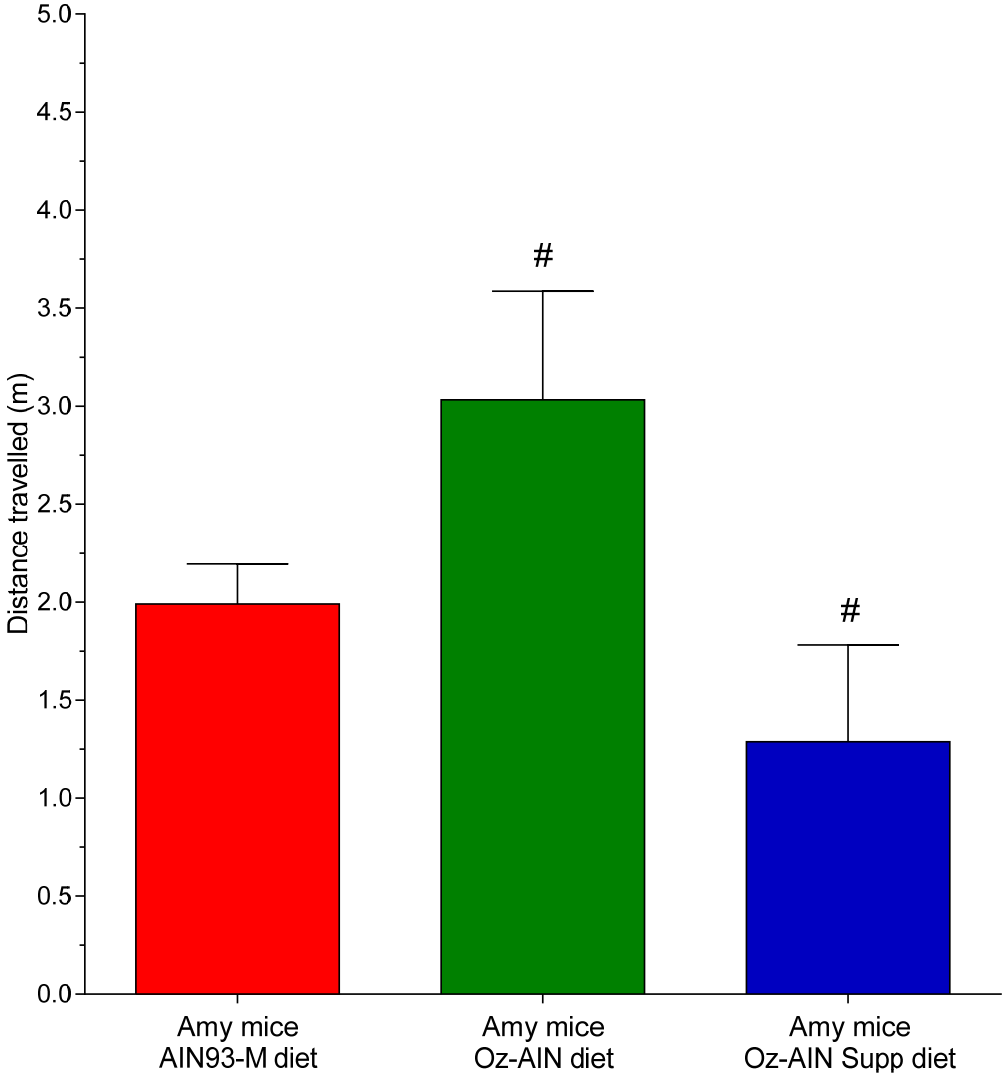


Figure 6. Amy mice fed the AIN93-M diet (red bar, n=9), Amy mice fed the Oz-AIN diet (green bar, n=9), Amy mice fed the Oz-AIN Supp diet (blue bar, n=8). Bars represent mean ±SEM. Bars with matching symbols were significantly different with Students *t*-test. (#) p=0.03.

The ability of nutrient supplements to prevent diet-type effects on speed (m/s).

A one-way ANOVA revealed significant diet-type effects on the speed travelled (m/s) by 6 month old Amy mice whilst looking for the buried chocolate ($p=0.01$, Figure 7). Bonferroni multiple comparison post tests revealed that these differences could be attributed to Amy mice that were fed the Oz-AIN Supp diet travelling significantly slower than either Amy mice that were fed the AIN93-M diet ($p=0.04$, Figure 7) or the Amy mice that were fed the Oz-AIN diet ($p=0.02$). However, no significant differences were detected between Amy mice that were fed the AIN93-M diet or the Oz-AIN diet, by either Bonferroni post tests ($p>0.99$) or Students *t*-tests ($p=0.71$, Figure 7). This suggests that the comparisons between Amy mice that were fed the AIN93-M diet or the Oz-AIN diet for their latency (s) or distance travelled (m) to the buried chocolate are reliable, as there was no difference in average speeds travelled.

The finding that Amy mice that were fed the Oz-AIN Supp diet travelled slower than Amy mice that were fed the Oz-AIN diet or the AIN93-M diet supports the conclusion that they had better olfactory abilities than other dietary groups at 6 months, as it suggests that they may have been more careful when searching for the chocolate. This is discussed further on page 458-459.

Figure 7. The ability of nutrient supplements to prevent potential diet-type effects on the average speed travelled (m/s) by 6 month old Amy mice whilst searching for a buried chocolate.

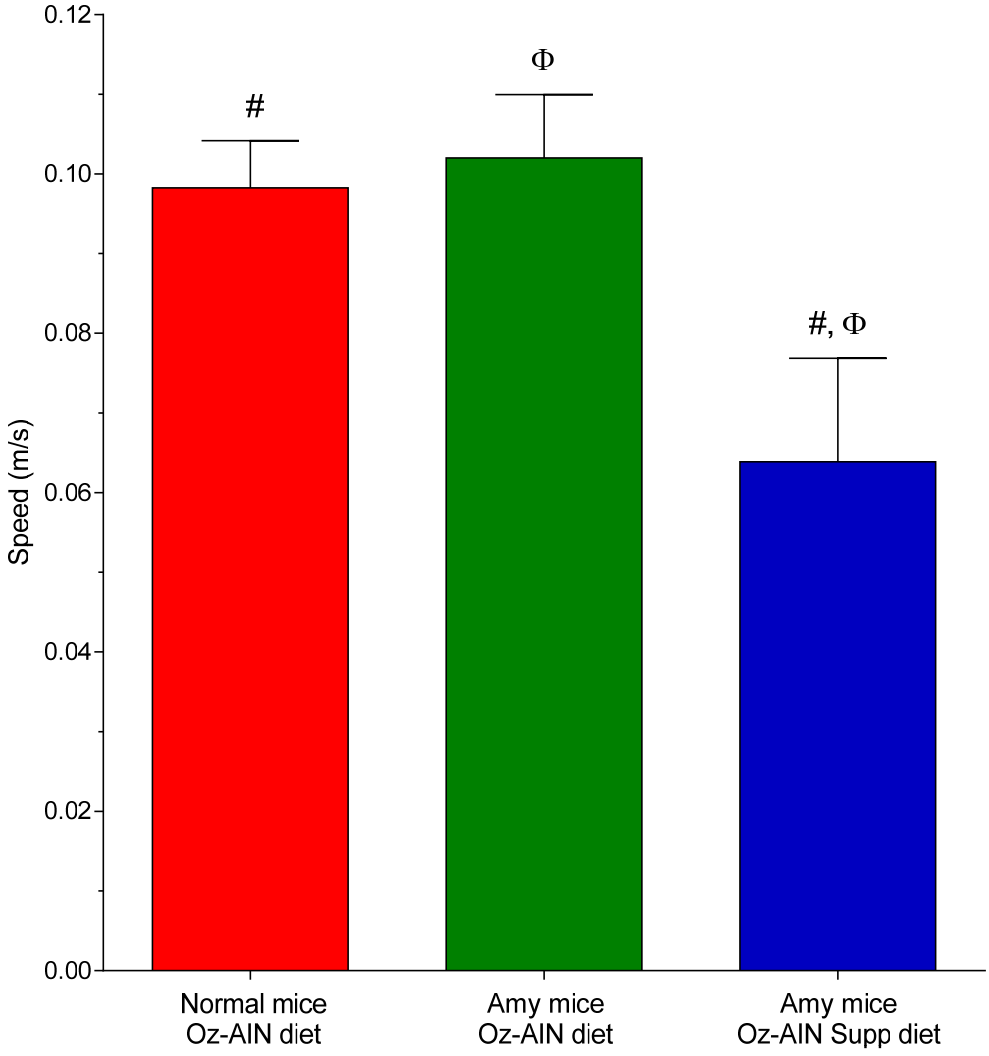


Figure 7. Amy mice fed the AIN93-M diet (red bar, n=9), Amy mice fed the Oz-AIN diet (green bar, n=9), Amy mice fed the Oz-AIN Supp diet (blue bar, n=8). Bars represent mean ±SEM. Bars with matching symbols were significantly different with Students *t*-test. (#) p=0.05. (Φ) p=0.02.

8.2.2. Mice at 12 months old.

8.2.2.1. The ability of nutrient supplements to prevent genotype effects on olfactory abilities of 12 month old mice in the Buried Chocolate Test.

The ability of nutrient supplements to prevent genotype effects on latency (s).

A one-way ANOVA did not detect significant differences in latency (s) for 15 month old normal and Amy mice to uncover a buried chocolate ($p=0.20$, Figure 8). Students *t*-tests did not detect differences between the latencies of the 15 month old normal mice and Amy mice that were fed the Oz-AIN diet to locate the buried chocolate (170.50 ±9.50 s and 125.30 ±27.33 s respectively, $p=0.31$, Figure 8). This confirmed that there were no genotype effects on the latency for 15 month old normal and Amy mice to uncover the buried chocolate.

There were non-significant trends that suggested that Amy mice that were fed the Oz-AIN Supp diet located the chocolate with shorter latencies than the normal mice that were fed the Oz-AIN diet (87.45 ±25.50 s and 170.50 ±9.5 s respectively, $p=0.07$, Figure 8) or the Amy mice that were fed the Oz-AIN diet (87.45 ±25.50 s and 125.30 s respectively, $p=0.09$, Figure 8). However, the purpose of these comparisons was to demonstrate that nutrient supplements can prevent *genotype* effects. While the mice that were fed the Oz-AIN Supp diet may have located the buried chocolate faster than other mice, this is unlikely to be attributed to genotype. As discussed in the paragraph above, there was no effect of genotype on olfactory abilities of 6 month old Amy mice. The beneficial effects of the nutrient supplements on olfactory abilities of 15 month old Amy mice is discussed in the context of the effects of diet on page 525-528.

Figure 8. The ability of nutrient supplements to prevent potential genotype effects on latency (s) for 15 month old normal and Amy mice to locate a buried piece of chocolate.

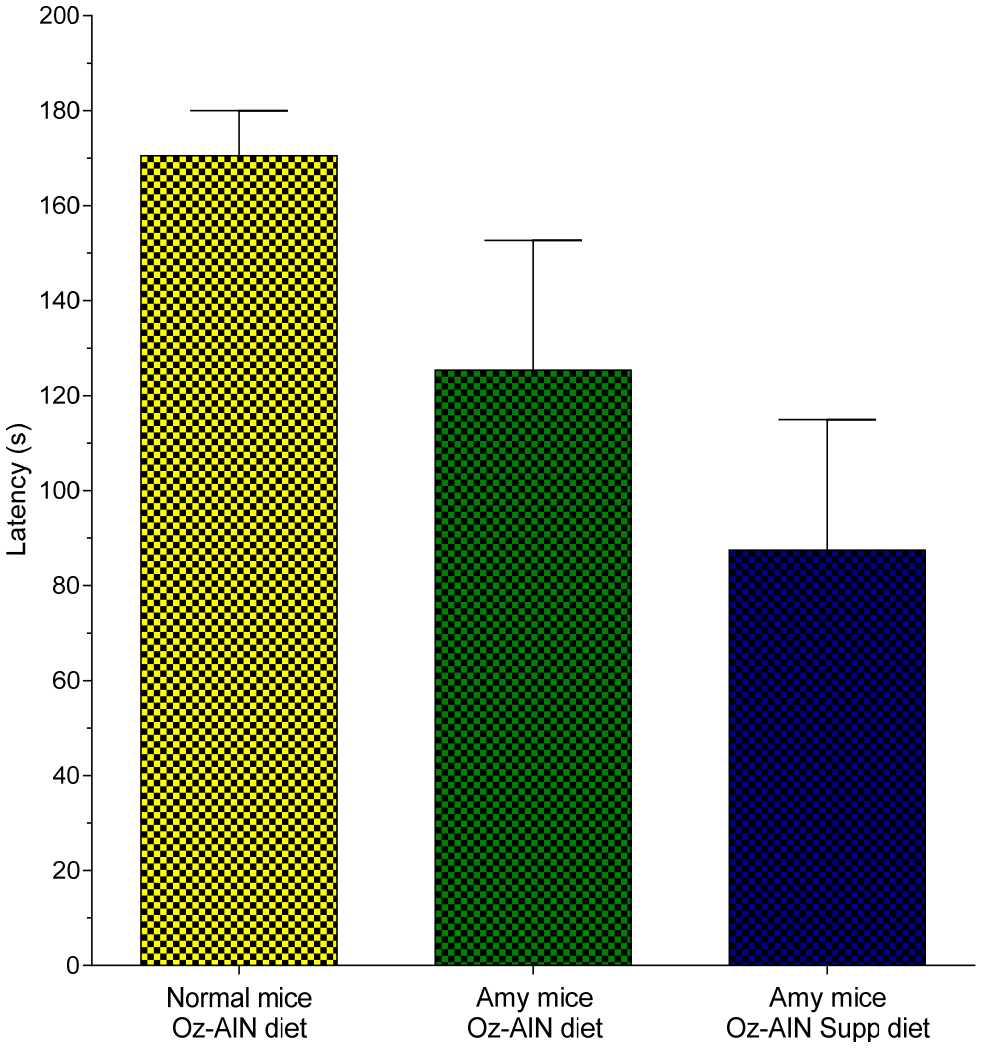


Figure 8. Normal mice fed the Oz-AIN diet (yellow bar, n=6), Amy mice fed the Oz-AIN diet (green bar, n=9), Amy mice fed the Oz-AIN Supp diet (blue bar, n=8). Bars represent mean ±SEM.

The ability of nutrient supplements to prevent genotype effects on distance (m).

A one-way ANOVA did not detect significant genotype effects on the distance travelled by the 15 month old normal mice that were fed the Oz-AIN diet, Amy mice that were fed the Oz-AIN diet and Amy mice that were fed the Oz-AIN Supp diet before locating a buried chocolate ($p=0.43$, Figure 9). Students *t*-tests also did not detect significant differences between the distance travelled by normal and Amy mice that were fed the Oz-AIN diet (4.65 ±0.15 m and 4.01 ±1.02 m respectively, $p=0.69$, Figure 9). This confirms that genotype does not have an effect on distance travelled by 15 month old normal or Amy mice before locating a buried chocolate.

A Students *t*-test between normal mice that were fed the Oz-AIN diet and Amy mice that were fed the Oz-AIN Supp diet indicated that the Amy mice that were fed the Oz-AIN Supp diet were able to locate the buried chocolate within shorter distances (4.65±0.15 m and 2.93 ±0.59 m respectively, $p=0.07$, Figure 9). While not significant, the Amy mice that were fed the Oz-AIN Supp diet also found the buried chocolate within shorter distances than Amy mice that were fed the Oz-AN diet (2.93 ±0.59 m and 4.01 ±1.02 m respectively, $p=0.39$, Figure 9). However, the purpose of these comparisons was to determine whether or not nutrient supplements prevent *genotype* effects on distance travelled (m) by 15 month old mice before locating the buried chocolate. There were no genotype effects observed in the current study, which suggests that while mice fed the Oz-AIN Supp diet performed better than other mice in the BCT, this was not to do with preventing genotype effects. The beneficial effects of the Oz-AIN Supp diet on olfactory function are discussed further in the context of the effect of diet on olfactory abilities of mice, on page 525-528.

Figure 9. The ability of nutrient supplements to prevent potential genotype effects on the distance travelled (m) by 15 month old normal and Amy mice before locating a buried piece of chocolate.

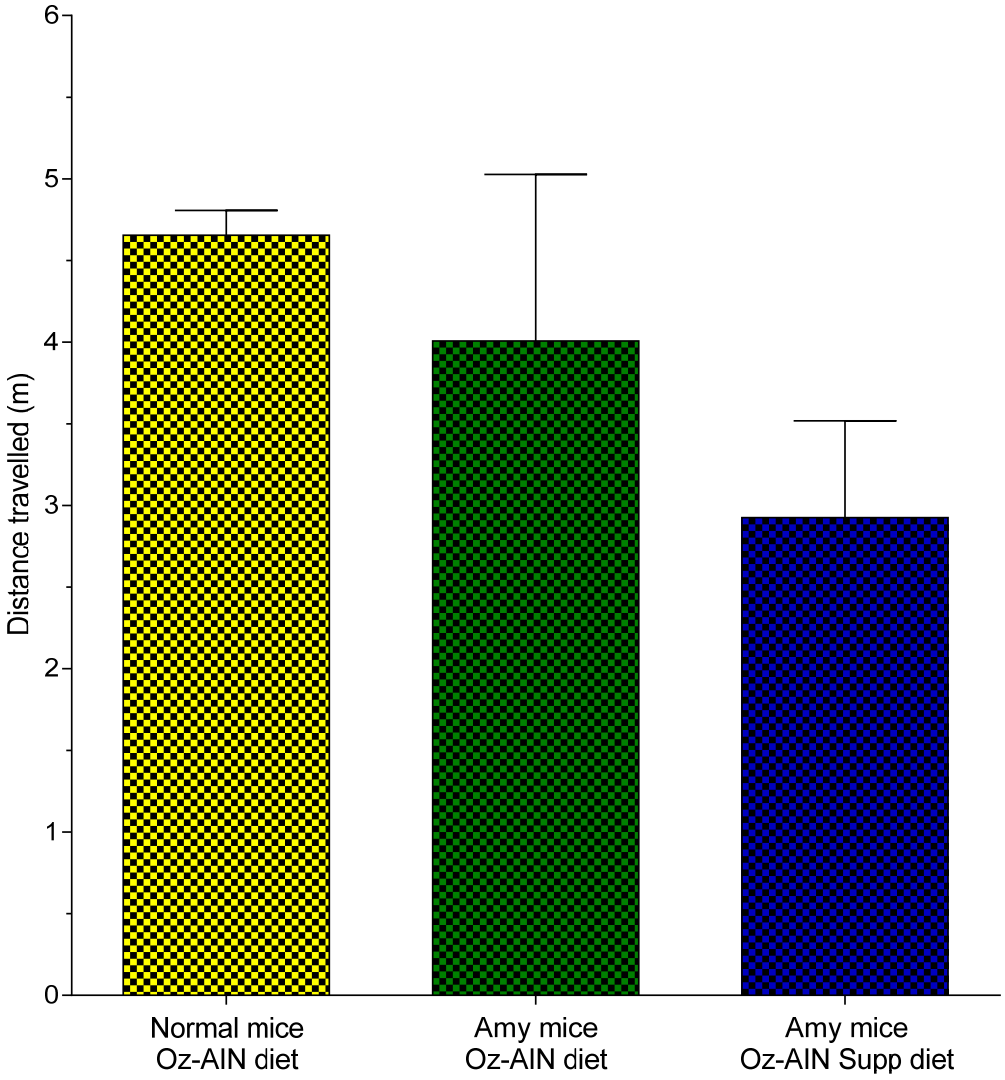


Figure 9. Normal mice fed the Oz-AIN diet (yellow bar, n=6), Amy mice fed the Oz-AIN diet (green bar, n=9), Amy mice fed the Oz-AIN Supp diet (blue bar, n=8). Bars represent mean ±SEM.

The ability of nutrient supplements to prevent genotype effects on speed (m/s).

A one-way ANOVA did not detect significant genotype effects on the average speed travelled (m/s) of normal mice that were fed the Oz-AIN diet, Amy mice that were fed the Oz-AIN diet or Amy mice that were fed the Oz-AIN Supp diet ($p=0.32$, Figure 10). Although the normal mice that were fed the Oz-AIN diet moved slower than Amy mice that were fed the Oz-AIN diet, this was not significant with a Students *t*-test (0.028 ±0.002 m/s and 0.040 ±0.006 m/s respectively, $p=0.25$, Figure 10). This confirms that there were no genotype effects on average speed travels (m/s) by 15 month old normal and Amy mice whilst searching for a buried chocolate.

The Amy mice that were fed the Oz-AIN Supp diet moved faster than the normal mice that were fed the Oz-AIN diet (0.046 ±0.008 m/s and 0.028 ±0.002 m/s respectively, $p=0.14$) and the Amy mice that were fed the Oz-AIN diet (0.046 ±0.008 m/s and 0.040 ±0.006 m/s, $p=0.56$, Figure 10). However, as suggested on page 490 this is unlikely to be due to prevention of genotype effects.

Figure 10. The ability of nutrient supplements to prevent potential genotype effects on the average speed travelled (m/s) by 15 month old Amy mice whilst searching for a buried piece of chocolate.

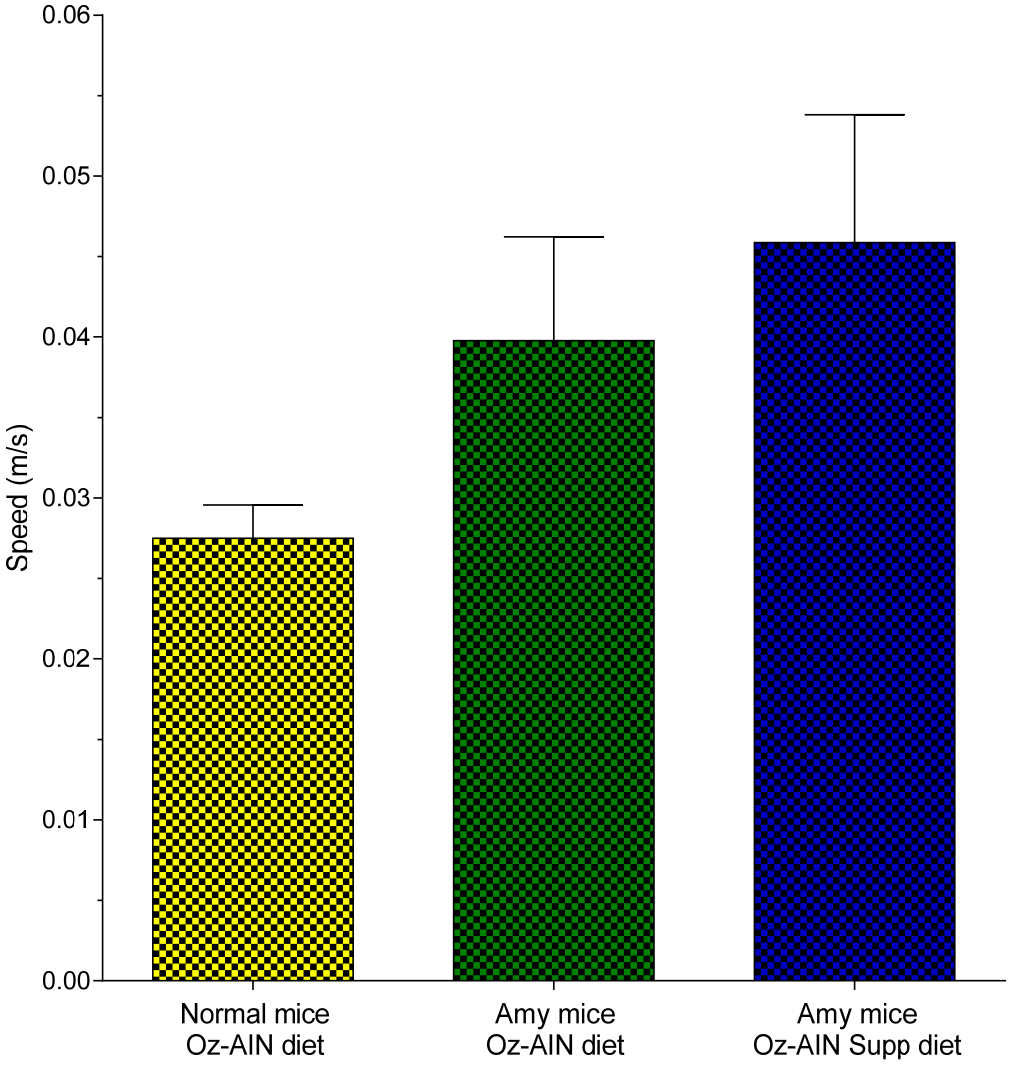


Figure 10. Normal mice fed the Oz-AIN diet (yellow bar, n=6), Amy mice fed the Oz-AIN diet (green bar, n=9), Amy mice fed the Oz-AIN Supp diet (blue bar, n=8). Bars represent mean ±SEM.

8.2.2.2. The ability of nutrient supplements to prevent diet-type effects on olfactory abilities of 12 month old mice in the Buried Chocolate Test.

The ability of nutrient supplements to prevent diet-type effects on latency (s).

A one-way ANOVA detected a significant effect of diet-type on the latency for 15 month old Amy mice to reach a buried chocolate ($p=0.03$, Figure 11). Bonferroni post tests revealed that Amy mice that were fed the AIN93-M diet located the chocolate significantly faster than Amy mice that were fed the Oz-AIN diet (35.38 ±8.37 s and 125.30 ±27.33 s respectively, $p=0.03$, Figure 11). This was confirmed with a Student's *t*-test ($p=0.006$, Figure 11). This suggests that there are dietary effects on olfactory abilities of 15 month old Amy mice, and that Amy mice that were fed the Oz-AIN diet had poorer olfactory abilities than Amy mice that were fed an optimal AIN93-M diet.

The Amy mice that were fed the Oz-AIN Supp diet located the chocolate with shorter latencies than Amy mice that were fed the Oz-AIN diet (87.45 ±27.50 s and 125.30 ±27.33 s respectively, Figure 11). While this is not significant at $p<0.05$, it would be significant at $p<0.10$. This suggests that there were trends for the nutrient supplements to prevent the detrimental effects of the Oz-AIN diet. However, the nutrient supplements did not completely prevent diet-type effects on the latencies to locate the chocolate. The Amy mice that were fed the Oz-AIN Supp diet took longer to locate the chocolate than Amy mice that were fed the AIN93-M diet (87.45 ±27.50 s and 35.38 ±8.37 s respectively, $p=0.08$). Similarly, this is not significant at $p<0.05$, but would be at $p<0.10$, suggesting that there were trends for the mice that were fed the nutrient supplements to take longer to locate the chocolate.

These data suggest that there were diet-type effects on the latency for 15 month old Amy mice to locate a buried chocolate, but that they were not completely prevented with nutrient supplements.

Figure 11. The ability of nutrient supplements to prevent potential diet-type effects on the latency (s) for 15 month old Amy mice to locate a buried piece of chocolate.

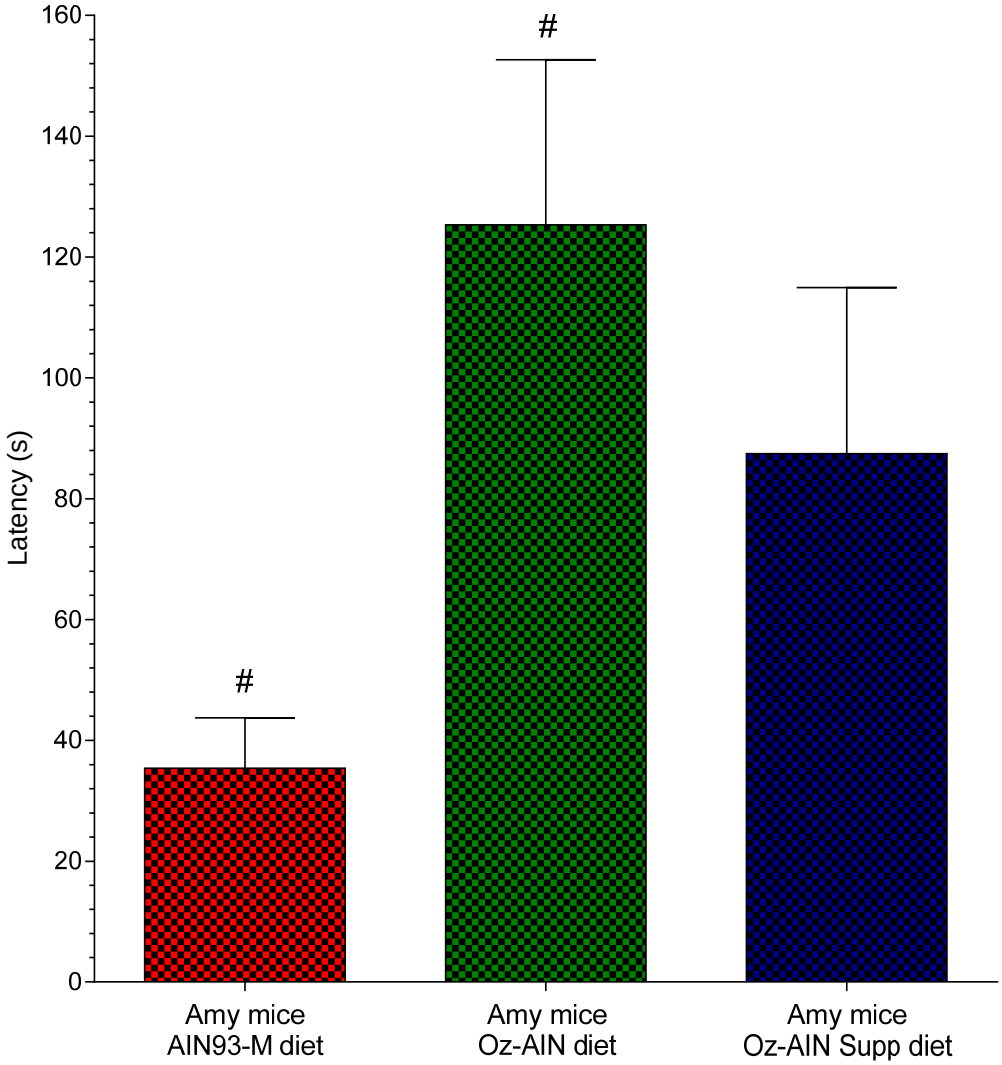


Figure 11. Amy mice fed the AIN93-M diet (red bar, n=9), Amy mice fed the Oz-AIN diet (green bar, n=9), Amy mice fed the Oz-AIN Supp diet (blue bar, n=8). Bars represent mean ±SEM. Bars with matching symbols were significantly different with Students *t*-test. (#) p=0.006.

The ability of nutrient supplements to prevent diet-type effects on distance (m).

A one-way ANOVA did not detect significant diet-type effects on distance travelled before uncovering a buried chocolate (p=0.11, Figure 12). Students *t*-test indicated that there were non-significant trends for Amy mice that were fed the Oz-AIN diet to travel further before uncovering the chocolate than Amy mice that were fed the AIN93-M diet (4.01 ±1.02 m and 1.73 ±0.48 m respectively, p=0.06, Figure 12). There were no trends to suggest that the Amy mice that were fed the Oz-AIN Supp diet travelled different distances to either the Amy mice that were fed the Oz-AIN diet (2.93 ±0.59 m and 4.01 ±1.02 m respectively, p=0.39, Figure 12) or the Amy mice that were fed the AIN93-M diet (2.93 ±0.59 m and 1.73 ±0.48 m respectively, p=0.14, Figure 12). This suggests that, while the mice that received the nutrient supplements did not have better olfactory abilities than mice that received the sub-optimal diet, they did not have poorer olfactory abilities than mice that were fed the ideal diet. Therefore, the nutrient supplements may have had a beneficial effect on distance travelled before locating the buried chocolate.

Figure 12. The ability of nutrient supplements to prevent potential diet-type effects on the distance travelled (m) by 15 month old Amy mice before locating a buried piece of chocolate.

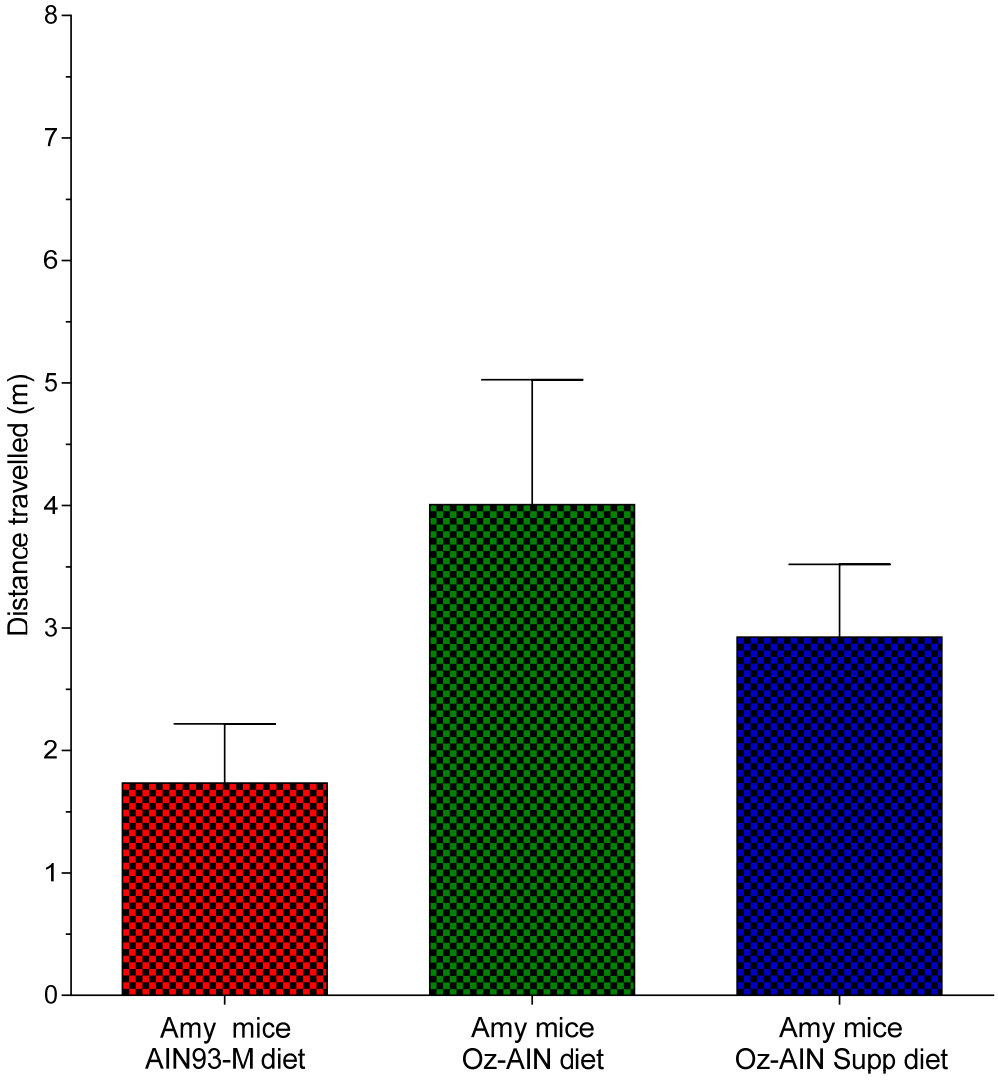


Figure 12. Amy mice fed the AIN93-M diet (red bar, n=9), Amy mice fed the Oz-AIN diet (green bar, n=9), Amy mice fed the Oz-AIN Supp diet (blue bar, n=8). Bars represent mean ±SEM.

The ability of nutrient supplements to prevent diet-type effects on speed (m/s).

A one-way ANOVA revealed that there were no significant effects of diet-type on average speeds (m/s) travelled by 15 month old Amy mice whilst looking for a buried chocolate ($p=0.68$, Figure 13). A Students *t*-test indicated that there were no differences in the average speed travelled by Amy mice that were fed the AIN93-M diet or the Oz-AIN diet (0.049 ± 0.008 m/s and 0.040 ± 0.006 m/s respectively, $p=0.40$, Figure 13). This suggests that the Oz-AIN diet does not have a detrimental effect on speed travelled whilst searching for a buried chocolate.

Similarly, Students *t*-test revealed that the Amy mice that were fed the Oz-AIN Supp diet did not travel at speeds that were significantly different to either the Amy mice that were fed the AIN93-M diet ($p=0.77$, Figure 13) or Amy mice that were fed the Oz-AIN diet ($p=0.55$, Figure 13). This demonstrates that the nutrient supplements did not have an effect on average speeds travelled by 15 month old Amy mice whilst searching for the buried chocolate.

The finding that there were no differences in the speeds travelled by 15 month old Amy mice that were fed the AIN93-M diet, the Oz-AIN diet or the Oz-AIN Supp diet indicate that the comparisons of latency (s) and distance travelled (m) are true reflections of olfactory abilities of Amy mice, and have not been confounded by factors such as mobility.

Figure 13. The ability of nutrient supplements to prevent potential diet-type effects on the average speed travelled (m/s) by 15 month old Amy mice whilst searching for a buried chocolate.

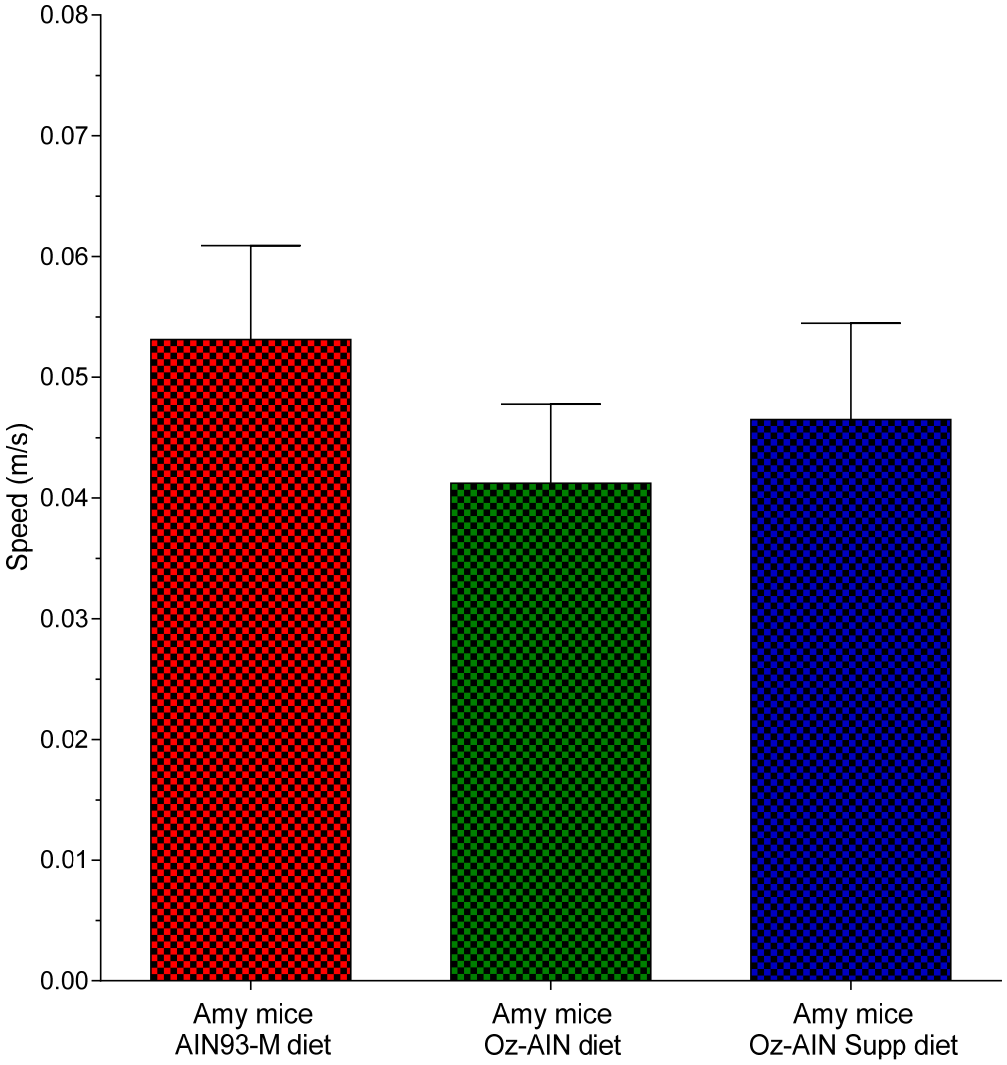


Figure 13. Amy mice fed the AIN93-M diet (red bar, n=9), Amy mice fed the Oz-AIN diet (green bar, n=9), Amy mice fed the Oz-AIN Supp diet (blue bar, n=8). Bars represent mean ±SEM.

8.2.3. Comparison of the performances of 6 and 15 month old mice in the Buried Chocolate Test.

Section 8.2.1 described the potential preventative effects of nutrient supplements on the genotype and diet-type effects on olfactory abilities of 6 month old mice. Section 8.2.2 described the potential preventative effects of nutrient supplements against the genotype and diet-type effects on olfactory function in 15 month old mice. The current section investigates the changes that occur with aging. First, comparisons are made *within groups* to determine whether or not aging from 6 to 15 months has an effect on olfactory abilities of mice. Then a comparison of the *change* of olfactory abilities when mice age from 6 to 15 months is made *between groups*. Diet-type and genotype effects on change in latency (s), distance travelled (m) or average speeds (m/s) are described in the *between groups* comparisons.

8.2.3.1. The effect of aging from 6 to 15 months on change in latency (s), distance (m) and average speed (m/s) before uncovering a buried chocolate.

Latency (s).

Irrespective of diet or genotype, all mice took longer to locate the buried chocolate at 15 months, compared to their performance at 6 months old (Figure 14). Paired Student *t*-tests revealed that this was significant for normal mice that were fed the Oz-AIN diet (170.50 ±9.50 s and 25.27 ±5.36 s respectively, p=0.0005, Figure 14), Amy mice that were fed the Oz-AIN diet (125.30 ±27.33 s and 31.76 ±7.22 s respectively, p=0.006, Figure 14) and Amy mice that were fed the Oz-AIN Supp diet (87.45 ±27.50 s and 16.20 ±3.90 s respectively, p=0.03, Figure 14). However, the increase in latency to locate the buried chocolate when Amy mice that were fed the AIN93-M

diet aged from 6 to 15 months was not significant (20.64 ±2.69 s and 35.38 ±8.37 s respectively, p=0.11, Figure 14). This indicates that age did not impair olfactory abilities of Amy mice that were fed the AIN93-M diet. The AIN93-M diet was the only diet that did not contain high levels of fat, which suggests that diet may have had an effect on age-related olfactory dysfunction. This is explored further in the comparisons of the change in olfactory abilities with aging *between* dietary groups (pp. 516-520).

Figure 14. A comparison of the latency (s) to uncover a buried chocolate by 6 and 15 month old normal and Amy mice.

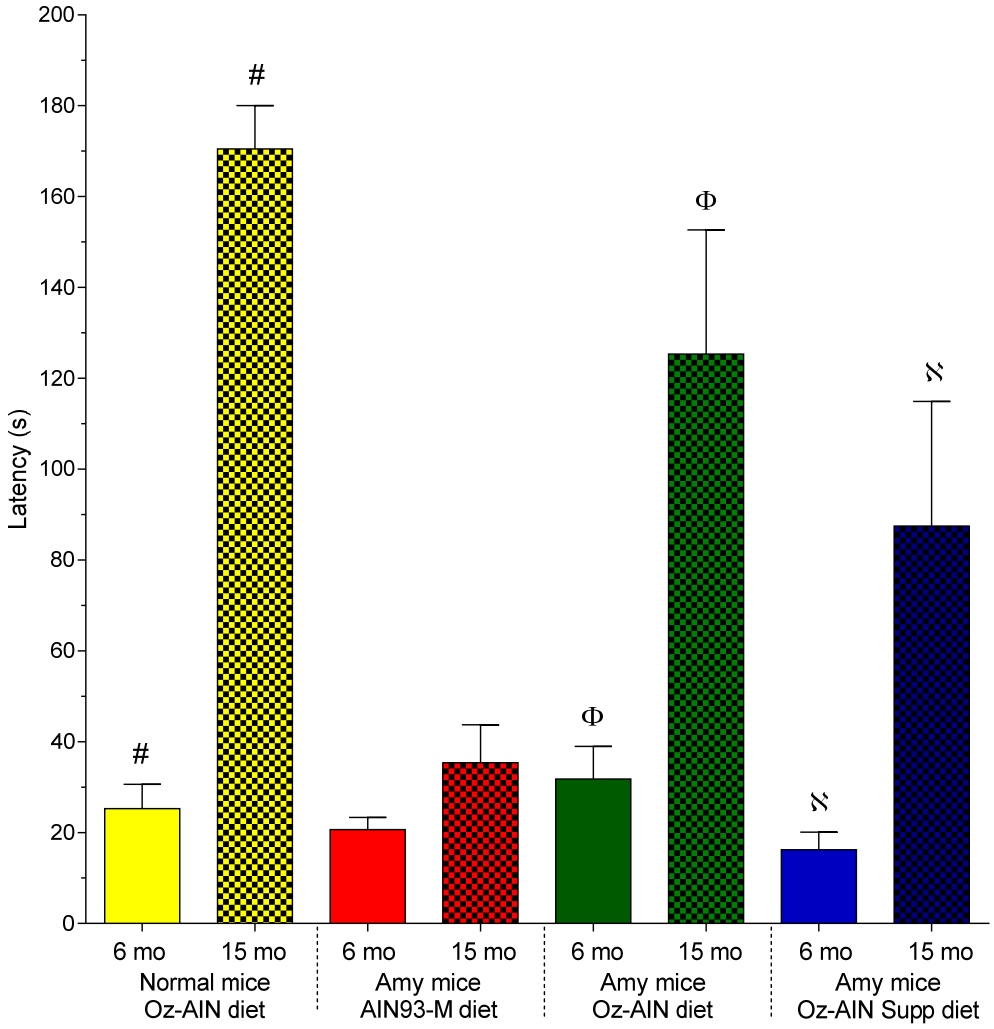


Figure 14. A comparison of the latency (s) to uncover a buried piece of chocolate between 6 month (solid bars) and 15 month old (checked bars) mice. Normal mice fed the Oz-AIN diet (yellow solid and checked bars, n=6), Amy mice fed the AIN93-M diet (red solid and checked bars, n=9), Amy mice that were fed the Oz-AIN diet (green solid and checked bars, n=9), and Amy mice that were fed the Oz-AIN Supp diet (blue solid and checked bars, n=8). Bars are mean ±SEM. Bars with matching symbols were significantly different with paired Students *t*-test. (#) p=0.0005. (Φ) p=0.006. (ϰ) p=0.03

Distance travelled (m).

A paired students *t*-test revealed that the normal mice that were fed the Oz-AIN diet travelled significantly further before locating the chocolate at 15 months compared to 6 months (4.65 ±0.15 m and 2.71 ±0.53 m respectively, p=0.03, Figure 15). Similarly, the Amy mice that were fed the Oz-AIN Supp diet travelled significantly further at 15 months compared to 6 months (2.93 ±0.59 m and 1.29 ±0.49 m respectively, p=0.02, Figure 15). This indicates that age had an effect on the olfactory abilities of the normal mice that were fed the Oz-AIN diet and Amy mice that were fed the Oz-AIN Supp diet.

While the Amy mice that were fed the Oz-AIN diet may have taken significantly longer to locate the buried chocolate when they were 15 months old compared to when they were 6 months old (p=0.006), they did not significantly increase distance travelled (4.01 ±1.02 m compared to 3.03 ±0.55 m respectively, p=0.41, Figure 15). This suggests that age may not have impaired olfactory abilities of Amy mice that were fed the Oz-AIN diet.

The distances travelled at 6 and 15 months were not significantly different for the Amy mice that were fed the AIN93-M diet (1.99 ±0.21 m and 1.73 ±0.48 m respectively, p=0.64, Figure 15). Taken with the data describing the change in latency with aging, this suggests that aging did not have an effect on olfactory abilities of Amy mice that were fed the AIN93-M diet.

Figure 15. A comparison of the distance travelled before uncovering a buried chocolate between 6 and 15 month old normal and Amy mice.

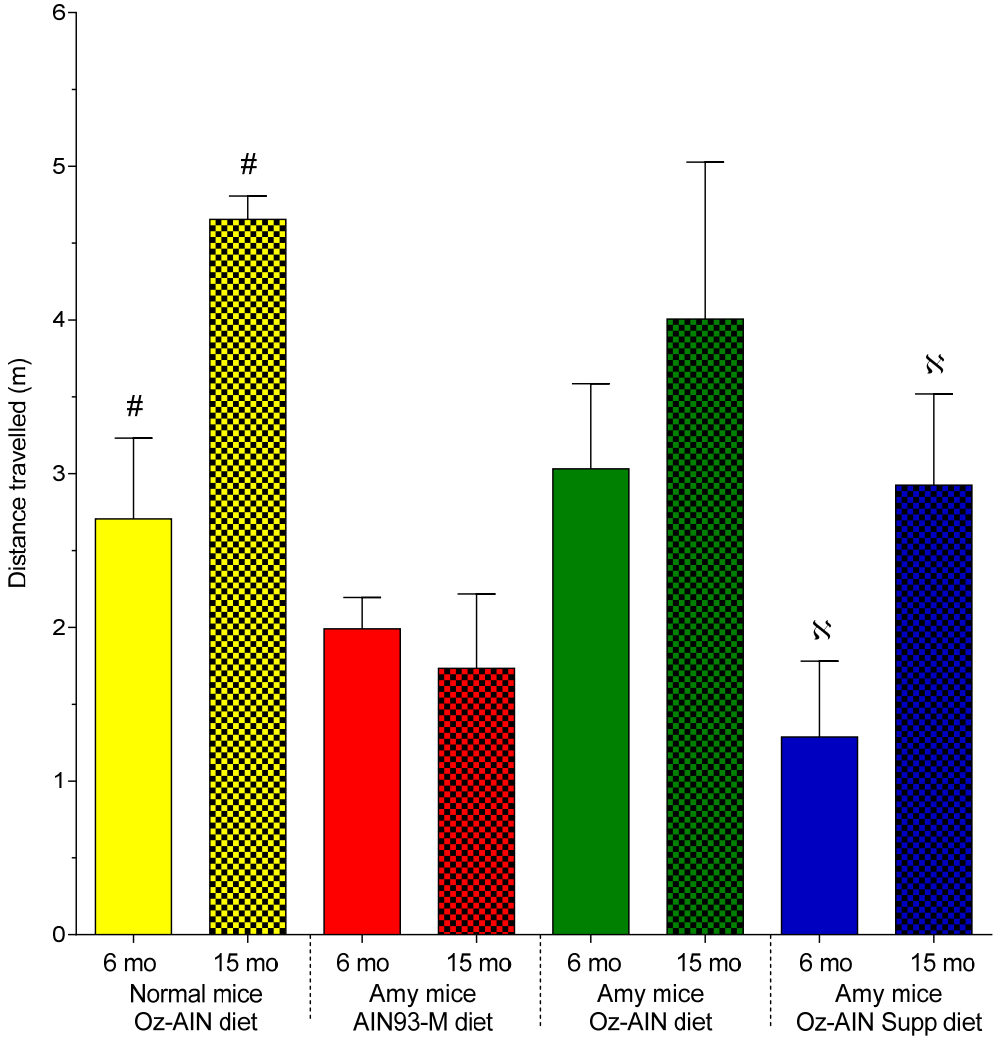


Figure 15. A comparison of the distance travelled (m) before uncovering a chocolate between 6 month old (solid bars) and 15 month old (checked bars) normal and Amy mice. Normal mice fed the Oz-AIN diet (yellow solid and chequered bars, n=6), Amy mice fed the AIN93-M diet (red solid and checked bars, n=9), Amy mice that were fed the Oz-AIN diet (green solid and checked bars, n=9), and Amy mice that were fed the Oz-AIN Supp diet (blue solid and checked bars, n=8). Bars are mean ±SEM. Bars with matching symbols were significantly different with paired Students *t*-test. (#) p=0.03. (ϰ) p=0.02.

Average speed travelled (m/s).

While all groups travelled slower at 15 months compared to 6 months, this was not significant for Amy mice that were fed the Oz-AIN Supp diet (0.061 ±0.013 m/s and 0.047 ±0.008 m/s respectively, p=0.12, Figure 16). The finding that the Amy mice that were fed the Oz-AIN Supp diet moved at comparable speeds when they were 6 and 15 months indicates that the change in latency (s) and distance travelled (m) reflects change in olfactory ability, rather than change in confounding factors such as mobility.

Speeds travelled at 15 months were significantly slower than those travelled at 6 months for the normal mice that were fed the Oz-AIN diet (p=0.003, Figure 16), Amy mice that were fed the Oz-AIN diet (p=0.0005, Figure 16) and Amy mice that were fed the AIN93-M diet (p=0.002, Figure 16). These significant changes with aging indicate that other age-related changes, such as mobility, may have had an effect on latency to locate the buried chocolate (Figure 14). However, mobility does not account for the significant increases of distance travelled (Figure 15). Therefore, these data may still be interpreted as reflecting change in olfactory abilities with age.

Figure 16. A comparison of the average speed travelled (m/s) by normal and Amy mice at 6 and 15 months whilst searching for a buried chocolate.

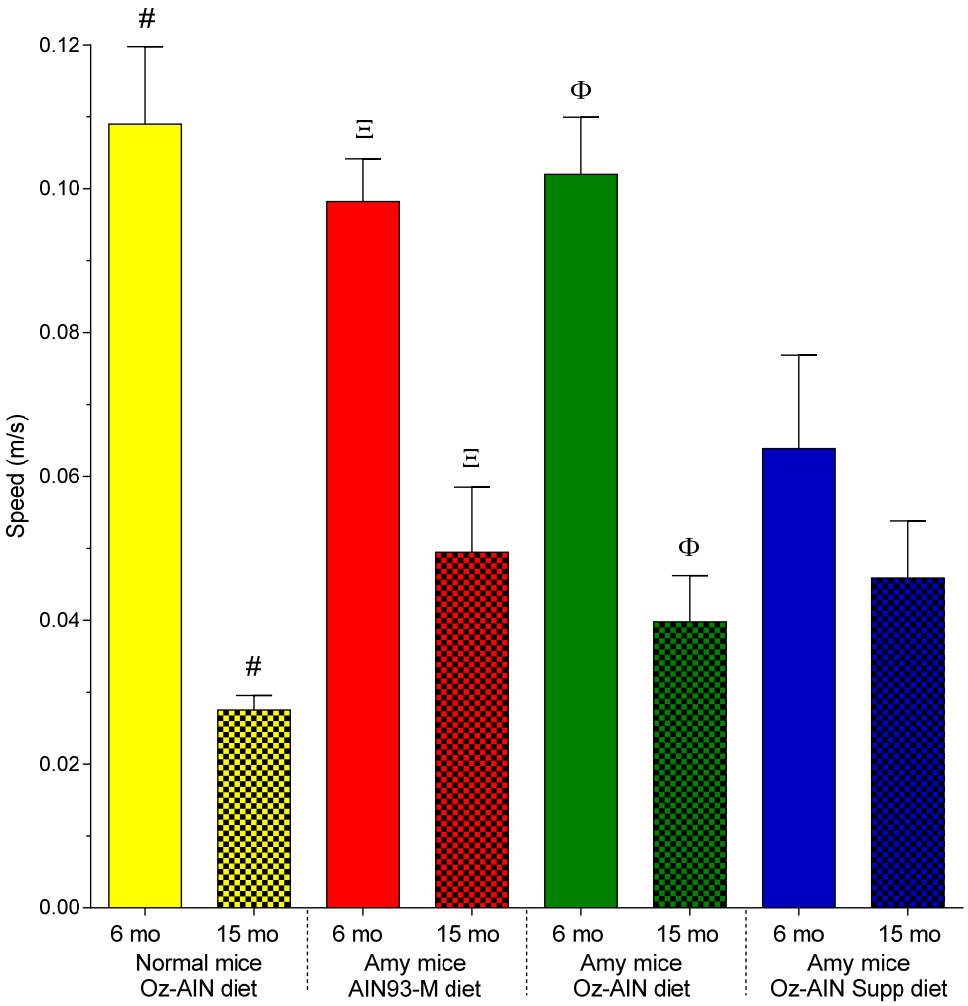


Figure 16. A comparison of the average speed travelled (m/s) by normal and Amy mice at 6 months old (solid bars) and 15 months old (checked bars) whilst searching for a buried chocolate. Normal mice fed the Oz-AIN diet (yellow solid and checked bars, n=6), Amy mice fed the AIN93-M diet (red solid and checked bars, n=9), Amy mice that were fed the Oz-AIN diet (green solid and checked bars, n=9), and Amy mice that were fed the Oz-AIN Supp diet (blue solid and checked bars, n=8). Bars are mean ±SEM. Bars with matching symbols were significantly different with Students *t*-test. (#) p=0.003. (Ξ) p=0.002. (Φ) p=0.0005.

8.2.3.2. The effects of genotype on the changes in latency (s), distance travelled (m) and average speed travelled (m/s) whilst searching for a chocolate at 6 and 15 months.

Two different measures were used to make comparisons *between* groups to determine the effect of age on latency (s), distance travelled (m) and speed (m/s). The absolute change of latency (s), distance (m) or speed (m/s) was calculated to provide an evaluation of the size of the change of each variable with age. However, the significant differences in some of these measured at 6 months (see section 7.2.1.) indicates that some mice started with better olfactory abilities than other mice. The consequence of this is that comparisons of change with aging have been made relative to different baselines, which may mask real differences between groups. To overcome this limitation, the change of each variable with aging has also been examined as a variable of 15 months compared to 6 months. These two measured enabled a comparison of the absolute change in latency (s), distance (m) and speed (m/s) as mice aged, and also enabled comparison of the degree of change relative to how mice performed at 6 months of age.

Latency (s).

One-way ANOVA's indicated that there was no effect of genotype on the absolute change in latency as mice aged from 6 to 15 months ($p=0.23$, Table 2) or the ratio of change in latency to the chocolate ($p=0.59$, Table 2). However, the normal mice that were fed the Oz-AIN diet increased latency to the chocolate by 145.20 ±8.96 s, which was almost twice that of Amy mice that were fed the Oz-AIN Supp diet (71.25 ±27.29 s, $p=0.09$, Students *t*-test, Table 2) or the Amy mice that were fed the Oz-AIN diet (76.56 ±23.64 s, $p=0.10$, Students *t*-test, Table 2). This suggests that the

normal mice that were fed the Oz-AIN diet may have been more affected by aging than Amy mice in terms of latency to uncover the buried chocolate.

The latencies for the 15 month old Amy mice that were fed the Oz-AIN diet to uncover the chocolate were 4.95 ±1.52 times greater than when they were 6 months old. The 15 month old normal mice that were fed the Oz-AIN diet took 7.41 ±1.14 times longer to uncover the buried chocolate compared to when they were 6 months (Table 2). This suggests that the normal mice that were fed the Oz-AIN diet may be more susceptible to age-related effects on latency to uncover the buried chocolate than Amy mice. However, there was no significant difference in the ratios of the change in latency between normal mice or Amy mice that were fed the Oz-AIN diet, suggesting that there were no real genotype effects on the variation of latency to uncover a buried chocolate as Amy mice aged from 6 to 15 months.

Table 2. Genotype effects on the difference and ratios of the change in the latency (s) for normal and Amy mice to uncover the buried chocolate at 6 and 15 months.

	Normal mice Oz-AIN diet	**Amy mice Oz-AIN diet**	**Amy mice Oz-AIN Supp diet**
6 months (mean latency)	25.27 ±5.36	31.76 ± 7.22	16.20 ±3.90
15 months (mean latency)	170.50 ±9.50	125.30 ±7.22	87.45 ±27.50
Difference (s)	**-145.20 ±8.96**	**-76.56 ±23.64**	**-71.25 ±27.29**
Ratio (15 : 6)	**7.41 ±1.14**	**4.95 ±1.52**	**7.49 ±2.54**

All numbers are mean ±SEM. Ratios are a comparison of latency at 15 months relative to 6 months. Differences and ratios were calculated individually for each mouse and averaged to determine mean ±SEM.

Distance travelled (m).

A one-way ANOVA did not detect significant differences in the absolute difference (p=0.77, Table 3) or the ratio (15:6 mo, p=0.10, Table 3) describing the change of distance travelled by mice with aging.

Student's *t*-tests did not detect differences between normal and Amy mice that were fed the Oz-AIN diet. The size of the change in distance travelled at 6 and 15 months for the Amy mice that were fed the Oz-AIN diet was similar to that of normal mice that were fed the Oz-AIN diet (0.97 ±1.12 and -1.95 ±0.48 m respectively, p=0.59, Table 3). Furthermore, the Amy mice that were fed the Oz-AIN diet and the normal mice that were fed the Oz-AIN diet increased distance travelled with similar ratios (1.76 ±0.68 and 1.89 ±0.32, p=0.90, Table 3). This suggests that there were no genotype effects on age-associated changes in distance travelled before locating a buried piece of chocolate.

Table 3. Genotype effects on the difference and ratio of the change in distance travelled before locating the buried chocolate at 6 and 15 months.

	Normal mice Oz-AIN diet	**Amy mice Oz-AIN diet**	**Amy mice Oz-AIN Supp diet**
6 months (mean distance)	2.71 ±0.53	3.03 ±0.55	1.29 ±0.49
15 months (mean distance)	4.65 ±0.15	4.01 ±1.02	2.93 ±0.59
Difference (m)	**-1.95 ±0.48**	**-0.97 ±1.12**	**-1.64 ±0.57**
Ratio (15 : 6)	**1.89 ±0.32**	**1.76 ±0.68**	**5.79 ±2.04**

All numbers are mean ±SEM. Ratios are a comparison of distance travelled at 15 months relative to 6 months. Differences and ratios were calculated individually for each mouse and averaged to determine mean ±SEM.

Average speed (m/s).

A one-way ANOVA detected significant differences in the overall change in speeds travelled by normal mice that were fed the Oz-AIN diet, Amy mice that were fed the Oz-AIN diet and Amy mice that were fed the Oz-AIN Supp diet ($p=0.004$, Table 4). However, Bonferroni multiple comparison tests did not detect significant differences between normal mice and Amy mice that were fed the Oz-AIN diet, suggesting that genotype did not affect age-associated change in speeds travelled ($p=0.87$, Table 4). Bonferroni post tests did detect significant differences in the change in speeds travelled by Amy mice that were fed the Oz-AIN Supp diet and either normal mice that were fed the Oz-AIN diet ($p=0.007$, Table 4) or Amy mice that were fed the Oz-AIN diet ($p=0.02$, Table 4). These comparisons were also significant using Students - *t*-tests (Table 4). This suggests that there may have been diet-type effects on the overall change in speeds travelled with aging.

A one way ANOVA revealed that the ratios of speeds at 15 months compared to 6 months were also significantly different between normal mice that were fed the Oz-AIN diet, Amy mice that were fed the Oz-AIN diet and Amy mice that were fed the Oz-AIN Supp diet ($p=0.009$, Table 4). Neither Bonferroni post tests, nor Student *t*-tests detected significant differences between the normal mice and the Amy mice that were fed the Oz-AIN diet ($p>0.99$ and $p=0.87$ respectively). This suggests that there were no genotype effects on the change in speed travelled between 6 and 15 months old.

Bonferroni post tests indicated that the Amy mice that were fed the Oz-AIN Supp diet had a significantly smaller ratio than either the normal mice that were fed the Oz-AIN diet ($p=0.02$, Table 4) or Amy mice that were fed the Oz-AIN diet ($p=0.03$, Table 4). These comparisons were also significant using Students *t*-tests (Table 4).

This indicates that the effect of aging on average speeds travelled by Amy mice that were fed the Oz-AIN Supp diet was smaller than that for normal mice of Amy mice that were fed the Oz-AIN diet.

Table 4. Genotype effects of the difference and ratio of the change in average speed travelled (m/s) before uncovering the buried chocolate at 6 and 15 months.

	Normal mice Oz-AIN diet	**Amy mice Oz-AIN diet**	**Amy mice Oz-AIN Supp diet**
6 months (mean speed)	0.109 ±0.011	0.102 ±0.0008	0.064 ±0.013
15 months (mean speed)	0.028 ±0.002	0.040 ±0.006	0.046 ±0.008
Difference (m/s)	**0.082 ±0.009** $^{\Xi}$	**0.062 ±0.011** $^{\Delta}$	**0.018 ±0.010** $^{\Xi, \Delta}$
Ratio (15 : 6)	**0.25 ±0.01** $^{\#}$	**0.41 ±0.08** $^{\Theta}$	**0.89 ±0.17** $^{\#, \Theta}$

All numbers are mean ±SEM. Ratios are a comparison of speed at 15 months relative to 6 months. Differences and ratios were calculated individually for each mouse and averaged to determine mean ±SEM. Statistical comparisons are made across rows. Numbers with matching symbols are significantly different with Students *t*-tests. (Ξ) p=0.003. (Δ) p=0.02. (Θ) p=0.02. (#) p=0.03.

8.2.3.3. The effects of diet-type on the changes in latency (s), distance travelled (m) and average speed travelled (m/s) whilst searching for a chocolate at 6 and 15 months.

Latency (s).

A one-way ANOVA did not detect significant diet-type effects on age-associated changes in latency to uncover the buried chocolate (p=0.23, Table 5). However, there were large differences between groups that may still be meaningful. The Amy mice that were fed the AIN93-M diet increased the latency to uncover the chocolate by 18.86 ±8.09 s as they aged from 6 to 15 months (Table 5). This was less than a third of the increases in latency of the Amy mice that were fed the Oz-AIN diet (-76.56

±23.64 s, p=0.06 with Student's *t*-test, Table 5) or the Amy mice that were fed the Oz-AIN Supp diet (-71.25 ±27.29 s, p=0.09 with Student's *t*-test, Table 5). While this is not significant at $p<0.05$, it is significant at $p<0.10$. This indicates that weak trends may have existed.

The 15 month old Amy mice that were fed the ideal AIN03-M diet took 1.86 ±0.42 times longer to locate the buried chocolate compared to when they were 6 months old. The Amy mice that were fed the Oz-AIN diet or the Oz-AIN Supp diet increased latency by 4.95 ±1.52 and 7.49 ±2.54 times respectively (Table 5). While comparisons between these differences were not significant with a one-way ANOVA (p=0.08), a Student's *t*-test revealed that this was significant between Amy mice that were fed the AIN93-M diet and Amy mice that were fed the Oz-AIN Supp diet (p=0.03, Table 5).

Collectively, these data suggest that diet may have an effect on the age-associated changes in the latency for Amy mice to locate a chocolate. Specifically, mice that were fed an optimal diet appeared to be protected against age-associated changes in latency to the chocolate, whilst Amy mice that were fed nutrient supplements were more severely affected.

Table 5. Diet-type effects on the difference and ratio of the change in latency (s) for Amy mice to uncover the buried chocolate at 6 and 15 months.

	Amy mice AIN93-M diet	**Amy mice Oz-AIN diet**	**Amy mice Oz-AIN Supp diet**
6 months (mean latency)	20.64 ±2.69	31.76 ± 7.22	16.20 ±3.90
15 months (mean latency)	35.38 ±8.37	125.30 ±7.22	87.45 ±27.50
Difference (s)	**-18.86 ±8.09**	**-76.56 ±23.64**	**-71.25 ±27.29**
Ratio (15 : 6)	**1.86 ±0.42$^{\Phi}$**	**4.95 ±1.52**	**7.49 ±2.54 $^{\Phi}$**

All numbers are mean ±SEM. Ratios are a comparison of latency at 15 months relative to 6 months. Differences and ratios were calculated individually for each mouse and averaged to determine mean ±SEM. Statistical comparisons are made across rows. Numbers with matching symbols were significantly different with Students *t*-tests. (Φ) p=0.03.

Distance travelled (m).

A one-way ANOVA did not detect significant diet-type effects on age-associated changes in distance travelled before locating a buried chocolate (p=0.26, Table 6). However, a Students *t*-test revealed that the difference between distances travelled at 6 and 15 months was significantly smaller for Amy mice that were fed the AIN93-M diet compared to Amy mice that were fed the Oz-AIN Supp diet (p=0.02, Table 6). This suggests that the AIN93-M diet may be protective against the age-associated increase in distance travelled or that the nutrient supplements may enhance age-associated impairments.

A one-way ANOVA revealed that there were significant diet-type effects on the ratios of distance travelled at 15 months compared to 6 months (p=0.02, Table 6). Bonferroni post tests revealed that these variances could be attributed to Amy mice that were fed the Oz-AIN Supp diet having significantly higher ratios than Amy mice that were fed the AIN93-M diet (p=0.03, Table 6). The Amy mice that were fed the

Oz-AIN Supp diet also had a higher ratio of change than Amy mice that were fed the Oz-AIN diet (Table 6). However, this was not detected as significant by either Bonferroni tests (p=0.07) or Students *t*-tests (p=0.07).

Collectively, these data suggest that the Oz-AIN Supp diet had a detrimental effect on the age-associated change in distance travelled and on the ratio of distances travelled at 6 and 15 months before locating the buried chocolate.

Table 6. Diet-type effects on the difference and ratio of the distance travelled (m) before Amy mice uncovered the buried chocolate at 6 and 15 months.

	Amy mice AIN93-M diet	**Amy mice Oz-AIN diet**	**Amy mice Oz-AIN Supp diet**
6 months (mean distance)	1.99 ±0.21	3.03 ±0.55	1.29 ±0.49
15 months (mean distance)	1.73 ±0.48	4.01 ±1.02	2.93 ±0.59
Difference (m)	**0.26 ±0.53** $^{\Xi}$	**-0.97 ±1.12**	**-1.64 ±0.57** $^{\Xi}$
Ratio (15 mo : 6 mo)	**1.01 ±0.28** $^{\Phi}$	**1.76 ±0.68**	**5.79 ±2.04** $^{\Phi}$

All numbers are mean ±SEM. Ratios are a comparison of distance travelled at 15 months relative to 6 months. Differences and ratios were calculated individually for each mouse and averaged to determine mean ±SEM. Statistical comparisons are made across rows. Numbers with matching symbols are significantly different with Students *t*-tests. (Ξ) p=0.02. (Φ) p=0.03.

Average speed (m/s).

A one-way ANOVA indicated that there were significant diet effects on age-associated changes in speed travelled whilst searching for the chocolate ($p=0.03$, Table 7). Bonferroni post tests revealed that there were no significant differences in overall change in speed of Amy mice that were fed either the AIN93-M diet or the Oz-AIN diet ($p>0.99$, Table 7). This suggests that the diet-effect on age-associated change in speed travelled is not attributed to the high-fat content of the Oz-AIN diet. However, Bonferroni post tests revealed that the Amy mice that were fed the Oz-AIN Supp diet made a significantly smaller decline in speed travelled with aging than the Amy mice that were fed the Oz-AIN diet ($p=0.03$, Table 7). This suggests that the nutrient supplements were able to alleviate age-associated decline in speed.

There were similar differences observed between groups for the ratios of the change in speed at 6 and 15 months. A one-way ANOVA detected that there were significant diet-type effects on ratios ($p=0.03$, Table 7), and Bonferroni multiple comparisons tests revealed that this was due to significant differences between Amy mice that were fed the Oz-AIN diet or the Oz-AIN Supp diet ($p=0.03$, Table 7). However, there were no significant differences in the ratios of change in speed between Amy mice that were fed the Oz-AIN diet or the AIN93-M diet. These patterns were also observed through comparisons with Student *t*-tests.

Collectively, these data suggest that a high-fat diet does not affect change in speed or the ratio of speed at 6 and 15 months, and that nutrient supplements may protect against age-associated decline in speed travelled.

Table 7. Diet-type effects on the difference and ratio of average speed (m/s) before Amy mice uncovered the buried chocolate at 6 and 15 months.

	Amy mice AIN93-M diet	Amy mice Oz-AIN diet	Amy mice Oz-AIN Supp diet
6 months (mean distance)	0.098 ±0.006	0.102 ±0.008	0.064 ±0.013
15 months (mean distance)	0.049 ±0.009	0.040 ±0.006	0.046 ±0.008
Difference (m/s)	**0.049 ±0.11**	**0.062 ±0.011** $^{\Phi}$	**0.018 ±0.010** $^{\Phi}$
Ratio (15 : 6)	**3.40 ±0.15**	**3.47 ±0.79**	**1.50 ±0.31**

All numbers are mean ±SEM. Ratios are a comparison of mean speed at 15 months relative to 6 months. Differences and ratios were calculated individually for each mouse and averaged to determine mean ±SEM. Statistical comparisons are made across rows. Numbers with matching symbols were significantly different with Students *t*-tests. (Φ) p=0.01.

8.3. Discussion.

Two studies that address olfactory dysfunction in Amy mice have been presented in this thesis. The first study focussed on the potentially detrimental effects of genotype and the Oz-AIN diet on olfactory function in normal and Amy mice, and was presented in Chapter 7. The second study, which has been presented in the current chapter, has focussed on the potentially protective effects of nutrient supplements against olfactory dysfunction in Amy mice that have been fed the Oz-AIN diet.

The potentially beneficial effect of nutrient supplements against genotype effects on olfactory abilities of Amy mice.

Consistent with the first aim of this study, the potential preventative effect of nutrient supplements on the genotype effect on olfactory function in normal and Amy mice was investigated at 6 and 15 months of age.

Olfactory decline is one of the earliest behavioural deficits to occur in AD [504, 528, 529], and olfactory deficits in Amy mice have been reported to present by 3 months, long before cognitive deficits occur [122, 518]. However, in the current study there were no genotype effects on the olfactory abilities of normal and Amy mice at either 6 or 15 months of age. While this was unexpected, there have been similar reports from other studies [123, 508].

In a longitudinal study of olfactory abilities, Phillips *et al.* compared *tau* transgenic mice, mice over-expressing amyloid precursor protein and normal mice [508]. Phillips *et al.* report that even by 18 months of age, there were no significant differences in the olfactory performances of mice [508]. Phillips *et al.* suggest that either the AD mouse model or olfactory test used could account for the discrepancies between their results and reports from others [508]. Neither of the AD-mouse models assessed by Phillips *et al.* develop β-amyloid deposits in the olfactory bulb [508], whilst mice that have demonstrated olfactory deficits do develop β-amyloid deposits throughout the olfactory bulbs prior to β-amyloid deposits in the hippocampus or cortex [505, 518]. This suggests that olfactory deficits are caused by β-amyloid deposition and neuronal loss in the olfactory tract [508], which has been proposed by others [518]. On the other hand, behavioural tests where olfactory deficits have been demonstrated involved motivation, digging or motor skills, which may all also be impaired by age [525]. If this has occurred, it suggests that age-associated changes that are not related to olfactory abilities may have confounded results, and in fact, AD-mice do not develop olfactory dysfunction.

However, neither of the suggestions proposed by Phillips *et al.* can fully account for the discrepancies between the current study and those of others that have reported olfactory dysfunction. While β-amyloid load or neuronal cell counts were not

measured specifically in the olfactory bulbs and tract of the Amy mice in the current study, there are reports from others that AD-type mice that carry the same mutations as Amy mice develop β-amyloid deposits in the olfactory bulb [120]. Furthermore, the BCT that was used to assess olfactory abilities in the current study required mice to dig, and may therefore have confounded the current findings.

The absence of a genotype effect on olfactory abilities contrasts with the finding of Chapter 7. Chapter 7 proposed that the genotype-induced effects on olfactory dysfunction in Amy mice that were fed the Oz-AIN diet may have been due to β-amyloid accumulation and subsequent neurodegeneration in regions of the brain that are associated with olfaction, which may have been accelerated by the high-fat content of the Oz-AIN diet. If this was the case, it is possible that that the high fat content of the Oz-AIN diet may have also had a detrimental effect on olfactory abilities of normal mice, which would mask any genotype effects that did exist. However, β-amyloid deposition and cell counts in the olfactory bulbs of Amy mice were not measured in this project, and need to be carried out before these conclusions can be made.

It was hypothesised that the genotype effect on olfactory dysfunction could be prevented with nutrient supplements. This was based on reports from others that nutrient supplements that have been used in the present study, such as docosahexaenoic acid in fish oil, B vitamins, and polyphenolic compounds have protective effects on other behavioural characteristics that are specific to AD [202, 488]. No significant genotype-effects on olfactory functioning have been reported in the current study, and therefore no such prevention can be offered by nutrient supplements.

However the Amy mice that were fed the Oz-AIN Supp performed better than normal mice that were fed the Oz-AIN diet at 6 and 15 months of age. This suggests that while genotype-effects of olfactory abilities between normal and Amy mice that were fed the Oz-AIN diet were not clearly demonstrated, the nutrient supplements somehow put Amy mice at an advantage so that they had better olfactory abilities than normal mice.

Adjei *et al.* report that neuronal populations change in size and number in the olfactory tubercle as a normal component of aging, and that this may be the cause of aging-related changes in olfactory abilities [530]. Therefore, for the Oz-AIN Supp diet to have enabled Amy mice to have better olfactory abilities than normal mice, supplements may have prevented neuronal loss in regions that naturally deteriorate with age, such as the olfactory tubercle. Douaud *et al.* reported that B vitamin supplements for 2 years prevented grey matter atrophy in brain regions that were particularly susceptible to degeneration in AD, and that this was potentially a result of reducing homocysteine levels [531]. This offers a potential mechanism through which the Oz-AIN Supp diet may have prevented neuronal loss in Amy mice, and enabled olfactory abilities that were greater than those of normal mice. Potentially, the B vitamins supplements in the Oz-AIN Supp diet (vitamin B12 and folate), reduced homocysteine levels and prevented neuronal loss in the olfactory bulbs of Amy mice.

Alternately, the beneficial effects of the Oz-AIN Supp diet may have been mediated through curcumin and docosahexaenoic acid. Both of these nutrients reduce β-amyloid deposition and β-amyloid-induced insulin resistance in triple transgenic AD-type mice [217]. It is possible that these compounds reduced β-amyloid deposition in the olfactory bulbs of Amy mice. Curcumin and docosahexaenoic acid may have also

prevented diet-induced obesity, which has been linked to anosmia [121, 512]. The consequence of this would be that the normal mice that were fed the Oz-AIN diet would have gained more weight than Amy mice that were fed the Oz-AIN Supp diet, and may therefore be more susceptible to diet-induced-obesity-related anosmia. This however, did not happen. Chapter 3 reported that weight gain between normal mice that were fed the Oz-AIN diet and Amy mice that were fed the Oz-AIN Supp diet was almost identical. However, this does not mean that other obesity-related events that are also associated with anosmia, such as insulin resistance, were not modified by the curcumin and docosahexaenoic acid in the Oz-AIN Supp diet, preventing olfactory dysfunction. However, this conclusion requires comparisons that include normal mice that have been fed the Oz-AIN Supp diet.

Collectively, these results do not support the hypothesis that genotype has an effect on the olfactory abilities of normal and Amy mice. These results do suggest that nutrient supplements may enhance the olfactory abilities of Amy mice that are fed the Oz-AIN Supp diet so that they are better than normal mice that are fed the Oz-AIN diet. Potential mechanisms for this to occur may be through curcumin and docosahexaenoic acid preventing diet-induced obesity and is subsequent detrimental effects on olfactory pathways, or through B vitamin mediated neuroprotection. However, these conclusions may require neuronal counts or comparisons that include normal mice fed the Oz-AIN Supp diet.

The potentially beneficial effect of nutrient supplements against diet-type effects on olfactory abilities of Amy mice.

Consistent with the second aim of this study, the potential preventative effect of nutrient supplements on the diet-type effect on olfactory function Amy mice was investigated at 6 and 15 months of age.

Similar to Chapter 7, no significant differences were detected between the olfactory abilities of 6 month old Amy mice that were fed either the AIN93-M or the Oz-AIN diet. However, diet-type effects were observed still observed, as the 6 month old Amy mice that were fed the Oz-AIN Supp diet had better olfactory abilities than Amy mice that were fed either the AIN93-M diet or the Oz-AIN diet.

Doorn *et al.* report that the anterior olfactory nucleus in AD patients, which is significantly impaired in AD mouse models [518], contains a significantly greater number of microglia than normal brains [532]. Furthermore, these microglia were closely associated with β-amyloid deposits, indicating that β-amyloid deposition in AD olfactory bulbs is closely associated with increased inflammation [532]. Potentially, in the current study, the anti-inflammatory capabilities of eicosapentaenoic acid and docosahexaenoic acid supplements within the Oz-AIN Supp diet may have counteracted the up-regulation of microglia and inflammatory response in the olfactory tract, therefore preventing neurodegeneration.

As discussed in Chapter 7, the diet-induced effects on olfactory dysfunction in Amy mice may be due to a combination of high-fat diet induced aggregation of β-amyloid [198, 472, 505, 519] and diet induced obesity [121]. Similarly, in the current study, Amy mice that were fed the Oz-AIN diet were significantly heavier than Amy mice that were fed the AIN93-M diet at both ages that they were tested in the BCT, supporting the proposal that the diet effect on olfactory function may be attributed to the relationship between diet-induced obesity, malnourishment and olfactory dysfunction. This relationship was discussed in detail in Chapter 7.

As suggested earlier in this chapter, the docosahexaenoic acid and curcumin in the nutrient supplemented diet may have been responsible for the superior olfactory abilities of the Amy mice. Docosahexaenoic acid and curcumin reduce β-amyloid

deposition in regions of the brain associated with olfaction, and prevent diet induced obesity in AD-type mice, both of which are suggested causes for the olfactory dysfunction in the Amy mice that were fed the Oz-AIN diet [217]. The current study does not have measurements of β-amyloid in the olfactory bulb, nor has it measured the activity of receptors in the nasal epithelium for hormones associated with obesity, such as ghrelin or leptin, both of which are up-regulated in diet induced obesity and anosmia [512]. Nonetheless, the potential of docosahexaenoic acid and curcumin to reduce these events may have potentially prevented olfactory dysfunction in 6 month old Amy mice.

In contrast to the abilities of docosahexaenoic acid and curcumin to reduce β-amyloid deposition in mouse models of AD, high-fat diets increase β-amyloid deposition and neuronal loss in Amy mice [197, 198]. In the context of a high fat diet, polyunsaturated fats such as docosahexaenoic acid are rapidly oxidised, and may therefore not always be neuroprotective [191]. Amy mice that were fed either the Oz-AIN diet or Oz-AIN Supp diet, both of which are high-fat diets, had olfactory dysfunction at 15 months. It is possible that the high-fat diets accelerated β-amyloid deposition and neurodegeneration in regions of the brain required for olfaction. Overtime, this leads to enhanced oxidative damage throughout the brain, which may be exacerbated by the rapidly oxidised docosahexaenoic acid. This would need to be confirmed over two studies. The first study would compare β-amyloid levels and neuron counts in the olfactory regions of brains of Amy mice that were fed the AIN93-M diet, the Oz-AIN diet or the Oz-AIN Supp diet. This would give an indication of whether or not the high fat diets did increase β-amyloid and neurodegeneration in olfactory regions of the brain in Amy mice. Secondly, to determine whether or not the beneficial effects of docosahexaenoic acid were lost through its oxidation in the high-fat diet, oxidative damage levels and brain DHA

levels would need to be assessed in the brains of Amy mice fed either the AIN93-M diet, the Oz-AIN diet or the Oz-AIN Supp diet. These studies could provide valuable mechanistic insight into the effect of diet on olfactory abilities in Amy mice.

Collectively, these results support the hypothesis that Amy mice that have been fed the Oz-AIN diet have poorer olfactory functioning than Amy mice that have been fed the AIN93-M diet. While this was prevented with nutritional supplementation at 6 months, nutrient supplements were unable to prevent olfactory dysfunction in 15 month old Amy mice. It is proposed that this is due to high-fat diet-induced accelerated β-amyloid deposition and neuron loss. However more tests need to be done to confirm this.

The effect of aging on olfactory abilities of normal and Amy mice.

Consistent with the third aim of this study, olfactory abilities of 6 and 15 month old Amy mice were compared in an attempt to characterise olfactory function at different stages of life.

In the current study, age-associated olfactory dysfunction was observed in normal mice that were fed the Oz-AIN diet, Amy mice that were fed the Oz-AIN diet and Amy mice that were fed the Oz-AIN Supp diet, but not in Amy mice that were fed the AIN93-M diet. This suggests that the AIN93-M diet may have been protective against age-associated olfactory decline.

Direct comparisons of the change in latency and distance travelled for normal mice that were fed the Oz-AIN diet, Amy mice that were fed the Oz-AIN diet or the Amy mice that were fed the Oz-AIN Supp diet suggest that the normal mice are more affected by age. Normal mice increased latency to the chocolate by almost twice that of Amy mice that were fed either the Oz-AIN or the Oz-AIN Supp diet. However,

comparisons of the ratios of latencies and distances travelled at 15 months relative to 6 months suggest that Amy mice that were fed the Oz-AIN supp were more affected by age than normal mice that were fed the Oz-AIN diet.

It is possible that these differences may be because Amy mice that were fed the Oz-AIN diet had better olfactory abilities than normal mice at 6 months. Therefore, any change in olfactory abilities may have a greater impact on the ratio of overall change. Nonetheless, these results indicate that the Amy mice that were fed the Oz-AIN Supp diet and the normal mice that were fed the Oz-AIN diet were more affected by age than Amy mice that were fed the Oz-AIN diet.

Unexpectedly, the only Amy mice that did demonstrate significant age-related olfactory decline were the Amy mice that were fed the Oz-AIN Supp diet. This may have been because the Amy mice that were fed the Oz-AIN Supp diet performed so well at 6 months of age that they were more vulnerable to age-related changes. However, it is also possible that by preventing genotype effects on olfactory ability at 6 months of age made the olfactory pathways more like that of normal mice.

Saiz-Sanches *et al.* report that in early life there is rapid decrease in interneurons in the olfactory bulbs of Amy mice which is accompanied by β-amyloid deposition [533]. If a similar event is occurring here, it is possible that the nutrient supplements were able to prevent the loss of interneurons in the olfactory bulb in early life. However, in doing so, the olfactory bulbs remained similar to that of normal mice. Consequently, loss of neurons within the olfactory bulb may have occurred at the same rate as seen in normal mice, which is why the ratio of change of latency to the chocolate at 15 compared to 6 months was similar between normal mice and Amy mice that received the Oz-AIN Supp diet. However, in order to demonstrate that these events are occurring, olfactory abilities of mice need to be assessed from an

earlier age, and assessments need to occur frequently. Concurrently, olfactory bulb measurements would need to be made with each test, possibly using MRI to give an idea of olfactory bulb volume. Until such studies are carried out, the proposal outlined above remains speculative. Similar to Chapter 6, the olfactory abilities of Amy mice that were fed the AIN93-M diet did not appear to change significantly with age. Comparison between groups revealed that while the age-related changes in latency and distance travelled of Amy mice that were fed the AIN93-M diet were different to those of Amy mice that were fed the Oz-AIN diet or the Oz-AIN Supp diet, this was only significant when compared with mice that were fed the Oz-AIN diet. It is suggested that this is due to the previously discussed detrimental effects of a high-fat diet on abilities in Amy mice.

8.4. Conclusion.

Taken together, these studies have demonstrated that diet and age have significant effects on olfactory abilities of Amy mice. At 6 months of age, Amy mice that are fed the Oz-AIN diet performed worse in the BCT than Amy mice that were fed the AIN93-M or the Oz-AIN Supp diet. This indicates that a sub-optimal diet impairs olfactory abilities in 6 month old Amy mice, and that this is prevented with nutrient supplements. At 15 months of age, the nutrient supplements no longer protected against the detrimental effects of the high-fat diet on olfactory abilities. The 15 month old Amy mice that were fed the Oz-AIN diet or the Oz-AIN Supp diet performed worse in the BCT than Amy mice that were fed the AIN93-M diet. The consequence of this was that age-associated olfactory decline appeared more severe in Amy mice that were fed the Oz-AIN Supp diet than all other treatment groups. The only mice that did not demonstrated age-related olfactory decline were the Amy

mice that were fed the AIN93-M diet. This suggested that age associated olfactory decline is associated with high-fat diet.

Chapter 9: Characterising the effect of genotype, diet-type and life-stage on telomere sequence length and oxidative base damage in the brains of Amy mice.

9. Background

Telomeres are specialised sequences of DNA (TTA GGG) that cap the ends of chromosomes and distinguish between the natural end of a chromosome or a DNA strand break which may trigger senescence. Owing to the inability of DNA polymerases to synthesize to the 3' end of the DNA sequence, telomeres shorten with every cellular division [228, 231, 237]. Consistent with this, telomere length decreases with age in proliferative tissues such as such as white blood cells, liver, heart, fat and skin [240, 534].

It has been reported that telomere length does not decrease with age in brains of aging rats [240]. This has been attributed to the fact that neurons are in a post-mitotic state, and therefore not likely to undergo age-mediated telomere attrition [240]. However, cellular events, such as oxidative stress can accelerate telomere attrition [230, 535], and neurons and glial cells are highly vulnerable to oxidative damage [536]. Therefore, it is arguable that as oxidative stress increases with age in the brain, telomere attrition should also occur in the aging human and rat brain via oxidative base damage mediated DNA breaks leading to terminal deletions of chromosomes. Furthermore, microglia and astrocytes undergo changes in telomere length *in vitro* and *in vivo* [537]. Accordingly, it has also been demonstrated that telomere length decreases with age in brain tissues of rats and humans [537, 538].

Telomere attrition has been reported in neurons and microglia of AD brains [248, 537-539]. Flanary *et al.* report that not only does telomere length in microglial cells decrease with age in rats but also that telomeres in microglia from human AD brains that had a high amyloid load, were shorter than those in microglia from normal brains [538]. They suggest that telomere length in microglia from AD patients is accelerated by β-amyloid and therefore, microglia from AD brains enter cellular senescence earlier then microglia from normal brains [538]. This leads to impaired functioning of microglia, reduced ability to clear β-amyloid and failure to provide support to neurons and results in the neuro-atrophy that is characteristic of AD brains [538].

There are other groups that agree that while telomere shortening does occur in the aging brain, the effects are different in normal and AD brains. Rolyan *et al.* report that aged telomerase knockout mice with short telomeres (G3*Terc*$^{-/-}$) have activated microglia and neuronal loss throughout the frontal cortex and hippocampus as well as the behavioural deficits that are similar to those observed in AD [233]. However, the telomerase knockout had the opposite effect in AD-type mice [233]. AD mice with short telomeres due to a telomerase knockout had conserved cognitive functions and had reduced β-amyloid pathology. This suggests telomere attrition with aging may be protective against β-amyloid neuropathology and behavioural deficits [233].

In contrast to the studies above, Coviello-McLaughlin & Prowse demonstrated that unlike other peripheral tissues, telomere length increases with age in normal mouse brains [234]. Telomere length in the brains of normal mice increases in the first 5 months of life and maintained at that length thereafter [234]. Furthermore, while telomere attrition rates were different in young and adult mice, telomere maintenance was not attributed to telomerase activity or change in cell number [234]. This

suggests that telomere dynamics in the brain may be more complicated than the replicative senescence hypothesis suggests.

Thomas *et al.* report that while telomeres in the peripheral tissues of AD patients are shorter than normal healthy controls, telomere length in hippocampal tissue is significantly longer [241]. They suggest that this may be a result of reduced proliferative capacity of cells in the hippocampus of AD patients, rather than an active lengthening of telomere sequence [241].

In a separate study, Thomas *et al.* report that telomeres in the olfactory bulbs of AD-type mice are shorter than in normal mice. This telomere attrition could be alleviated by dietary supplementation with the polyphenolic compound curcumin, suggesting that dietary supplementation can prevent DNA damage events in the AD-mouse brain [106].

The olfactory bulb and the dentate gyrus of the hippocampus are both proliferative regions of the brain. Therefore, it may initially appear fair to attempt to compare the two studies by Thomas *et al*. However, in their study of human brains, Thomas *et al*. did not specifically measure telomere length in the dentate gyrus, but used brain pieces of the whole hippocampus [241]. Therefore, they may have also been assessing non-proliferative tissues in other regions of the brain. Nonetheless, the finding in their latter study that dietary supplementation was able to alter changes in telomere length that were associated with AD [106], suggest that it may be useful to determine whether or not dietary supplementation can alter telomere dynamics throughout the brain.

The role of the present chapter is to describe the results of experiments that aimed to characterise genotype effects and diet-type effects on telomere length and oxidative base damage in brain tissue from adult normal and Amy mice.

While it was not within the original scope of this study, the opportunity arose to investigate telomere length in the brains of AD and normal mice at two stages of adulthood and in so doing, compare telomere length in late aging. This change to the study design enabled comparison of telomere length in the brains of mice at ages that were equivalent to 10 human years apart, long after the β-amyloid neuropathology had been established [132].

The aims of the study described in this chapter are to:

1. **Investigate the effect of genotype on telomere length in the brains of 15 and 18 month old Amy mice.**

   This was achieved by making three comparisons:

   (i) Telomere length in the brains of 15 month old normal mice and Amy mice that were fed the Oz-AIN diet was measured and compared using real-time q-PCR (RT-qPCR).

   (ii) Telomere length in the brains of 18 month old normal mice and Amy mice that were fed the Oz-AIN diet was measured and compared using RT-qPCR.

   (iii) An age-genotype interaction on telomere length was also investigated through comparison of the telomere length in brains from 15 and 18 month old normal and Amy mice that had been fed the Oz-AIN diet.

2. **Investigate the effect of diet on telomere length in the brains of 15 and 18 month old Amy mice.**

   This was achieved by making three comparisons:

   (i) Telomere length in the brains of 15 month old Amy mice that were fed the AIN93-M diet, the Oz-AIN diet or the Oz-AIN Supp diet was measured and compared using RT-qPCR.

   (ii) Telomere length in the brains of 18 month old Amy mice that were fed the AIN93-M diet or the Oz-AIN diet was measured and compared using RT-qPCR.

   (iii) An age-diet interaction on telomere length was also investigated through comparison of the telomere length in brains from 15 and 18 month old Amy mice that had been fed the AIN93-M diet or the Oz-AIN diet.

3. **Investigate the effects of genotype, diet-type, or aging on oxidative base damage in the brains of 15 and 18 month old Amy mice.**

   This was achieved by making three sets of comparisons:

   (i) A genotype effect was investigated through comparisons of oxidative base damage in the brains of normal mice and Amy mice that were fed the Oz-AIN diet.

   (ii) A diet-type effect was investigated through comparisons of oxidative base damage in the brains of 15 month old Amy mice that had been fed the AIN93-M diet, the Oz-AIN diet or the Oz-AIN Supp diet.

   (iii) The effect of aging was investigated through comparisons of the oxidative base damage in the brains of 15 and 18 month old diet-type and genotype matched mice.

Telomere length and oxidative base damage were both measured with RT-qPCR. The protocols for both assessments are described in the results sections 9.1.5. and 9.1.6.

The exploratory study that is described in this chapter reports that diet-type, genotype, and age affect telomere length in the brains of Amy mice. Surprisingly, while telomere length decreased with age in the brains of normal mice, aging had the opposite effect on telomere length in the brains of Amy mice. However, this appeared to be dependent on diet, as telomere length only increased with age in the brains of Amy mice that were fed the Oz-AIN diet. It is suggested that this may be a consequence of changes in cell population in the brains of aging Amy mice, or due to over compensation by telomerase.

9.1. Methods.

9.1.1. Animals.

All experiments were approved by the Commonwealth Scientific and Industrial Research Organisation (CSIRO) Animal Welfare Committee, Australia in accordance with National Health and Medical Research Council guidelines.

Female Amy (APPswe/PSEN1dE9) mice and their female normal (C57bl/6) littermates were bred at and provided by Flinders University Animal Facility, Bedford Park, South Australia. Genotype was confirmed by PCR and agarose gel electrophoresis, as described in Appendix I. Mice were housed ($n<6$) in cages lined with sawdust with easy access to food and water.

Mice were separated into two studies (Table 1, Figure 1). The first study focussed on the potentially detrimental effects of the Oz-AIN diet on telomere length and

oxidative base damage in normal and Amy mouse brains. This was a two-by-two factorial design where normal and Amy mice were fed either the AIN93-M diet or the Oz-AIN diet. Mice were fed their respective diets from weaning until they were 18 months old (Table 1, Figure 1A).

The second study was designed to demonstrate the potentially beneficial effects of nutrient supplements on telomere length and oxidative base damage in the brains of normal and Amy mice. Amy mice were randomly divided into one of three groups and fed the AIN93-M diet, the Oz-AIN diet or the Oz-AIN Supp diet until they were 15 months old. Ten normal mice that were fed the Oz-AIN diet from weaning until they were 15 months old and were used as controls (Table 1, Figure 1B). There were difficulties managing mice over grooming each other after 15 months of age. Therefore, the second study only ran for 15 months.

While it was not the original intention to have first and the second studies end at different ages, this created an opportunity to compare telomere length at two different ages that were the equivalent of 10 human years apart, well after β-amyloid neuropathology is established [132, 160, 366, 367].

Table 1 outlines the total numbers of brains that were analysed in each study. The first study was not designed to investigate the effect of nutrient supplements. Therefore, there are no 18 month old Amy mice that were fed the Oz-AIN Supp diet. Similarly, the second study did not contain any normal mice that were fed the AIN93-M diet, as they were not required as a control. As a result, there are no 15 month old normal mice that were fed the Oz-AIN diet.

Table 1. The numbers of mice that were used in the two studies designed to investigate the effects of genotype and diet on telomere length and oxidative base damage in Amy mouse brains.

	Normal mice AIN93-M diet	**Normal mice Oz-AIN diet**	**Amy mice AIN93-M diet**	**Amy mice Oz-AIN diet**	**Amy mice Oz-AIN Supp diet**
15 months	n = 10	n = 8	n = 9	n = 7	- - N/A - -
18 months	- - N/A - -	n = 10	n =11	n = 14	n = 11

Figure 1A. The design of Study 1: The effect of an the Oz-AIN diet on telomere length and oxidative base damage in mouse brains.

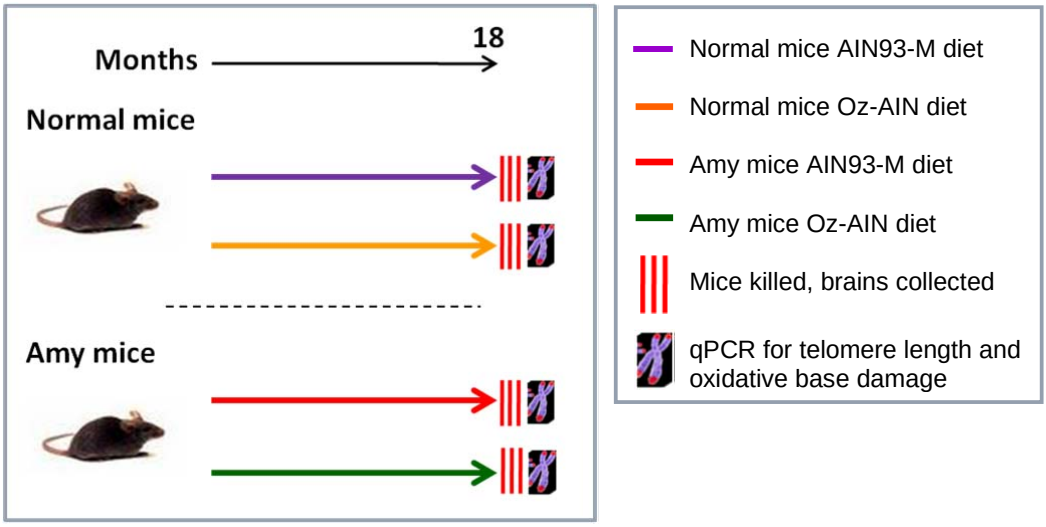


Figure 1A. Normal mice and Amy mice were separated into two groups each, and fed either the AIN93-M diet or the Oz-AIN diet for 18 months. At 18 months, mice were killed and their brains removed. Telomere length and oxidative base damage were assessed with qPCR. Normal mice that were fed the AIN93-M diet (purple arrow, n=10), normal mice fed the Oz-AIN diet (orange arrow, n=8), Amy mice fed the AIN93-M diet (red arrow, n=9), Amy mice fed the Oz-AIN diet (green arrow, n=7).

Figure 1B. The design of Study 2: The potentially beneficial effects of nutrient supplements on telomere length and oxidative base damage in mouse brains.

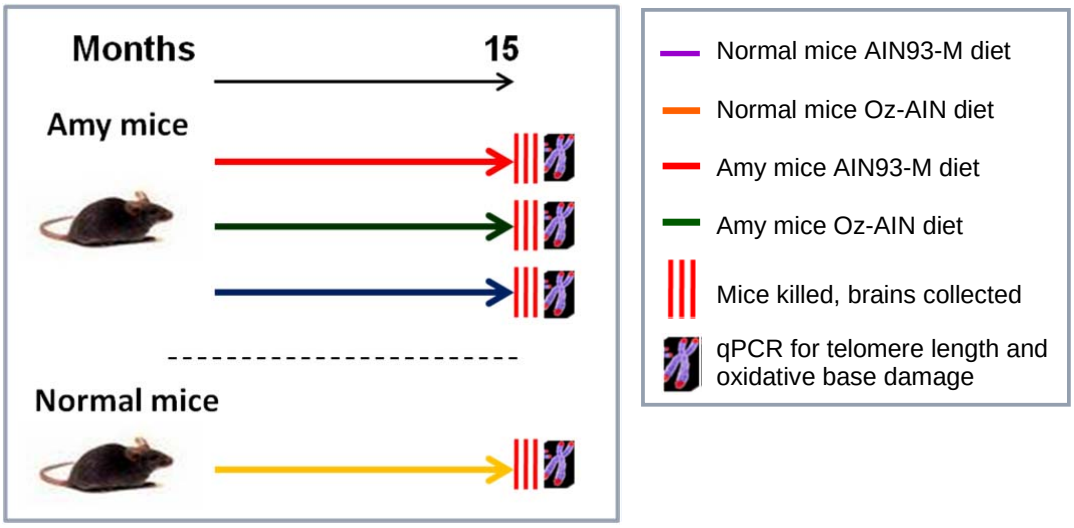


Figure 1B. Amy mice were separated into three groups and then fed either the AIN93-M diet (red arrow, n=11), the Oz-AIN diet (green arrow, n=14) or the Oz-AIN Supp diet (blue arrow, n=11). A fourth group of normal mice that were fed the Oz-AIN diet (orange arrow, n=10) were controls. Mice were fed their respective diets for 15 months and then were killed and their brains removed. Telomere length and oxidative base damage were assessed using qPCR.

9.1.2. Tissue collection and storage.

Mice were sacrificed at 15 and 18 months of age. Mice were anaesthetised with isoflurane and killed by exsanguination from the abdominal aorta. Mice were perfused with PBS before brains were removed, weighed and halved. The right brain hemisphere was snap frozen in liquid nitrogen and stored at -80°C for DNA analysis.

9.1.3. DNA isolation from mouse brain tissue.

Whole brain hemispheres, rather than specific regions, were used for DNA extraction.

DNA was extracted from mouse brain tissue using the DNeasy Blood and Tissue Kit (69506, © QIAGEN) as per the protocol outlined in the DNeasy® Blood and Tissue Handbook (QIAGEN, 2006) and is described below.

9.1.3.1. Protocol.

A rocking incubator (Orbital mixer incubator, Ratek instruments, Australia) was pre-heated to 56°C.

Tissues were thawed on ice and cut into small 25 mg pieces before being placed in a 1.5 ml microcentrifuge tube that had been labelled with the corresponding mouse number.

180 µL Buffer ATL (Tissue Lysis Buffer, 19076, QIAGEN) and 20 µL proteinase K (19131, QIAGEN) were added to each of the labelled microcentrifuge tubes containing brain tissue. To ensure that buffer ATL, proteinase K and brain tissues were thoroughly mixed, samples were vortexed for 30 s each at room temperature using a Townson Tru-Mix vortex (Townson Tru-Mix, Townson & Mercer Pty. Ltd.).

Samples were placed in the rocking incubator and incubated at 56°C, until brain tissues were completely lysed (3-5 hours). To aid lysis, tissues were vortexed mid-way through incubation.

Buffer AL (Lysis Buffer, 19075, QIAGEN) and ethanol (E7023, Sigma-Aldrich) were combined 1:1, as per Table 2 to make the Buffer AL+ethanol mix. 400µL of the Buffer AL+ethanol mix was added to each sample, which was then immediately vortexed for 30 s at room temperature.

Table 2. Sample calculations for the amount of Buffer AL and ethanol that are required for 24 samples.

Reagent	**Amount per microcentrifuge tube**		**Number of samples**	**Amount added to master mix**
Buffer AL	200 µL	x	24	4,800 µL
Ethanol (96%)	200 µL	x	24	4,800 µL
TOTAL	**400 µL**	x	24	**9,600 µL**

Samples were each transferred to a DNeasy spin column (QIAGEN, Australia), which was placed in a 2 ml collection tube (19201, QIAGEN, Australia) and centrifuged at 8,000 rpm for 1 min at room temperature in a microcentrifuge (Eppendorf centrifuge 5415R, Eppendorf). The flow-through solution and collection tubes were discarded and the sample, which remained in the DNeasy spin column, was placed into a fresh 2 ml collection tube (19201, QIAGEN, Australia).

500 µL Buffer AW1 (Wash Buffer (1), QIAGEN) (in 96% ethanol) was added to each sample. Samples were centrifuged at 8,000 rpm for 1 min at room temperature in a microcentrifuge (Eppendorf centrifuge 5415R, Eppendorf). The flow-through

solution and collection tube were discarded, and the sample, which remained in the DNeasy spin column, was placed into a fresh 2 ml collection tube (19201, QIAGEN, Australia).

500 µL Buffer AW2 (Wash Buffer (2), QIAGEN) (in 96% ethanol) was added to each sample. Samples were centrifuged at 14,000 rpm for 3 min at room temperature in a microcentrifuge (Eppendorf centrifuge 5415R, Eppendorf) to dry the DNeasy membrane. The flow-through solution and collection tube were discarded, and the sample, which remained in the DNeasy spin column, was placed into a fresh 2 ml collection tube (19201, QIAGEN, Australia).

200 µL Buffer AE (Elution Buffer, 19077, QIAGEN) was added carefully and directly onto the spin column membrane in each spin column that contained sample. Samples incubated at room temperature for 1 min. Samples were centrifuged at 8,000 rpm for 1 min at room temperature in a microcentrifuge (Eppendorf centrifuge 5415R, Eppendorf). The flow-through solution from the spin column contained the DNA extract.

To ensure maximum DNA yield, the flow-through was collected and re-added to the spin column. Samples were then centrifuged at 8,000 rpm for 1 min at room temperature in a microcentrifuge (Eppendorf centrifuge 5415R, Eppendorf).

Samples were transferred to labelled 1.5 ml eppendorf tubes and stored at 4°C.

9.1.4. Measuring DNA in purified DNA samples.

A nanodrop® spectrophotometer that was connected to a laptop running NanoDrop 1000 software (version 3.6.0, Thermo Fisher Scientific, USA) was used to measure the amount of DNA in the purified DNA samples. All samples and reagents were measured using plugged sterile pipette tips (10 µL).

Prior to use, the nanodrop® spectrophotometer was cleaned by dropping 2µL ultra pure water (UPW) onto the platform and wiped with an anti-static tissue (KimWipes, Kimtech science, Kimberly Clark, USA).

To form a baseline reading, 2µL of Buffer AE (Elution Buffer, 19077, QIAGEN) was transferred onto the nanodrop® spectrophotometer platform. Buffer AE was used to form the baseline reading because all DNA samples were in Buffer AE solution (see 9.1.3.1.). The baseline measurement was set by selecting "blank" in the main screen of the nanoDrop 1000 software (version 3.6.0, Thermo Fisher Scientific, USA).

To measure the amount of DNA in each sample, 1 µL of DNA sample was added to the nanodrop® spectrophotometer platform and "measure" was selected on the main screen of the nanoDrop 1000 software (version 3.6.0, Thermo Fisher Scientific, USA). Between each measurement, the nanodrop® spectrophotometer platform was cleaned with 2 µL UPW in between sample measurements.

The measurement of DNA content (ng/µL), that was provided by the nanoDrop 1000 software (version 3.6.0, Thermo Fisher Scientific) was used to calculate the amount of UPW that was required to make the DNA solution up to 5 ng/µL. DNA content was re-measured using the nanodrop® spectrophotometer to ensure accuracy of dilutions.

The 260/280 reading that is provided by the nanoDrop 1000 software (version 3.6.0, Thermo Fisher Scientific) is a measure of DNA purity. Samples that are pure have a 260/280 reading between 1.7 and 1.9.

DNA samples were stored at 4°C until use for RT-qPCR.

9.1.5. RT-qPCR for analysis of absolute telomere length.

The RT-qPCR to assess telomere length is carried out in two PCRs. The first PCR provided a measurement of the total amount of telomere sequence per 4 ng of DNA sample. The second PCR provided a measure of the number of genome copies per DNA sample by measuring the amount of a single copy gene. Telomere length per DNA sample is calculated by dividing the absolute telomere length by the amount of single copy gene. The following sections describe the procedures for each of these two PCRs.

9.1.5.1. Primers.

The primers that were used in each of the two PCRs required to measure telomere length are listed in Table 3. Primers and standards were purchased from GeneWorks (GeneWorks Pty. Ltd., Hindmarsh, SA). Each primer was made up into a stock solution (20nmol) which was then diluted into a working solution (2 nmol).

Table 3. Sequences of primers and telomere standard that were used in the RT-qPCR for the analysis of telomere length in mouse brain.

	Sequence
TeloFWD	CGG TTT GTT TGG GTT TGG GTT TGG GTT TGG GTT TGG GTT
TeloRev	GGC TTG CCT TAC CCT TAC CCT TAC CCT TAC CCT TAC
36b4FWD	ACT GGT CTA GGA CCC GAG AAG
36b4REV	TCA ATG GTG CCT CTG GAG ATT
Telomere Standards	TTA GGG TTA GGG TTA GGG TTA GGG TTA GGG TTA GGG TTA GGG TTA GGG TTA GGG TTA GGG TTA GGG TTA GGG TTA GGG

9.1.5.1.1. Calculations and dilutions to make STOCK and WORKING solutions for each primer used in the two PCR's to measure telomere length.

<u>TeloFwd STOCK solution</u>

585 µg of teloFwd primer was purchased from GeneWorks (GeneWorks Pty. Ltd.) at a concentration of 47.8 nmol.

To make a STOCK solution of 20 nmol:

100 nmol = 4.78 nmol x 10

= 478 µL UPW

20 nmol = 478 / 5

= 95.6 µL UPW

- 95.6 µL UPW was added to the geneworks vial to obtain a TeloFwd STOCK solution (20 nmol) and stored at -20°C.

TeloFwd WORKING solution

The TeloFwd WORKING solution (2 nmol) was a 1:10 dilution of the TeloFwd STOCK solution (20 nmol).

- 95 µL of TeloFwd STOCK solution (20 nmol) was added to 855 µL UPW and stored at -20°C.

TeloRev STOCK solution

830 µg TeloRev was purchased from GeneWorks (GeneWorks Pty. Ltd.) at a concentration of 71.3 nmol.

To make a STOCK solution of 20 nmol:

100 nmol = 71.3 x 10

= 713 µL UPW

20 nmol = 713 / 5

= 142.6 µL UPW

- 142.6 µL UPW was added to the GeneWorks tube to obtain a TeloRev STOCK (20 nmol) and stored at -20°C.

TeloRev WORKING solution

The TeloRev WORKING solution (2 nmol) was a 1:10 dilution of TeloRev STOCK solution (20nmol).

- 100 µL of TeloRev STOCK solution (20 nmol) was added to 900 µL UPW and stored at -20°C.

36b4Fwd STOCK solution

494 µg 36b4Fwd was purchased from GeneWorks (GeneWorks Pty. Ltd.) at a concentration of 76.3 nmol.

To make a STOCK solution of 20 nmol:

100 nmol = 76.3 x 10

= 763 µL UPW

20 nmol = 763 / 5

= 152.6 µL UPW

- 152.6 µL UPW was added to the GeneWorks tube to obtain a 36b4Fwd STOCK solution (20 nmol) and stored at -20°C.

36b4Fwd WORKING solution

The 36b4Fwd WORKING (2 nmol) was a 1:10 dilution of 36b4Fwd STOCK solution (20nmol).

- 100 µL of 36b4Fwd STOCK solution (20 nmol) was added to 900 µL UPW and stored at -20°C.

36b4Rev STOCK solution

577 µg 36b4Rev was purchased from GeneWorks (GeneWorks Pty. Ltd.) at a concentration of 89.4 nmol.

To make a 36b4Rev STOCK solution of 20 nmol:

100 nmol = 89.4 x 10

= 894 µL UPW

20 nmol = 894 / 5

= 178.8 µL UPW

- 178.8 µL UPW was added to the GeneWorks tube to obtain a 36b4Rev STOCK solution (20 nmol) and stored at -20°C.

36b4Rev WORKING solution

The 36b4Rev WORKING solution (2 nmol) was a 1:10 dilution of 36b4Rev STOCK solution (20nmol).

- 100 µL of 36b4Rev STOCK solution (20nmol) was added to 900 µL UPW and stored at -20°C.

9.1.5.1.2. Calculations to make telomere standards.

A standard curve is generated with each plate to ensure that telomeric DNA sequence is uniform between samples. To generate the standard curve, 1022 µg Telomere Standard primer (TeloStd) was purchased from GeneWorks (GeneWorks Pty. Ltd.), and then diluted in 1022 µL UPW to make a stock concentration of 1 µg/µL.

Serial dilutions (of 1:100 and then 1:30) of stock were made to obtain a standard stock of 300 pg / µL. Standard stock was made into working standards (Std) in serial dilutions of 10 as per Table 4. Standards were stored at 4°C in an 8 well strip until use.

Table 4. Serial dilutions of telomere standard primer.

Std Sample	Volume of Std Sample Added	Volume of UPW added	Final Concentration
Standard STOCK			300 pg / µL
Std 1	60 µL of STOCK	1140 µL	15 pg/µL
Std 2	120 µL Std 1	1080 µL	1.5 pg / µL
Std 3	120 µL Std 2	1080 µL	0.15 pg / µL
Std 4	120 µL Std 3	1080 µL	0.015 pg / µL
Std 5	120 µL Std 4	1080 µL	0.0015 pg / µL
Std 6	120 µL Std 5	1080 µL	0.00015 pg / µL

9.1.5.2. PCR master mix.

All reagents for the PCR (excluding DNA) were combined to make a master mix (Table 5) in the biohazard hood. PCR master mix was made to fill all wells of a 96-well plate (4ti-0770/C, FrameStar®96, 4titude®, UK), and allow for 4% pipetting error (i.e. enough for 100 wells).

When making the PCR master mix, the SYBR 1 mastermix (#4367396 Applied Biosystems), which contains AmpliTaq Gold DNA polymerase, dNTPs, SYBR I Green Dye, optimised buffers and passive reference dye (ROX) was added to the mixture slowly, as it contains detergent and may bubble if handled roughly. Master-mix was made up immediately prior to each PCR and used fresh.

Table 5. Calculations for reagents in the PCR master mix to assess absolute telomere length.

	Amount	x	number of wells + 5% (error)	TOTAL
UPW	4 µL	x	100 (full plate)	400 µL
SYBR 1 master mix	10 µL	x	100 (full plate)	1000 µL
TeloFwd (2 µmol)	1 µL	x	100 (full plate)	100 µL
TeloRev (2 µmol)	1 µL	x	100 (full plate)	100 µL
TOTAL				1600 µL

9.1.5.3. Plate set up for RT-qPCR to assess the total amount of telomere sequence per 4 ng of mouse DNA.

16 µL PCR master mix was added to each well of a 96-well plate (4ti-0770/C, 4titude®) in the biohazard hood.

PCR plates were removed from the hood and 4 µL of DNA sample (20 ng), standard, non-template control (UPW), or long telomere positive control was then added to the plate in triplicate as per the example in Figure 2.

The long telomere positive control DNA was 1301 (cell line 1301; accession number 01051619, European Collection of Cell Cultures, UK EQUIPMENT).

All samples were added to the plate in triplicate. The first three columns contained the controls (UPW and 1301) and standards. The remainder of wells on the plate contained DNA samples in triplicate, and each PCR gave a reading for telomere

length from 24 DNA samples. Figure 2 is a representative plate that has been set up for PCR to assess the total amount of telomere sequence per 4 ng of DNA sample.

The 96 well plates were loaded and then spun in a centrifuge (Hettich Zentrifugen Rotanta 460R) for 2 min at 1000 rpm at 4°C to ensure that all samples were combined with the master mix.

Figure 2. Example of a 96-well plate set up for RT-qPCR to assess the total amount of telomere sequence per 4 ng of mouse DNA.

	1	2	3	4	5	6	7	8	9	10	11	12
A	UPW			#1			#2			#3		
B	1301			#4			#5			#6		
C	S1			#7			#8			#9		
D	S2			#10			#11			#12		
E	S3			#13			#14			#15		
F	S4			#16			#17			#18		
G	S5			#19			#20			#21		
H	S6			#22			#23			#24		

Figure 3. Example of a 96-well plate set up for qPCR to assess the amount of 36b4 per 4 ng of mouse DNA.

	1	2	3	4	5	6	7	8	9	10	11	12
A	#1			#2			#3			#4		
B	#5			#6			#7			#8		
C	#9			#10			#11			#12		
D	#13			#14			#15			#16		
E	#17			#18			#19			#20		
F	#21			#22			#23			#24		
G	#25			#26			#27			#28		
H	#29			#30			#31			#32		

9.1.5.4. Master mix and plate set up for RT-qPCR to assess the amount of 36b4 per 4 ng of mouse DNA.

The master mix and plate set up for the RT-qPCR to assess genome copies per sample were the same as those for the RT-qPCR to assess the total amount of telomere sequence per sample (9.1.5.2 and 9.1.5.3) with some modifications.

TeloFwd and TeloRev were replaced with 36b4Fwd and 36b4Rev in the master mix (Table 6).

Telomere standards were not run on the 96-well plate for the RT-qPCR for 36b4. This allowed for measurement of 32 samples (Figure 3).

Table 6. Calculations for reagents in the PCR master mix to assess genome copies per sample.

	amount	x	Number of wells + 5% (error)	TOTAL
UPW	4 µL	x	100 (full plate)	400 µL
SYBR 1 master mix	10 µL	x	100 (full plate)	1000 µL
36b4Fwd (2 µmol)	1 µL	x	100 (full plate)	100 µL
36b4Rev (2 µmol)	1 µL	x	100 (full plate)	100 µL
TOTAL				1600 µL

9.1.5.5. RT-qPCR cycling conditions for measurement of absolute telomere length and the number of genome copies per sample.

The RT-qPCR cycling conditions were the same for the assessment of absolute telomere length and number of genome copies per sample.

Plates were placed in an ABI 7300 Sequence Detection System (Applied Biosystems), which was attached to a laptop with sequence detection software (7300 system SDS software, Applied Biosystems).

Sample identifiers such as detectors (SYBR 1 mastermix), non-template control, sample names, and standards (including standard quantities) were entered into the sequence detection software.

The PCR cycling conditions were: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 95°C for 15 s then 60°C for 1 min. This was completed with a dissociation step of 95°C for 15 s, 60°C for 30 s and 95°C for 15 min.

9.1.6. Measurement of oxidative base damage by RT-qPCR.

Oxidative base damage was assessed by measuring the amount of 8-Oxo-2'-deoxyguanosine (8-oxo-dG) per 4 ng of DNA sample. DNA samples were digested with formamidopyrimidine DNA glycolase (FPG), which is a bacterial enzyme that recognises and excises oxidised bases. RT-qPCR was carried out on digested and non-digested DNA samples, which were then compared to provide a measure of oxidised bases in the mouse brain. All samples, oligimers and reagents were measured pipettes (1-10 μL) and plugged sterile pipette tips (10 μL).

9.1.6.1. Overnight digestion with Fpg.

Digest mix was made containing 12 U FPG (#M0240, BioLabs Ipswich, MA) per sample in 1 x NEB buffer (B7001, BioLabs, Ipswich, MA). Non-digest control mix was made by replacing FPG with UPW. Extra mix was made to allow for pipetting error (Table 7).

Table 7. Calculation for the overnight digestion (FPG-containing) and non-digestion (UPW-containing) mixes for 45 DNA samples.

	µL		Number of samples + 1	Total (µL)
BSA	0.3	X	46	13.8
NEB	3.0	X	46	138.0
FPG or UPW	1.0	X	46	46.0
TOTAL				197.8

4.3 µL of either digest (FPG-containing) or non-digest (UPW-containing) mixes were added each well on a 96-well plate ((4ti-0770/C, 4titude®)), so that half the wells contained 4.3 µL of digest mix, and half the wells contained 4.3 µL non-digest mix (Figure 4).

300 ng DNA was diluted in 15.7 µL UPW. DNA was then added to each well in the 96-well plate ((4ti-0770/C, 4titude®)), so that all wells contained 300 ng DNA in 20 µL of either digest or non-digest mix. DNA samples were added to one digest mix-containing and one non-digest mix containing well each (Figure 4).

Figure 4. Example of a 96-well plate set up for overnight digestion with FPG.

		1	2	3	4	5	6	7	8	9	10	11	12
DIGESTED	A	#1	#2	#3	#4	#5	#6	#7	#8	#9	#10	#11	#12
	B	#13	#14	#15	#16	#17	#18	#19	#20	#21	#22	#23	#24
	C	#25	#26	#27	#28	#29	#30	#31	#32	#33	#34	#35	#36
	D	#37	#38	#39	#40	#41	#42	#43	#44	#45	NTC	NTC	NTC
NON-DIGESTED	E	#1	#2	#3	#4	#5	#6	#7	#8	#9	#10	#11	#12
	F	#13	#14	#15	#16	#17	#18	#19	#20	#21	#22	#23	#24
	G	#25	#26	#27	#28	#29	#30	#31	#32	#33	#34	#35	#36
	H	#37	#38	#39	#40	#41	#42	#43	#44	#45	NTC	NTC	NTC

Well plates were spun for 1 min at 1,000 rpm at 4°C in a centrifuge (Hettich Zentrifugen Rotanta 460R).

Samples were incubated overnight in a rocking incubator (Orbital mixer incubator, Ratek instruments, Australia) that was maintained at 37°C. The reaction was stopped by a 10 min incubation period at 65°C in an incubator (Orbital mixer incubator, Ratek instruments, Australia).

9.1.6.2. Protocol for the RT-qPCR to measure oxidative base damage in DNA samples from mouse brain.

All reagents for the RT-qPCR (excluding DNA) were combined in the biohazard hood to make PCR master mix (Table 8). The TeloFwd and TeloRev primers in the PCR master mix are the same as those used to measure telomere length (9.1.5.1., Table 3). SYBR 1 mastermix (#4367396 Applied Biosystems), which contains AmpliTaq Gold DNA polymerase, dNTPs, SYBR I Green Dye, optimised buffers

and passive reference dye (ROX) was added to the mixture slowly, as it contains detergent and may bubble if handled roughly. Master-mix was made up immediately before each PCR and used fresh. Enough PCR master mix was made for all wells of a 96-well plate, and to allow for 4% pipetting error (Table 8).

Table 8. Calculations for reagents in the PCR master mix to assess oxidative base damage.

	amount	x	Number of wells + 4% (error)	TOTAL
UPW	4 µL	x	100 (full plate)	400 µL
TeloFwd (2 µmol)	1 µL	x	100 (full plate)	100 µL
TeloRev (2 µmol)	1 µL	x	100 (full plate)	100 µL
SYBR 1 mastermix	10 µL	x	100 (full plate)	1000 µL
TOTAL				1600 µL

9.1.6.3. Plate set up to measure oxidative base damage.

16 µL PCR master mix was added to each well of a 96-well plate ((4ti-0770/C, 4titude®)) (Figure 5). The 96-well plate was virtually divided into two halves so that digested and undigested samples could be run on the same plate. Rows A-D were designated "Digested Samples". Rows E-H were designated "Non-Digested samples" (Figure 5).

4 µL of each DNA sample (from the digested or non-digested samples) was added to the plate in triplicate and 4 µL UPW added as non-template control. The 96-well plate was spun in a centrifuge (Hettich Zentrifugen Rotanta 460R) at 1000 rpm for 2 mins, to ensure that all samples were thoroughly combined with the master mix.

Figure 5. Example of a 96-well plate set up for qPCR to assess oxidative base damage after FPG digestion in mouse tissue DNA.

		1	2	3	4	5	6	7	8	9	10	11	12
DIGESTED	A	#1			#2			#3			#4		
	B	#5			#6			#7			#8		
	C	#9			#10			#11			#12		
	D	#13			#14			NTC			UPW		
NON-DIGESTED	E	#1			#2			#3			#4		
	F	#5			#6			#7			#8		
	G	#9			#10			#11			#12		
	H	#13			#14			NTC			UPW		

9.1.6.4. RT-qPCR cycling conditions for the measurement of oxidative base damage.

Plates were placed in an ABI 7300 Sequence Detection System (Applied Biosystems, Foster City, CA), which was attached to a laptop with sequence detection software (7300 system SDS software, Applied Biosystems).

Sample identifiers such as detectors (SYBR 1 mastermix), non-template control, sample names, and standards (including standard quantities) were entered into the sequence detection software.

PCR cycling conditions were: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 95°C for 15 s then 60°C for 1 min. This was completed with a dissociation step of 95°C for 15 s, 60°C for 30 s and 95°C for 15 s.

9.1.7. Data analysis.

Results were analysed in sequence detection software (7300 system SDS software, Applied Biosystems), Microsoft Excel 2007 (for windows) and GraphPad Prism® version 5.0 (GraphPad Software).

Cyclic threshold (CT) values were calculated by the sequence detection software (7300 system SDS software, Applied Biosystems). CT values are inversely proportional to the amount target nucleic bases in the sample, and therefore are used to determine the amount of DNA sequence in each sample.

Standard curve were generated for each PCR plate to assess telomere length. Figure 6 shows the standard curves that were used to measure telomere length in mouse brains. Figure 6 confirms that all the standard curves were similar, confirming that

PCR results from different plates were comparable. The telomere length from mouse brains falls within the linear range of these standard curves (between 7.0 and 8.0 log(TL) kbp).

The total amount of telomere sequence per sample was measured from the standard curve generated from the same PCR plate. Telomere length was then calculated by dividing the total amount of telomere sequence by the total amount of 36b4 sequence for each sample.

It is possible to estimate the amount of telomere sequence per chromosome by dividing this value by 80 (laboratory mice have 40 chromosomes, with 2 telomeres per chromosome) [540, 541]. However, this was not done in the current analysis as telomere length was also to be compared to total oxidative DNA damage, rather than damage per chromosome.

The number of oxidised bases in mouse brains were measured by determining the difference in CT between the FPG-digested and non-digested DNA (ΔCT). The ΔCT was then inserted into the equation $x= (\Delta CT-0.3823)/0.0858$ to obtain the number of FPG sensitive lesions and provides a measurement of oxidised base damage.

Age-Genotype and Age-Diet-type interactions were determined with two-way ANOVA and Bonferroni multiple comparison post tests. Comparison amongst 15 month old mice were carried out with one-way ANOVA and Bonferroni post tests. Comparison amongst 18 month old mice groups was carried out with two-way ANOVA and Bonferroni post tests. For all comparisons, statistical significance was set at $p<0.05$.

Figure 6. Standard curve that was used to calculate the amount of telomere sequence per sample.

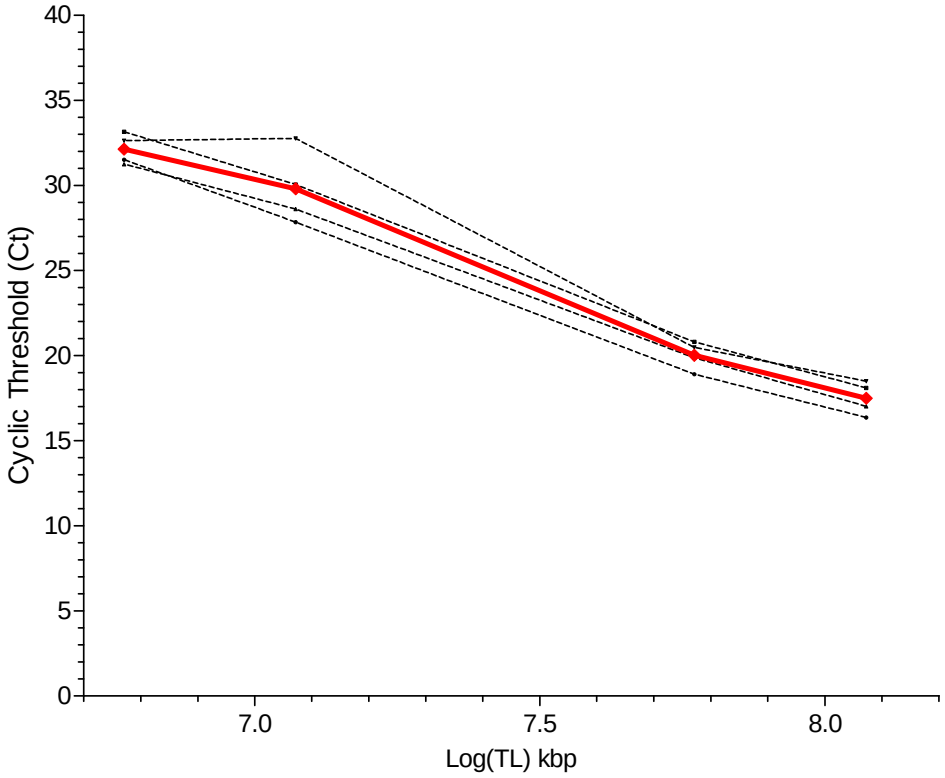


Figure 6. A standard curve was generated for each PCR plate that was run to assess the amount of telomere sequence per 20ng of DNA. The Y axis represents the Cyclic threshold at which SYBR green fluoresces in the PCR. The X axis represents the amount of telomere product (log(TL) kbp). The dotted lines are the standard curves that were generated for 15 month old mice. The red line represents the mean of all standard curves. The measurements for telomere length in mice fall within the linear range of the standard curve (between 7.0 and 8.0 Log(TL) kbp).

9.2. Results.

9.2.1. Telomere length in the brains of 15 and 18 month old Amy mice.

Telomere sequence length was measured in the brains of 15 and 18 month old normal and Amy mice. The quantitative real-time amplification (RT-qPCR) that was used to do this was carried out as per section 9.1.5. (pp. 546-557).

Table 9 provides the telomere length in the brains of 15 and 18 month old normal and Amy mice that were fed the AIN93-M diet, the Oz-AIN diet, or the Oz-AIN Supp diet. There are no measurements for telomere length in the brains of 15 month old normal mice that were fed the AIN93-M diet or 18 month old Amy mice that were fed the Oz-AIN Supp diet, as these treatment groups were not included in each of their studies (see 9.1.1., pp. 537-540). The following sections compare the telomere lengths that are described in Table 9 to demonstrate the effects of genotype and diet-type on age-induced changes in telomere length in the brains of normal and Amy mice.

Table 9. Absolute telomere length (kbp) in the brains of 15 and 18 month old normal and Amy mice.

	Normal mice AIN93-M diet	**Normal mice Oz-AIN diet**	**Amy mice AIN93-M diet**	**Amy mice Oz-AIN diet**	**Amy mice Oz-AIN Supp diet**
18 months	25.89×10^3 $\pm 9.45 \times 10^3$	11.27×10^3 $\pm 3.57 \times 10^3$	16.33×10^3 $\pm 6.00 \times 10^3$	41.38×10^3 $\pm 10.51 \times 10^3$	- - N/A - -
15 months	- - N/A - -	32.87×10^3 $\pm 4.28 \times 10^3$	16.80×10^3 $\pm 3.68 \times 10^3$	16.13×10^3 $\pm 4.19 \times 10^3$	14.63×10^3 $\pm 1.27 \times 10^3$

9.2.2. The effects of genotype, diet-type and a genotype-diet-type interaction on telomere length in the brains of 18 month old mice.

A two-way ANOVA revealed that diet-type and genotype only accounted for 1.08% and 4.20% respectively, of the overall variance of telomere length in the brains of 18 months old mice ($p=0.55$ and $p=0.24$ respectively, Figure 7), indicating that neither diet-type nor genotype had an effect on telomere length in the brains of 18 month old mice. However, there was a significant genotype-diet-type interaction that accounted for 15.65% of the overall variance ($p=0.03$, Table 9, Figure 7).

Bonferroni post tests indicated that there were weak genotype effects and that genotype effected telomere length more amongst mice than were fed the Oz-AIN diet compared to those fed the AIN93-M diet. The Amy mice that were fed the Oz-AIN diet had longer telomeres than normal mice that were fed the Oz-AIN diet ($p=0.18$, Table 9, Figure 7). The failure of this to achieve significance may be due to an outlier in the amongst the Amy mice that were fed the Oz-AIN diet who had extremely short telomeres (3.39 x 103 kbp, $p=0.04$ when removed). However, there were no significant differences in the telomere lengths of normal and Amy mice that were fed the AIN93-M diet ($p>0.99$, Table 9, Figure 7). This suggests that the genotype-diet-type interaction may be attributed to differences between mice that were fed the Oz-AIN diet.

There were no differences between telomere length in the brains of normal mice that were fed the AIN93-M diet or the Oz-AIN diet ($p>0.99$, Table 9, Figure 7). However, telomeres in the brains of Amy mice that were fed the Oz-AIN diet were longer than those of Amy mice that were fed the AIN93-M diet ($p=0.36$, Table 9,

Figure 7). This failed to achieve significance due to outliers amongst Amy mice that were fed the AIN93-M diet or the Oz-AIN diet who had telomere lengths of 3.39 x 10^3 kbp and 4.58 x 10^4, respectively ($p=0.05$ when removed). This indicates that Amy mice were more susceptible to the effects of diet than normal mice.

Summary of telomere length in the brains of 18 month old mice.

Collectively, these data indicate that there is a significant diet-genotype effect on telomere length in the brains of 18 month old Amy mice, whereby Amy mice that are fed the Oz-AIN diet have longer telomeres than either genotype matched or diet-type matched mice.

Figure 7. The effects of diet-type and genotype on telomere length (kbp) in the brains of 18 month old normal and Amy mice.

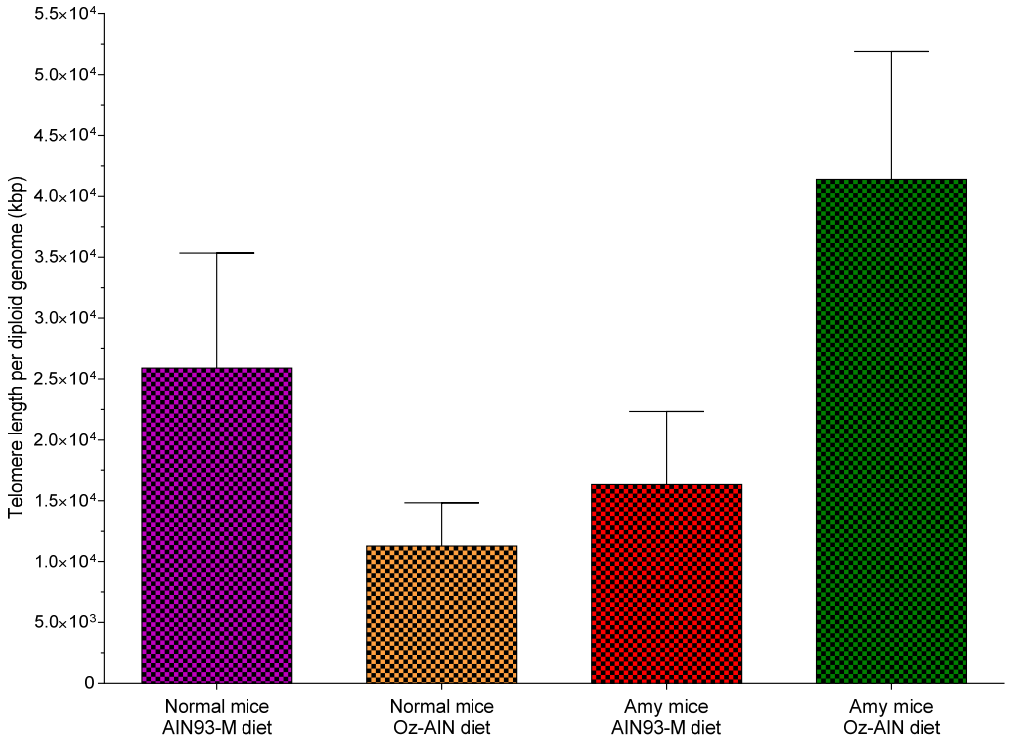


Figure 7. Telomere sequence length in brain tissue was measured and compared between normal and Amy mice that were fed the AIN93-M diet or the Oz-AIN diet. Normal mice that were fed the AIN93-M diet (purple checked bar, n=10), Normal mice that were fed the Oz-AIN diet (orange checked bar, n=8), Amy mice that were fed the AIN93-M diet (red checked bar, n=9), and Amy mice that had been fed the Oz-AIN diet (green checked bar, n=7). Bars represent mean ±SEM. While there was a significant genotype-diet-type interaction ($p=0.03$), Bonferroni post tests did not detect significant differences between groups.

9.2.3. The effect of genotype and diet-type on telomere length in the brains of 15 month old mice.

Genotype effects on telomere length.

An investigation into the effect of genotype on telomere length in 15 month old mouse brains was made through a comparison of the telomere length (kbp) in the brains of 15 month old normal and Amy mice that had been fed the Oz-AIN diet (Figure 8). Telomere length in the brains of 15 month old Amy mice that had been fed the Oz-AIN Supp diet were included in these comparisons to determine whether or not nutrient supplements can prevent genotype effects on telomere length in the Amy mouse brains (Figure 8).

A one-way ANOVA detected significant differences between the telomere lengths in the brains of 15 month old normal mice that were fed the Oz-AIN diet, Amy mice that were fed the Oz-AIN diet and Amy mice that had been fed the Oz-AIN Supp diet ($p=0.004$, Table 9, Figure 8). Bonferroni post tests confirmed that this may be attributed to a genotype effect as Amy mice that had been fed the Oz-AIN diet had significantly shorter telomeres than normal mice that were fed the Oz-AIN diet ($p=0.01$, Table 9, Figure 8). The effects were not prevented or alleviated by nutrient supplements. Bonferroni post tests revealed that the 15 month old Amy mice that were fed the Oz-AIN Supp diet also had shorter telomeres per diploid genome than normal mice ($p=0.008$, Table 9, Figure 8). Telomere length in the brains of Amy mice that were fed the Oz-AIN Supp diet was not different to that in the brains of Amy mice that had been fed the Oz-AIN diet ($p>0.99$, Table 9, Figure 8), which confirms the results above that nutrient supplements do not have an effect on telomere length in the brains of 15 month old Amy mice.

Figure 8. The effect of nutrient supplements on the genotype-induced difference of telomere length in brain tissue from 15 month old normal and Amy mice.

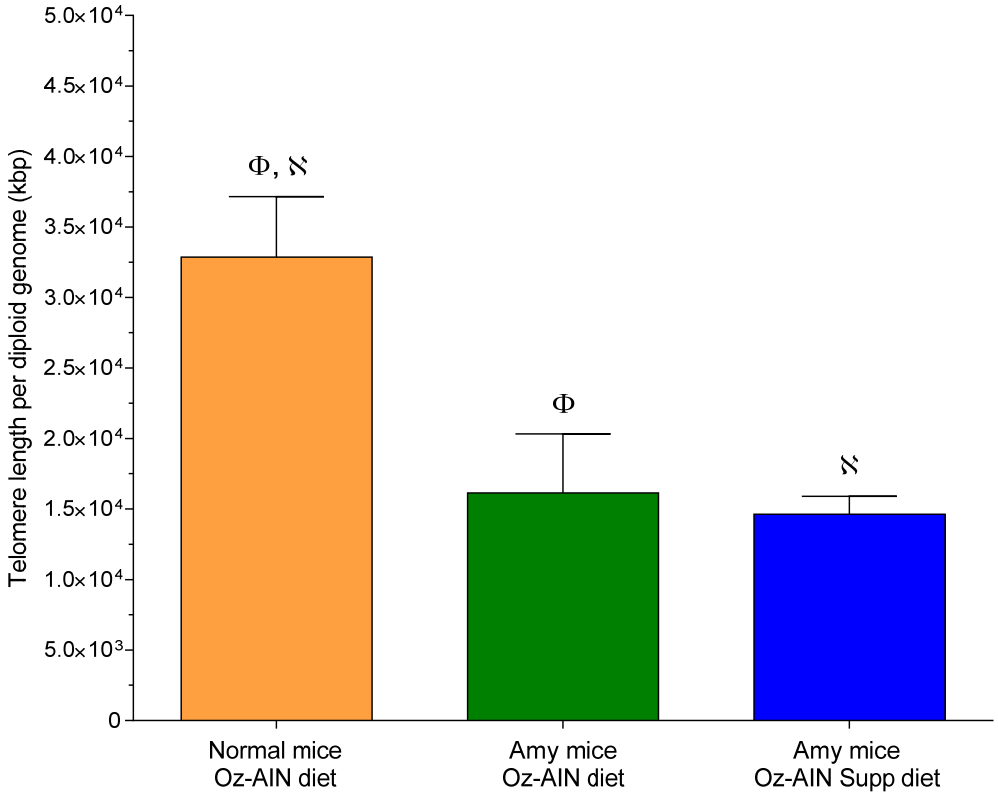


Figure 8. Telomere sequence length in brain tissue was measured and compared between 15 month old normal and Amy mice. Normal mice that were fed the Oz-AIN diet (orange bar, n=10), Amy mice that were fed the Oz-AIN diet (green bar, n=14), and Amy mice that were fed the Oz-AIN Supp diet (blue bar, n=11). Bars represent mean ±SEM. Bars with matching symbols are significantly different with Bonferroni post tests. (Φ) p=0.01. (ℵ) p=0.008.

Diet-type effects on telomere length.

An investigation into the effect of diet-type on telomere length in the brains of 15 month old Amy mice was made through a comparison of the telomere length (kbp) in the brains of 15 month old Amy mice that had been fed the AIN93-M diet or the Oz-AIN diet (Figure 9). Amy mice that were fed the Oz-AIN Supp diet were also included in these comparisons to demonstrate whether or not feeding Amy mice nutrient supplements call alleviate or prevent diet-type effects on telomere length in 15 month old Amy mice (Figure 9).

A one-way ANOVA did not detect significant differences in telomere lengths in the brains of Amy mice that were fed the AIN93-M diet, the Oz-AIN diet or the Oz-AIN Supp diet ($p=0.92$, Table 9, Figure 9). Bonferroni post tests confirmed that there were no differences in telomere length in the brains of 15 month old Amy mice that had been fed the AIN93-M diet or the Oz-AIN diet. This suggests that a high-fat diet does not alter telomere length in the Amy mouse brain. Bonferroni post tests also did not detect significant differences in telomere lengths in the brains from Amy mice that were fed the Oz-AIN diet or the Oz-AIN Supp diet, confirming that nutrient supplements do not alter telomere length in the brains of 15 month old Amy mice.

Summary of telomere length in the brains of 15 month old mice.

Collectively, these data indicated that genotype affects telomere length in brains of 15 month old Amy mice, and Amy mice have shorter telomeres than normal mice. These effects were not prevented with nutrient supplements. There were no differences in telomere lengths of Amy mice eating the AIN93-M diet, the Oz-AIN diet, or the Oz-AIN Supp diet. Telomere length in the brains of 15 month old Amy mice is not affected by a high-fat diet, sub-optimal micronutrient intake, or dietary supplementation.

Figure 9. The effect of diet-type on telomere length (kbp) in the brains of 15 month old Amy mice.

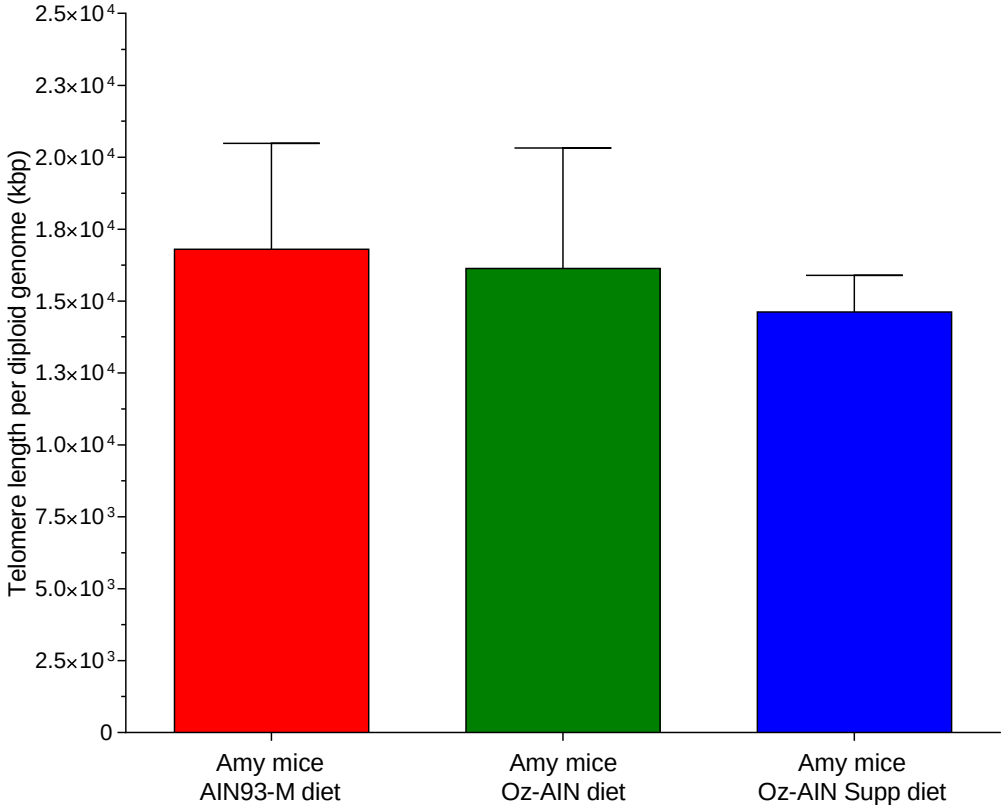


Figure 9. Telomere sequence length in brain tissue was measured and compared between 15 month old Amy mice that were fed the AIN93-M diet (red bar, n=11), Amy mice that were fed the Oz-AIN diet (green bar, n=14), and Amy mice that were fed the Oz-AIN Supp diet (bar, n=11). Bars represent mean ±SEM.

9.2.4. An Age-Genotype interaction on telomere length in the brains of 15 and 18 month old mice.

A two-way ANOVA revealed that age and genotype only accounted for 0.20% and 2.71% of the variance of telomere length in the brains of Amy and normal mice ($p=0.75$ and $p=0.25$ respectively, Figure 10). However, there was a significant age-genotype interaction that accounted for 33.33% of the overall variance of telomere length ($p=0.0002$).

At 18 months, telomeres in the brains of normal mice that were fed the Oz-AIN diet were significantly shorter than those in the brains of Amy mice that were fed the Oz-AIN diet ($p=0.01$, Table 9, Figure 10). However, at 15 months, telomeres in the brains of normal mice that were fed the Oz-AIN diet were longer than those of Amy mice that were fed the Oz-AIN diet, *albeit* non-significant ($p=0.10$, Table 9, Figure 10). This suggests that the genotype effect on telomere length in the brains of Amy mice is age-dependent.

Furthermore, aging appeared to have opposite effects on telomere length in normal and Amy mice. Telomeres in the brains of 18 month old Amy mice that were fed the Oz-AIN diet were significantly longer telomeres than those of 15 month old Amy mice that were fed the Oz-AIN diet ($p=0.02$, Table 9, Figure 10). However, telomeres in the brains of 18 month normal mice that were fed the Oz-AIN diet were shorter than those of 15 month old normal mice that were fed the Oz-AIN diet ($p=0.07$, Table 9, Figure 10). While this is not significant at $p<0.05$, it would be significant at $p<0.10$, suggesting that there were trends for telomere length to decrease with age in the brains of adult normal mice.

Figure 10. The Genotype-Age interaction on telomere length (kbp) in the brains of 15 and 18 month old normal and Amy mice.

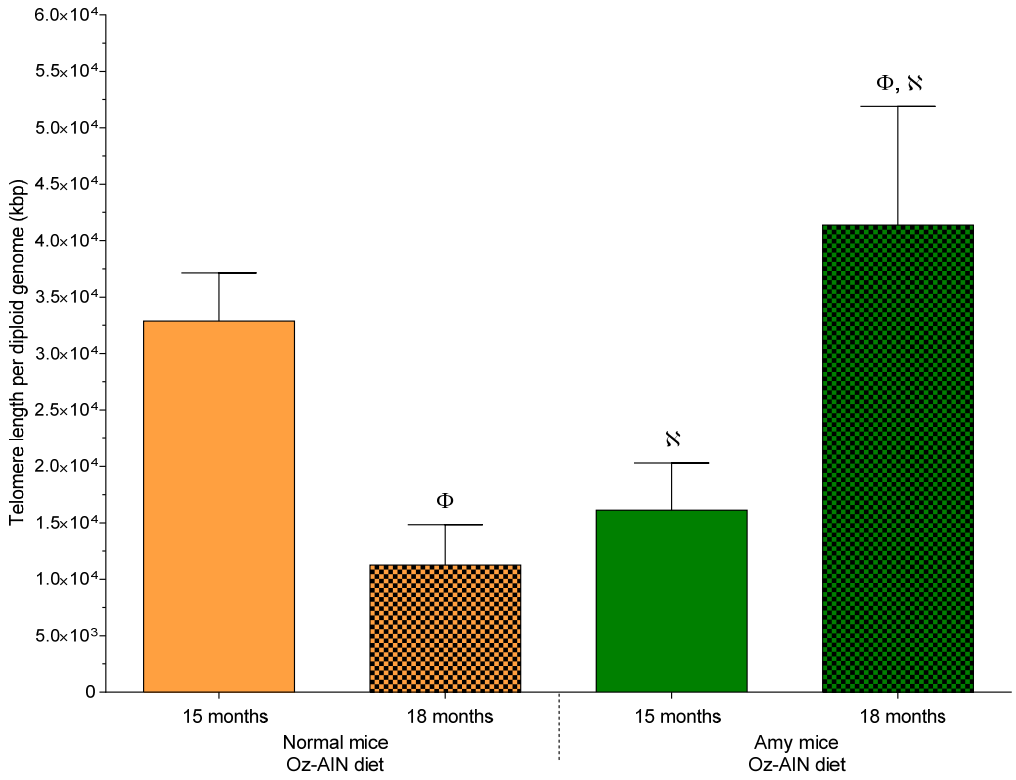


Figure 10. Telomere sequence length was measured in the brains of 15 and 18 month old normal and Amy mice that had been fed the Oz-AIN diet. 15 month old normal mice that were fed the Oz-AIN diet (plain orange bar, n=10), 18 month old normal mice that were fed the Oz-AIN diet (checked orange bar, n=8), 15 month old Amy mice that were fed the Oz-AIN diet (plain green bar, n=14), and 18 month old Amy mice that were fed the Oz-AIN diet (checked green bar, n=7). Bars represent mean ±SEM. There was a significant age-genotype interaction (p=0.0002). Bars with matching letters are significantly different with Bonferroni post tests. (Φ) p=0.01. (ℵ) p=0.02

9.2.5. An Age-Diet-type interaction on telomere length in the brains of 15 and 18 month old Amy mice.

A two-way ANOVA detected significant age and genotype effects that accounted for 9.99% and 9.67% of the variance of telomere length in the brains of Amy and normal mice ($p=0.04$ and $p=0.04$ respectively, Figure 11). There was also a significant age-genotype interaction that accounted for 10.75% of the overall variance of telomere length ($p=0.03$, Figure 11).

Age did not have an effect on telomere length in the brains of Amy mice that were fed the AIN93-M diet ($p>0.99$, Figure 11). However, age had a significant effect on telomere length in the brains of Amy mice that were fed the Oz-AIN diet. Telomeres in the brains of 15 month old Amy mice that were fed the Oz-AIN diet were significantly shorter than those in the brains of 18 month old Amy mice that were fed the Oz-AIN diet ($p=0.03$, Table 9, Figure 11). This suggests that the effect of age on telomere length in the brains of Amy mice is dependent on diet.

Furthermore, diet-type appeared to have different effects on telomere length at different ages. Diet-type did not have an effect on telomere length in the brains of 15 month old Amy mice ($p>0.99$, Table 9, Figure 11). However, the 18 month old Amy mice that were fed the Oz-AIN diet appeared to have longer telomeres than 18 month old Amy mice that were fed the AIN93-M diet ($p=0.06$, Table 9, Figure 11). While this is not significant at $p<0.05$, it would be significant at $p<0.10$, suggesting that diet-type had an effect on telomere length in the brains of 18 month old Amy mice.

Figure 11. The Diet-type-Age interaction on telomere length (kbp) in the brains of 15 and 18 month old normal and Amy mice.

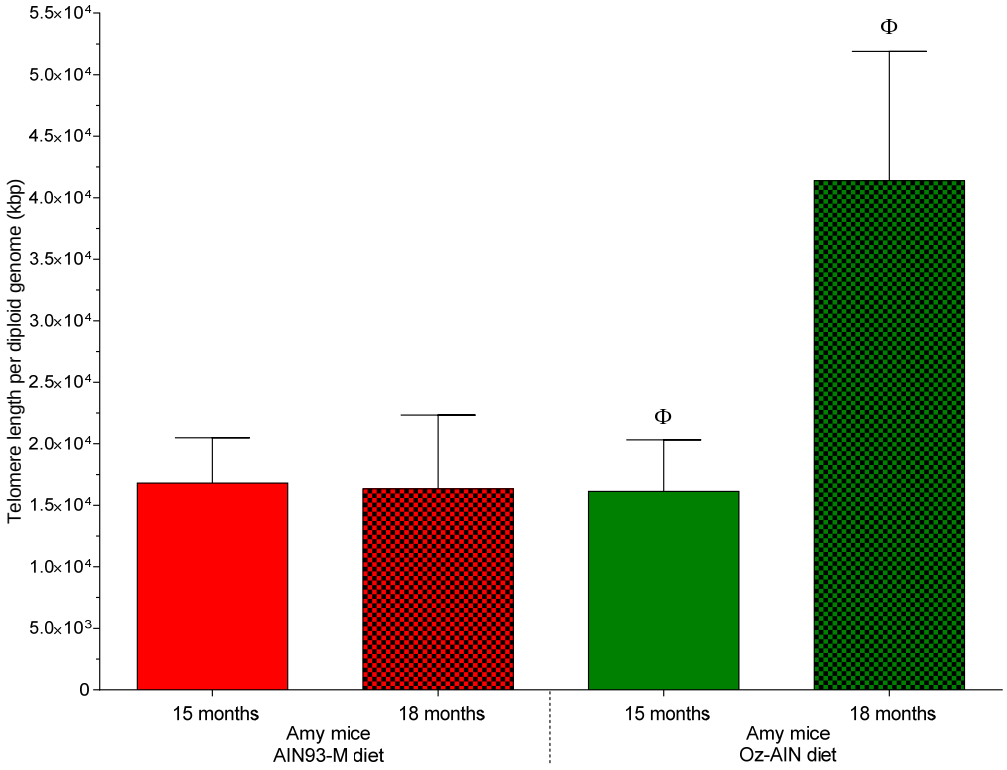


Figure 11. Telomere sequence length in brain tissue was measured and compared between diet-matched 15 and 18 month old Amy mice that had been fed either the AIN93-M diet or the Oz-AIN diet. 15 month old Amy mice that had been fed the AIN93-M diet (plain red bar, n=11), 18 month old Amy mice that had been fed the AIN93-M diet (checked red bar, n=9), 15 month old Amy mice that had been fed the Oz-AIN diet (plain green bar, n=14), and 18 month old Amy mice that had been fed the Oz-AIN diet (checked green bar, n=7). Bars represent mean ±SEM. There were significant diet-type effects (p=0.04), age effects (p=0.04) and a significant diet-type-age interaction (p=0.03). Bars with matching symbols are significantly different with Bonferroni post tests. (Φ) p=0.03.

9.2.6. Oxidative base damage in the brains of 15 and 18 month old normal and Amy mice.

Oxidative DNA damage was assessed in the brains of 15 and 18 month old 18 month old normal and Amy mice by measuring the number of 8-Oxo-2'-deoxyguanosine (8-oxo-dG) repeats using RT-qPCR as per section 9.1.6. (p. 556).

Table 10 shows the amount of 8-oxo-dG measured by RT-qPCR in the brains of 15 and 18 month old normal and Amy mice that were fed the AIN93-M diet, the Oz-AIN diet, or the Oz-AIN Supp diet. There are no measurements for 8-oxo-dG in the brains of 15 month old normal mice that were fed the AIN93-M diet or 18 month old Amy mice that were fed the Oz-AIN Supp diet (see 9.1.1., pp. 537-540). The following sections compare the values in Table 10 to demonstrate the effects of genotype and diet-type on age-induced changes in oxidative base damage in the brains of normal and Amy mice.

Table 10. Oxidative base damage in the brains of 15 and 18 month old normal and Amy mice.

	Normal mice AIN93-M diet	**Normal mice Oz-AIN diet**	**Amy mice AIN93-M diet**	**Amy mice Oz-AIN diet**	**Amy mice Oz-AIN Supp diet**
18 months	13.53 ±3.84	20.33 ±5.48	10.59 ±3.75	23.73 ±3.74	- - N/A - -
15 months	- - N/A - -	12.82 ±3.52	25.49 ±4.07	24.03 ±3.61	18.25 ±1.76

Oxidative base damage as indicated by the number of oxidised guanine bases (kb) in telomere repeats.

9.2.7. The effects of genotype, diet-type and a genotype-diet-type interaction on oxidative base damage in the brains of 18 month old mice.

Genotype effects on oxidative base damage in the brains of 18 month old normal and Amy mice.

A two-way ANOVA detected a significant diet-type effect on that accounted for 15.27% of the overall variance of oxidised base damage in telomeres in brains of 18 month old normal and Amy mice ($p=0.04$, Table 10, Figure 12). Genotype and a genotype-diet-type interaction only accounted for <0.1% and 1.54% of the overall variance respectively ($p=0.96$ and $p=0.50$, Table 10, Figure 12).

While Bonferroni post tests did not detect significant differences between groups, the mice that were fed the AIN93-M diet appeared to have less 8-oxo-dG than mice that were fed the Oz-AIN diet. This was not significant between normal mice ($p>0.99$, Table 10, Figure 12) or Amy mice ($p=0.41$, Table 10, Figure 12). This suggests that the AIN93-M diet may have had a protective effect against 8-oxo-dG accumulation in the brains of 18 month old normal and Amy mice.

There were no significant differences in the amount of oxidative damage measured in the brains of 18 month old normal and Amy mice that were fed the AIN93-M diet ($p>0.99$, Table 10, Figure 12), or between 18 month old normal and Amy mice that were fed the Oz-AIN diet ($p>0.99$, Table 10, Figure 12). This supports the findings of the two-way ANOVA described above, and suggests that irrespective of diet, genotype did not have an effect on oxidative base damage in the brains of 18 month old mice.

Figure 12. The effect of diet-type on oxidative base damage in the of 18 month old normal and Amy mice.

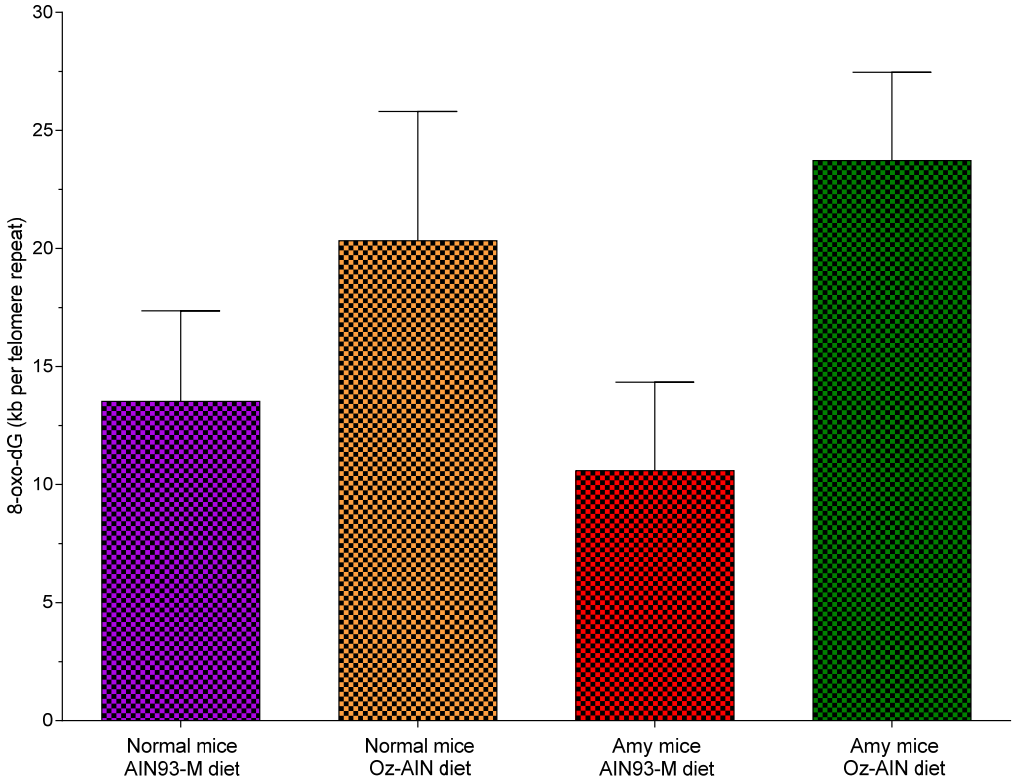


Figure 12. The number of oxidised guanine bases in telomere repeats was measured and compared between normal and Amy mice that were fed the AIN93-M diet or the Oz-AIN diet. Normal mice that were fed the AIN93-M diet (purple checked bar, n=10), Normal mice that were fed the Oz-AIN diet (orange checked bar, n=8), Amy mice that were fed the AIN93-M diet (red checked bar, n=9), and Amy mice that had been fed the Oz-AIN diet (green checked bar, n=7). Bars represent mean ±SEM. A two-way ANOVA detected significant diet-type effects (p=0.04). Bonferroni post tests did not detect significant differences between groups.

9.2.8. The effect of genotype and diet-type on oxidative base damage in the brains of 15 month old mice, and the potentially protective effects on nutrient supplements.

Genotype effects on oxidative base damage.

An investigation into the effect of genotype on oxidative base damage in 15 month old normal and Amy mice was made through a comparison of the amount of 8-oxo-dG per diploid genome in the brains of 15 month old Amy mice that had been fed the Oz-AIN diet (Table 10, Figure 13). The 15 month old Amy mice that had been fed the Oz-AIN Supp diet were included in these analyses to determine whether or not nutrient supplements can prevent genotype effects on oxidative base damage in the brains of 15 month old Amy mice.

A one-way ANOVA did not detect significant differences in the levels of oxidative base damage to telomeres in the brains of normal mice that were fed the Oz-AIN diet, Amy mice that were fed the Oz-AIN diet or Amy mice that were fed the Oz-AIN Supp diet ($p=0.06$, Table 10, Figure 13). However, while this is not significant at $p<0.05$, it would be significant at $p<0.10$, which suggested that there may be trends for some variation of oxidative base damage between groups. Further analysis with Bonferroni post tests revealed that there were trends for 15 month old Amy mice that were fed the Oz-AIN diet to have higher levels of oxidative base damage than the normal mice that were fed the Oz-AIN diet ($p=0.06$, Table 10, Figure 13).

These trends were not observed between Amy mice that were fed the Oz-AIN Supp diet and normal mice that were fed the Oz-AIN diet ($p=0.29$, Table 10, Figure 13). Oxidative base damage in the brains of 15 month old Amy mice that had been fed the Oz-AIN Supp diet was less than that of Amy mice that were fed the Oz-AIN diet

(p=0.29, Table 10, Figure 13), and greater than that of normal mice that were fed the Oz-AIN diet (p=0.29, Table 10, Figure 13). This suggests that, even though differences were not significant, nutrient supplements may tend to partially reduce oxidative damage in the Amy mice brain.

Figure 13. The effect of nutrient supplements on the genotype-induced difference in the levels of oxidative base damage in brain tissue from 15 month old normal and Amy mice.

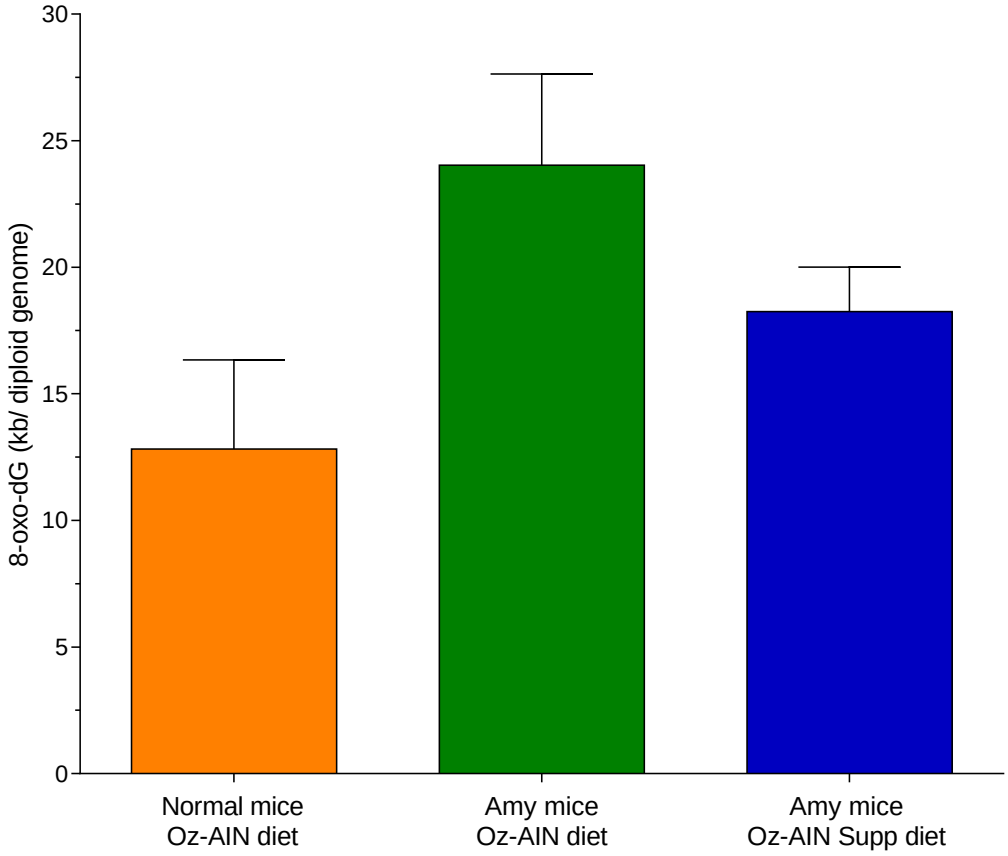


Figure 13. The number of oxidised guanine bases per diploid genome were measured and compared between 15 month old normal and Amy mice. Normal mice that were fed the Oz-AIN diet (plain orange bar, n=10), Amy mice that were fed the Oz-AIN diet (plain green bar, n=14), and Amy mice that were fed the Oz-AIN Supp diet (blue bar, n=11). Bars represent mean ±SEM.

Diet-type effects on oxidative base damage.

A one-way ANOVA did not detect significant diet-type effects on oxidative base damage in the brains of 15 month old Amy mice that had been fed the AIN93-M diet, the Oz-AIN diet, or the Oz-AIN Supp diet ($p=0.25$, Table 10, Figure 14). This suggests that feeding a sub-optimal diet to Amy mice does not have an effect oxidative base damage in the brains of Amy mice relative to Amy mice that were fed an ideal rodent diet.

There were no differences in the amount of 8-oxo-dG in the brains of Amy mice that were fed the AIN93-M diet or the Oz-AIN diet ($p>0.99$, Table 10, Figure 14). This indicates that a high-fat, suboptimal diet does not have an effect on the amount of oxidative damage in the brains of Amy mice.

While there appeared to be less 8-oxo-dG in the brains of Amy mice that were fed the Oz-AIN diet compared to mice that were fed either the AIN93-M diet or the Oz-AIN diet, this was not significant ($p=0.38$ and $p=0.60$ respectively, Table 10, Figure 14). The failure of this to achieve significance may be attributed to the great deal of variation within groups. It is suggested here that the nutrient supplements may have partially reduced oxidative base damage in the brains of Amy mice.

Summary of oxidative base damage in the brains of 15 month old Amy mice.

Collectively, these data suggest that there are trends for genotype to affect oxidative base damage in Amy mouse brains so that 15 month old Amy mice that have been fed the Oz-AIN diet have more oxidative base damage than normal mice that are fed the Oz-AIN diet. This may have partially been reduced by nutrient supplements, because although there were no significant differences in oxidative base damage between Amy mice that were fed the Oz-AIN diet or the Oz-AIN Supp diet, there

were also no significant differences or trends between Amy mice that were fed the Oz-AIN Supp diet and normal mice that were fed the Oz-AIN diet. Furthermore, Amy mice that were fed the Oz-AIN Supp diet may have had the least amount of oxidative damage when comparing oxidative damage in the brains of Amy mice that were fed the AIN93-M diet, Oz-AIN diet, or the Oz-AIN Supp diet. This suggests that while the high fat content of a diet did not affect telomere length, nutrient supplements may have been able to partially reduce oxidative damage in Amy mouse brains.

Figure 14. The effect of diet-type on oxidative damage in the brains of 15 month old Amy mice.

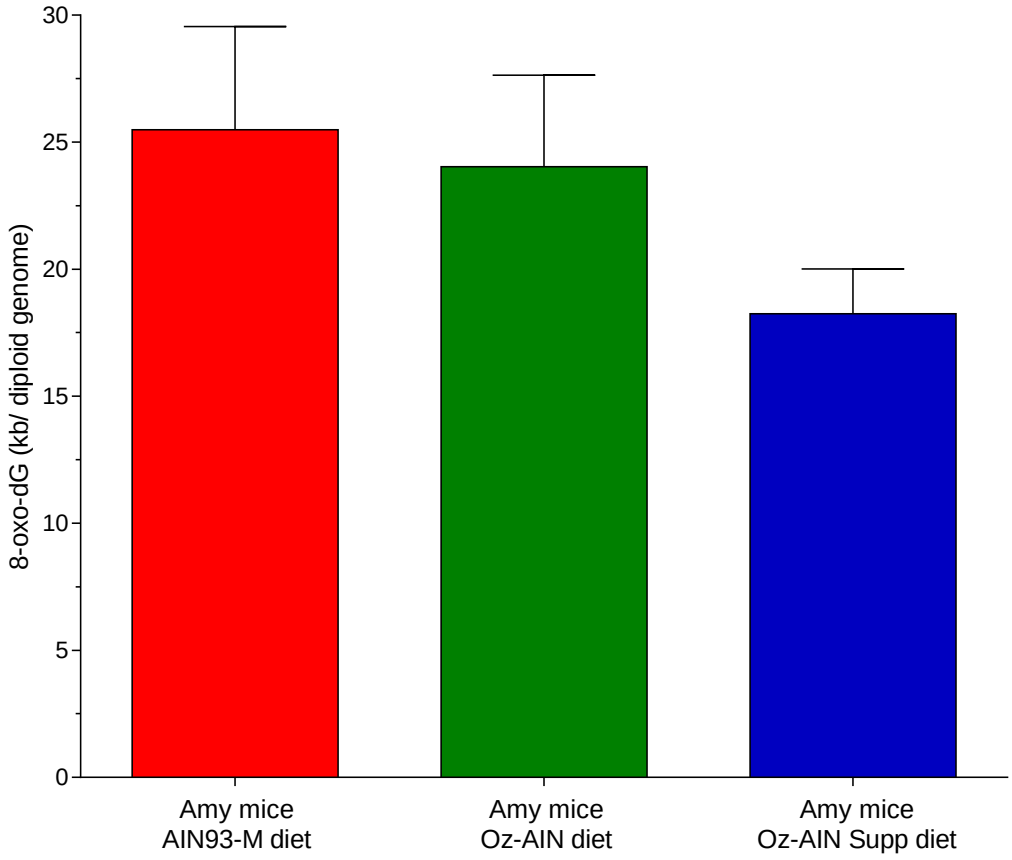


Figure 14. The number of oxidised guanine bases per diploid genome were measured and compared between 15 months old Amy mice that were fed either the AIN93-M diet (red bar, n=11), the Oz-AIN diet (green bar, n=14), or the Oz-AIN Supp diet (blue bar, n=11). Bars represent mean ±SEM.

9.2.9. An Age-genotype interaction on oxidative base damage in the brains of 15 and 18 month old mice.

A two-way ANOVA detected significant effects of genotype on oxidative base damage, which accounted for 10.73% of the overall variance between groups ($p=0.05$, Table 10, Figure 15). Age and an age-genotype interaction only accounted for 1.17% and 3.68% respectively, which was not considered to be significant ($p=0.50$ and 0.24, Table 10, Figure 15). However, Bonferroni multiple comparison post tests revealed that there were trends to suggest genotype effects on oxidative base damage between normal and Amy mice at 15 months but not 18 month old mice ($p=0.13$ and $p>0.99$ respectively, Table 10, Figure 15). This suggests that, although no significant interaction was detected by the two-way ANOVA, that genotype effects may be age dependent (Figure 15).

Bonferroni post tests confirmed that there were no significant differences between 15 and 18 month old normal mice ($p>0.99$, Table 10, Figure 15), or between 15 and 18 month old Amy mice ($p>0.99$, Table 10 Figure 15). This confirms the findings of the two-way ANOVA described above, that there were no age effects on the amount of oxidative base damage in the brains of normal or Amy mice.

Figure 15. The Age-Genotype interaction on oxidative base damage in the brains of 15 and 18 month old normal and Amy mice.

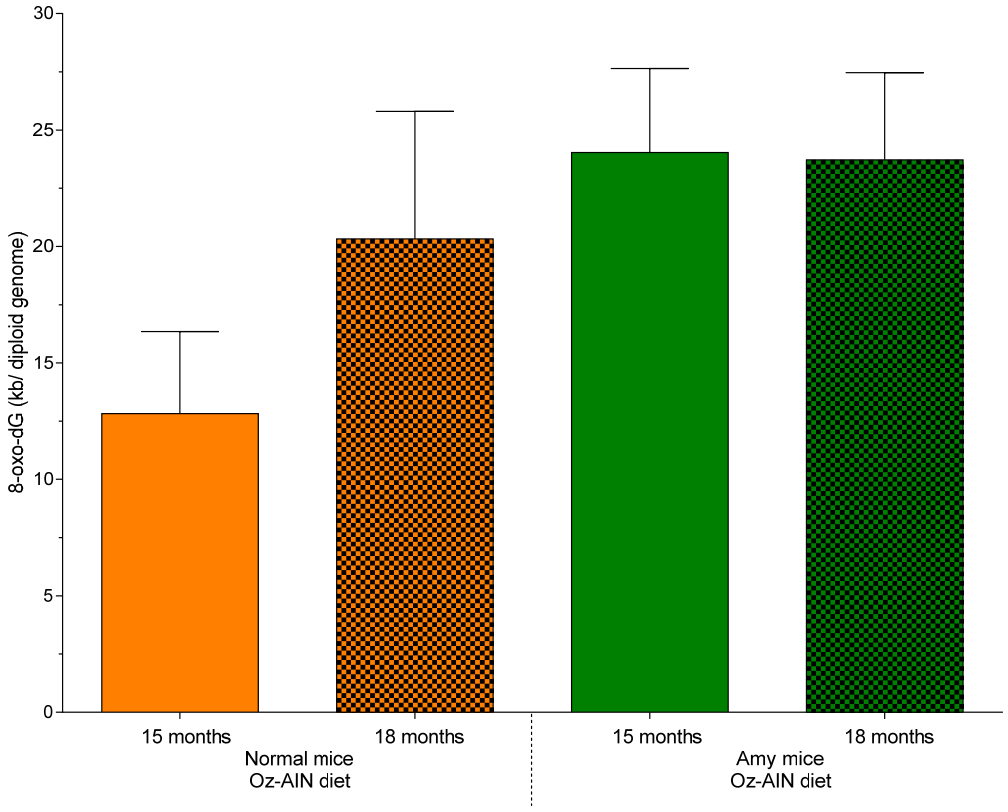


Figure 15. The number of oxidised bases per diploid genome was measured in the brains of 15 and 18 month old normal and Amy mice that were fed the Oz-AIN diet. 15 month old normal mice that were fed the Oz-AIN diet (plain orange bar, n=10), 18 month old normal mice that were fed the Oz-AIN diet (checked orange bar, n=8), 15 month old Amy mice that were fed the Oz-AIN diet (plain green bar, n=14), and 18 month old Amy mice that were fed the Oz-AIN diet (checked green bar, n=7). Bars represent mean ±SEM.

9.2.10. An age-diet-type interaction on oxidative base damage in the brains of 15 and 18 month old Amy mice.

A two-way ANOVA detected significant diet effects ($p=0.01$, Figure 16) and a significant diet-type-age interaction ($p=0.01$, Figure 16) on oxidative base damage in the brains of Amy mice. Diet-type accounted for 19.46% of the overall variation of oxidative base damage, and the diet-type-age interaction accounted for 18.49% of variation. Age effects only accounted for 0.55% of overall variation, and were not significant ($p=0.65$, Table 10, Figure 16).

Bonferroni post tests revealed that there was no significant differences in the brains of 15 month old Amy mice that had been fed the AIN93-M diet or the Oz-AIN diet ($p>0.99$, Table 10, Figure 16). However, there was significantly less oxidative base damage in the brains of 18 month old Amy mice that had been fed the AIN93-M diet compared to 18 month old mice that had been fed the Oz-AIN diet ($p=0.02$, Table 10, Figure 16). This indicates that the diet-type effects on oxidative base damage are age dependent, and that 18 month old mice are more susceptible to diet-type increases in oxidative base damage than 15 month old mice.

Figure 16. The Age-Diet-type interaction on oxidative damage in the brains of 15 and 18 month old normal Amy mice.

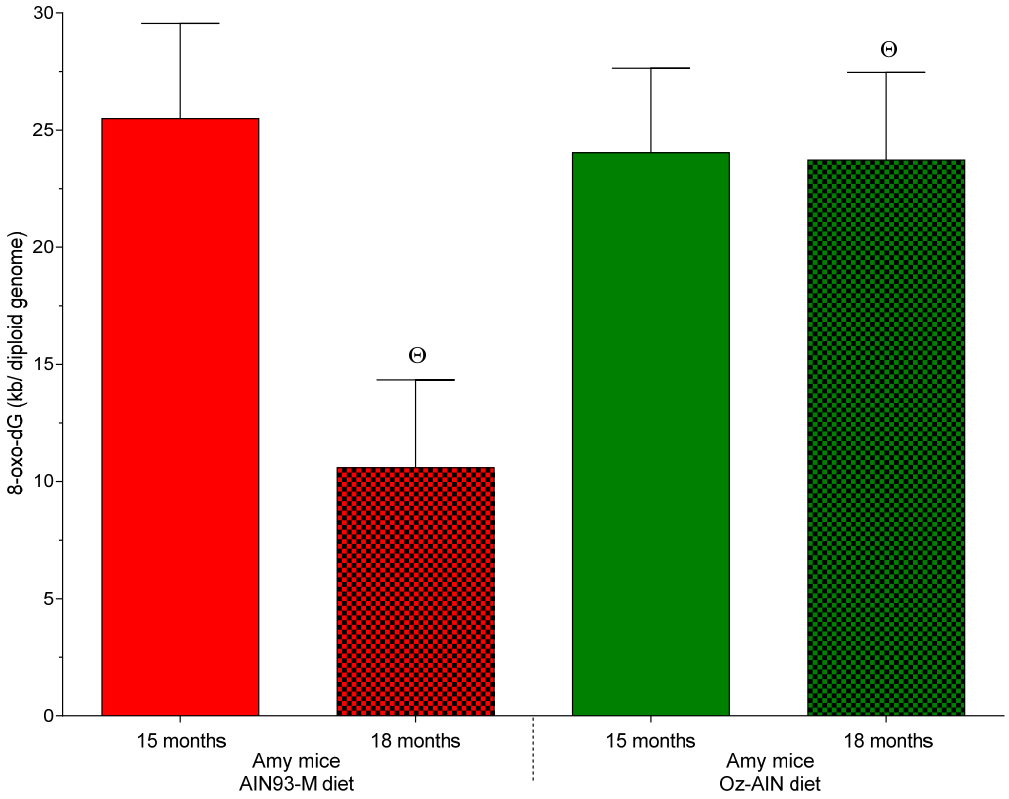


Figure 16. The number of oxidised bases per diploid genome was measured in the brains of diet-matched 15 and 18 month old Amy mice that were fed either the AIN93-M diet or the Oz-AIN diet. 15 month old Amy mice that had been fed the AIN93-M diet (plain red bar, n=11), 18 month old Amy mice that had been fed the AIN93-M diet (checked red bar, n=9), 15 month old Amy mice that had been fed the Oz-AIN diet (plain green bar, n=14), and 18 month old Amy mice that had been fed the Oz-AIN diet (checked green bar, n=7). Bars represent mean ±SEM. Bars with matching symbols are significantly different with Bonferroni post tests. (Θ) p=0.02.

9.2.11. Summary of results.

Table 12. Summary of results describing the effect of genotype on telomere length and oxidative base damage in the brains of normal and Amy mice.

	Telomere length	Oxidative base damage
15 months	There was a genotype effect on telomere length. Normal mice that were fed the Oz-AIN diet had longer telomeres than Amy mice that were fed the Oz-AIN diet. This was not prevented with nutrient supplements.	There were trends that suggested genotype affects oxidative base damage. Oxidative base damage in the brains of Amy mice that were fed the Oz-AIN diet was higher than that of normal mice. This may *partially* have been prevented with nutrient supplements.
18 months	There were no genotype effects on telomere length in the brains of Amy mice.	There were no genotype effects on oxidative base damage in the brains of Amy mice.
Age-Genotype interaction	There was a significant age-genotype interaction. The 18 month old Amy mice had longer telomeres than 15 month old Amy mice. However, no age effects were observed between 15 and 18 month old normal mice.	No significant interaction was detected, but genotype effects were age dependent. At 15 months, normal mice had less oxidative base damage than Amy mice. However, at 18 months of age, no genotype effects were observed.

Table 13. Summary of results describing the effect of diet-type on telomere length and oxidative base damage in the brains of normal and Amy mice.

	Telomere length	Oxidative base damage
15 months	There were no diet-type effects on telomere length in the brains of Amy mice.	There were no significant diet-type effects on oxidative base damage. However, there were trends that may have suggested that Amy mice fed the Oz-AIN Supp diet had less oxidative base damage than Amy mice fed the AIN93-M diet or the Oz-AIN diet.
18 months	There were no diet-type effects on telomere length in the brains of Amy mice.	There were trends for diet-type effects on oxidative base damage in the brains of Amy mice. Amy mice that were fed the AIN93-M diet had less oxidative base damage than Amy mice that were fed the Oz-AIN diet.
Age-Diet-type interaction	There was a diet-type-age interaction. 18 month old Amy mice fed the Oz-AIN diet had longer telomeres than 15 month old Amy mice fed the Oz-AIN diet. There were no differences between 15 and 18 month old Amy mice that were fed the AIN93-M diet.	There was a diet-type-age interaction. There was no difference in oxidative base damage between 15 month old Amy mice fed the AIN93-M diet or the Oz-AIN diet. However, at 18 months, Amy mice fed the Oz-AIN diet had more oxidative base damage than Amy mice fed the AIN93-M diet.

9.3. Discussion.

Genotype effects on telomere length in the brains of 15 month old mice.

The current study has reported that there was a genotype effect on telomere length in the brains of 15 month old normal and Amy mice that were fed the Oz-AIN diet. This could be reflective of microglial response in Amy and normal mouse brains. Flanary *et al.* report that telomeres in the brains from AD patients are shorter than those in the brains of healthy controls, despite both populations having similar levels of telomerase activity [538]. They propose that β-amyloid induced an inflammatory response in AD brains, increasing microglial proliferation. The accelerated microglial replication in AD brains enhances telomere attrition, and as a consequence microglia enter senescence long before microglia in the brains of normal, age-matched controls [538]. Flannery *et al.* propose that once microglia enter senescence, they become less effective at providing neurotrophic support or at clearing amyloid, which further accelerates β-amyloid neuropathology [538]. Similar events have also been observed in rats [538]. While inflammatory markers have not been assessed in the current study, it is possible that the same events are happening in the brains of the 15 month old Amy mice that were fed the Oz-AIN diet.

Chapter 4 reported that the majority of β-amyloid in the 15 month old Amy mouse brain was deposited as necrosis-associated β-amyloid deposits, and that these necrotic cells were most likely to be neurons. It is feasible to suggest that these neurons underwent necrosis as they no longer received microglial support. In the context of the reports from Flanary *et al.* it is possible that neurons may not have received microglial support as microglia had already entered cellular senescence.

However, without measurements of cell counts and microglial activation, this is hard to conclude.

An alternate suggestion for the cause of the genotype effects that were observed on telomere length in the brains of 15 month old mice may be due to oxidative damage. Oxidative stress is a characteristic feature of AD [242, 245]. Owing to the high number of guanine repeats in the telomere sequence (TTA GGG) telomeres are susceptible to oxidative DNA damage [245, 246]. This is consistent with the current study, where the brains of Amy mice that were fed the Oz-AIN diet had higher levels of oxidative base damage than normal mice.

Genotype effects on telomere length in the brains of 18 month old mice.

At 18 months of age, although differences were not significant, there were trends to suggest that Amy mice that were fed the AIN93-M diet had shorter telomeres than normal mice that were fed the AIN93-M diet. These trends are similar to the genotype effects that were observed in the brains of 15 month old mice that were fed the Oz-AIN diet. This suggests that the patterns for genotype effect on telomere length is maintained at 18 months of age between normal and Amy mice that are fed and ideal diet, compared to 15 month old mice that were fed a sub-optimal high-fat diet.

However, when mice were fed the sub-optimal Oz-AIN diet for 18 months, the inverse effect was observed. Rather than having shorter telomeres than normal mice, the 18 month old Amy mice that had been fed the Oz-AIN diet had significantly longer telomeres than normal mice that were also fed the Oz-AIN diet.

It is arguable that the calculated telomere length values represent telomere length per diploid genome, and therefore are reflective of telomere length of the remaining cells

in the mouse brain. If by 18 months of age, many of the microglial cells have undergone senescence, the relative percentage of neurons would have increased. Neurons, may be expected to have longer telomeres than other cells in the brain as they have not undergone high levels of proliferation. This is not to suggest that the number of neurons exceeds that of remaining glial cells. Microglia are one of the major cell types capable of replication in the CNS, and make up more than 10% of glial cells in the brain [116]. However, it suggests that neurons would make up a greater proportion of cells than they do at younger ages, which results in RT-qPCR detecting greater amounts of telomere sequence. This warrants future research, and could be addressed using confocal microscopy, to measure telomere content in nuclei, and co-localize these nuclei with different markers for neurons, glial cells and astrocytes in the brains of 18 month old Amy mice.

Another possibility is that telomerase, the enzyme responsible for maintaining telomere length, becomes overactive in microglia of Amy mouse brains, resulting in increased telomere length. Microglial activation and up regulation has been cited by many authors as a characteristic feature of the AD brain [542]. If there had been substantial up-regulation of microglia in the brains of Amy mice, then it would be expected that overall telomere length would rapidly decrease. This has been observed in some AD mouse models [538].

It may be possible that telomerase and other telomere repair machinery become overactive in response to the prolonged oxidative stress induced by the Amy genotype and by a high-fat diet. While this may result in repair of oxidative base damage so that it was the same as that observed in normal mice, the over compensation of repair machinery may have also resulted in increased telomere length. To demonstrate the effect of increased telomerase activity post-mitotic cells

in 15 and 18 month old mice, Bernardes de Jesus *et al.* injected normal mice with a virus that carried the mouse variant of telomerase and also had the ability to cross the blood brain barrier [543]. While they report that increased telomerase activity in the aging mouse brain did increase telomere length, the mice with longer telomeres were healthier, had better metabolisms, and improved cognitive functioning and physical performance [543]. This contrasts with the findings of the study described here, that the 18 month old Amy mice with increased telomere length demonstrated poorer cognitive performance and were obese. This does not necessarily mean that the increased telomeres in the Amy mouse brain were not associated with telomerase activity, but it does highlight the need to measure telomerase activity in the 18 month old Amy mouse brain before a conclusion can be made.

Telomere length at two different stages of aging.

Telomere length has been demonstrated to decrease with aging in the brains of normal rodents [538]. Flannery *et al.* attribute this to the loss of a sub-set of microglial cells that have long telomeres and are present in young, but not 18 month old brains [538]. This is consistent with the findings of the present study that telomere length decreased in the brains of aging normal mice that were fed the Oz-AIN diet.

However, as Amy mice that were fed the Oz-AIN diet aged, telomere length increased significantly. This demonstrates a clear genotype effect on age-related changes in telomere length in normal and Amy mice. As previously discussed this may be due to changes in cell population in the brains of Amy mice, or due to hyperactive DNA repair machinery in response to oxidative stress.

There were trends for increased oxidative base damage with aging in the brains of normal mice, but not in the brains of Amy mice. However, oxidative base damage

was never as high in the brains of normal mice as it was in the brains of Amy mice. This demonstrates a genotype effect on oxidative base damage in the brains of Amy mice. It is not clear whether or not this is a product of the β-amyloid pathology. It will be useful in the future to also measure oxidative base damage in the brains of younger mice, prior to the onset of β-amyloid pathology, to assess whether this is accelerated levels of oxidative damage that are attributed to aging or a product of DNA pathology.

The current data indicates effects of genotype on telomere length in the brains of 15 and 18 month old mice, and suggests that changes in telomere length with age differ dramatically between normal and Amy mice. However, this raises more questions than it answers. A possible explanation for the differences in telomere length with aging in normal and Amy brains may be differences in cell population. It will be important to discern in the future whether or not this decrease is associated with changes in glial or neuronal population, or whether it is due to some inherent feature within the cell itself. Telomerase activity in neuronal and microglial tissue of aging Amy and normal mouse brains may offer further insight into telomere maintenance changes that may be occurring, while a measure of the changing cell-type population numbers, and their specific telomere length, would also aid interpretation of these telomere length analyses.

Diet-type effects on telomere length in the brains of 15 month old mice.

Nutrient supplements and caloric restriction have been demonstrated to have an effect on telomere length in proliferative tissues of 18 month old mice. However, their effect on telomere length in the brains of 15 month old mice has not been demonstrated. The current study found no effect of diet on telomere length in the brains of 15 month old Amy mice.

Diet-type effects on telomere length in the brains of 18 month old mice.

While there was no significant effect of diet on telomere length in the brains of 18 month old mice, there were trends to suggest that mice that were fed the Oz-AIN diet may tend to have shorter telomeres. This suggests that the high-fat, sub-optimal Oz-AIN diet may have a detrimental effect on telomere length in the normal 18 month old mouse brain. Vera *et al.* recently demonstrated that reduced caloric intake was associated with increased telomere length in mice [244]. While they did not assess telomere length in the brain tissue of mice, they report that caloric restriction reduces age-related telomere attrition in peripheral blood leukocytes [244]. It may therefore be possible that the increased caloric intake of mice that were fed the Oz-AIN diet in the current study led to accelerated telomere shortening.

In contrast to the diet type effects on telomere length in 18 month old normal mice, the 18 month old Amy mice that were fed the Oz-AIN diet had significantly longer telomeres than Amy mice that were fed the AIN93-M diet. This suggests that although the sub-optimal diet appeared to increase telomere attrition in normal mice, it may increase telomere length in Amy mouse brains. As suggested earlier, this may be reflective of changes in cellular population in AD mouse brains.

Oxidative stress and inflammation are characteristic features in AD, and both elicit an increased microglial response. However, as microglia proliferate telomere length decreases and they lose their ability to protect neurons for increasing levels of oxidative stress. It is possible that in response to this, telomerase activity in neurons is increased, which then over-compensates for telomere attrition, and subsequently telomere length is increased. Marie-Egyptienne *et al*. demonstrated that telomerase inhibition leads to a rapid increase in expression of the catalytic subunit of telomerase, mTERT [544]. The compensatory response to telomerase deficiency

supports the current proposal that loss of telomerase in aging Amy mice may lead to an over-active response and increased telomere length. However, telomerase activity in the brains of 18 month old Amy mice that are fed either the AIN93-M diet or the Oz-AIN diet would need to be measured to confirm this hypothesis.

Changes in telomere length with aging later in life.

There were differing effects of diet-type on age-associated changes in telomere length in the brains of Amy mice. Telomere length in the brains of 15 month old Amy mice that were fed the AIN93-M diet were the same as those in the brains of 18 months old mice, despite there being lower levels of oxidative stress in the 18 month old mice. This most likely suggests that there is no correlation between oxidative base damage and age-associated changes in telomere length in the brains of mice that are fed a diet that meets all nutrient requirements.

However, the sub-optimal Oz-AIN diet did have an effect on age-associated changes in telomere length in the brains of Amy mice. Telomere length increased significantly with age in the brains of AD mice that were fed the Oz-AIN diet, despite there being no difference in oxidative base damage.

As previously discussed, it is possible that this is attributed to increased telomerase activity. On the other hand, it is also possible that the overall population of cells changed with aging, so that cell with longer telomeres (such as neurons, which are in a post-mitotic state) remain but other cells have died. To establish whether or not this has occurred, it would be necessary to first demonstrate that there were indeed sub-populations of cells that had longer telomeres, and then to determine whether or not they survived in Amy mouse brains until old age.

9.4. Conclusion.

Collectively, these data have shown that there are diet-type, genotype and aging effects on telomere length in the brains of normal and Amy mice. Telomere length decreases with age in the brains of normal mice that are fed a high-fat diet. Surprisingly, the opposite effect occurred in Amy mice. Although telomere length in the brains of Amy mice is shorter than that of normal mice at 15 months of age, telomeres in the brains of 18 month old Amy mice that were fed the Oz-AIN diet was significantly longer than those of normal mice. This suggests that telomere length increases with aging in the brains of Amy mice. It is suggested here that this may be a consequence of changes in cell population in the brains of aging mice, or due to over compensation by telomerase. Future studies are needed to determine this.

Chapter 10: Discussion and Conclusions

10.1. Evaluation of the diets designed in preparation of this thesis.

The current thesis had two aims. First, to establish the effect of an Australian-type diet on the behavioural deficits and β-amyloid neuropathology observed in a double transgenic mouse model of AD (APPswe/PSEN1dE9, called Amy mice). Second, to explore the potential for nutrient supplements to slow the progression of the behavioural deficits and β-amyloid neuropathology that are observed in the same mouse model. A small exploratory study on the effect of diet, genotype and age on telomere length and oxidative base damage to telomeres in the mouse brain has also been included in this project.

Commercially available synthetic 'Westernised' rodent diets are generally high in fat (30% - 40% kcal) at the expense of carbohydrates (40% - 50% kcal) [274, 280-282], and are considered to reflect the macronutrient intake of a diet typically consumed in Western cultures. However, these Westernised rodent diets do not necessarily characterise the *types* of fats in Western diets, which have 2.7 times more saturated fat than polyunsaturated fat, and an ω-3: ω-6 ratio of approximately 1:10 [262, 274, 280, 282]. Furthermore, the micronutrient content of synthetic Westernised rodent diets do not necessarily reflect the proportions of essential vitamins and minerals in the diets typically consumed in western cultures.

The Oz-AIN diet is an original rodent diet that has been developed in preparation of this thesis. The novelty of the Oz-AIN diet is that it reflects the macronutrient and micronutrient content of diets typically consumed by Australian women, who are twice as likely to develop AD by the time they are 65 than Australian men [172,

262]. The fat content has been carefully adjusted so that the Oz-AIN diet has a polyunsaturated: monounsaturated: saturated (P:M:S) ratio of 1.0: 2.4: 2.7, and an ω-3: ω-6 ratio of 1.0:10.0. Furthermore, micronutrient content of the Oz-AIN diet has been adjusted to reflect the degree that essential vitamins and minerals differ from recommended levels in the diets typically eaten by Australian women [260, 262]. This has created a high-fat diet that is low in essential nutrients such as calcium and folate, and high in essential nutrients such as niacin. Thus, the Oz-AIN diet is an ideal platform on which to test the effects of an Australian-type diet, alone or with nutrient supplements, on the β-amyloid neuropathology and behavioural deficits that are associated with AD in an AD mouse model.

A second novel rodent diet was constructed from the Oz-AIN diet and contained nutrient supplements that have been demonstrated to have beneficial effects against the β-amyloid neuropathology or behavioural deficits in AD in other dietary studies [86, 191, 214, 288, 296, 339]. This diet was called the Oz-AIN Supp diet.

The polyphenolic compounds curcumin and grape seed extract are powerful antioxidants that easily cross the blood brain barrier. These compounds are highly beneficial in reducing β-amyloid neuropathology when injected directly into the bloodstream of mice [545]. However, they are extremely hydrophobic and unstable, and therefore very little curcumin is absorbed from the diet [213]. Nonetheless, Wang *et al.* fed Amy mice diets that were supplemented with either grape seed extract or curcumin, and report that both supplements were able to reduce β-amyloid load and alleviate inflammation in Amy mouse brains [157]. Wang *et al.* suggest that these effects were mediated through interactions with aggregation and assembly of β-amyloid fibrils, however they do acknowledge that this was not observed in their previous studies [157]. Other research groups have also successfully demonstrated that dietary supplementation with curcumin or grape seed extract

reduce inflammation and β-amyloid burden in AD-type mice [332, 340]. These studies investigated the effects of supplementation against ideal or standard rodent diets. The benefits of curcumin and grape seed extract against β-amyloid deposition have not been investigated in the context of a typical Australian-type diet, which is high in total fats and contains sub-optimal levels of essential micronutrients. However, curcumin has been demonstrated to prevent cognitive dysfunction and improve synaptic plasticity in a mouse model of diet-induced metabolic disorder [546], suggesting that polyphenolic compounds may still exert beneficial neurological effects in the presence of a high-fat diet. Therefore, both curcumin and grape seed extract were added to the Oz-AIN Supp diet.

The ω-3 fatty acids docosahexaenoic acid and eicosapentaenoic acid and alpha-lipoic acid were also added to the Oz-AIN Supp diet, as they have anti-inflammatory properties and reduce cognitive decline in rodent models of AD [220, 346, 348]. Labrousse *et al.* report that feeding normal aged mice diets that are supplemented with docosahexaenoic acid and eicosapentaenoic acid for two months is enough to improve spatial memory and prevent age-related neuro-inflammation [220]. Furthermore, Moranis *et al*. report that feeding mice a diet that is adequate in polyunsaturated fatty acids prevents age-related emotional changes and conserved docosahexaenoic acid levels in the brain, but did not prevent age-related neuro-inflammation or decline in spatial memory [547]. This suggested that the ω-3 fatty acids play a role in spatial memory and that supplementation may have beneficial effects in AD.

The benefit of adding a combination of polyphenolic compounds and ω-3 fatty acids is that they may aid each other's roles in neuroprotection. The potent anti-oxidant capabilities of the polyphenolic compounds may reduce oxidation of docosahexaenoic acid, therefore reducing the risk of oxidative damage induced by

lipid oxidation. The use of fish oil may increase the bioavailability of curcumin, enhancing its antioxidant potential in the CNS [217, 225].

Vitamin B12 and folate have also been added to the Oz-AIN diet. However, this was done to explore the role of folate replenishment, rather than supplementation. Vitamin B12 or folate deficiency is associated with an increased risk of AD in humans and mice [290, 333, 336, 465]. This is thought to be primarily due to their roles in homocysteine metabolism. The Oz-AIN diet contains 57% of the recommended amount of folate. Both folate and vitamin B12 have been added to the Oz-AIN Supp diet to restore folate levels and promote homocysteine metabolism.

The Amy mice that were fed the Oz-AIN diet gained weight faster than normal mice that were fed the Oz-AIN diet or Amy littermates that were fed an optimal rodent diet, the AIN93-M diet. This indicated that both diet-type and genotype affect weight gain in Amy mice. Weight gain was alleviated with nutrient supplements, as the Amy mice that were fed the Oz-AIN Supp diet gained weight at the same rate as normal mice that were fed the Oz-AIN diet and Amy mice that were fed the AIN93-M diet.

Mody *et al.* investigated the effects of aging or a high-fat diet on weight gain of double transgenic (APP/PSEN1) and single transgenic (PSEN1) mice [305]. They report that, although aging did not have a significant effect on weight gain, APP/PSEN1 mice were more susceptible to high-fat diet induced weight gain than either normal or PSEN1 mice. Furthermore, markers for insulin resistance were elevated in APP/PSEN1 mice that were fed a control diet, and for all mice that were fed the high-fat diet. This indicated that the increased susceptibility to diet-induced obesity may be attributed to increased insulin resistance in APP/PSEN1 mice.

There is increasing momentum in the theory that AD and Diabetes Type II are related, and that they are both associated with insulin resistance and β-amyloid

secretion [548-550]. Insulin resistance leads to reduced glucose uptake and increases β-amyloid production and secretion from neurons [549, 550]. While insulin resistance was not assessed in the Amy mice that were fed the Oz-AIN diet, this provides a potential mechanism to explain the genotype effect on weight gain in Amy mice that were fed the Oz-AIN diet, and indicates a potential avenue for the protective effects of nutrient supplements.

The ω-3 fatty acid, eicosapentaenoic acid reduces weight gain and prevents insulin resistance in mice that are fed a high-fat diet [355]. Kalupahana *et al.* propose that adiponectin, which is an anti-inflammatory adipokine, plays a large role in the protective effects of eicosapentaenoic acid, as there is no difference in plasma adiponectin, glucose or insulin levels between mice fed a low-fat diet and mice fed the high-fat eicosapentaenoic acid supplemented diet [355]. In addition to the beneficial effects of ω-3 fatty acids against diet-induced insulin resistance, polyphenolic compounds have also been reported to alter glucose metabolism and prevent insulin resistance *in vitro* and *in vivo* [551]. While these studies have not utilized AD mouse models, they do suggest that ω-3 fatty acids and polyphenolic compounds have the potential to reduce insulin resistance and weight gain. In light of the reports from Mody *et al.* that APP mice are more susceptible to weight gain as a result of increased expression of markers for insulin resistance, this suggests that the ω-3 fatty acids and polyphenolic compounds in the Oz-AIN Supp diet may have played major roles in the prevention of genotype-induced obesity in Amy mice.

The ability of nutrient supplements to prevent diet-type effects on weight gain may be due to interactions between the ω-3 fatty acid and lipid metabolism. Long chain ω-3 fatty acid supplements increase lipid catabolism and reduce lipogenesis and prevent diet-induced obesity through regulation of adipose cell turnover [350, 352, 354]. Supplementing a high-fat diet with ω-3 fatty acids reduces inflammation and

oxidative stress that is associated with long term high-fat feeding [350-352, 355]. Cui *et al*. report that feeding mice dietary supplements of lipoic acid prevented diet-induced weight gain and upregulated expression of genes such as super oxide dismutase, peroxiredoxin-4 and glutathione peroxidise, all of which are involved in anti-oxidant defence systems [351].

In addition to this, B vitamins and polyphenolic compounds may have been able to prevent diet induced obesity through preventing high-fat diet-induced oxidative stress and inflammation. Park *et al*. fed mice high-fat diets that had been supplemented with *Sophora japonica L*, which is a plant enriched in polyphenolic compounds that is used in traditional Chinese medicine. They report that not only was there a dose-dependent decrease in weight gain, but the polyphenolic supplements also reduced serum cholesterol and low density lipoprotein levels [358]. This indicates that the ability of polyphenolic compounds to protect against high-fat diet induced weight gain may be through altered lipid metabolism. This is consistent with reports from others that polyphenolic compounds can accelerate lipid metabolism [360].

Weight gain and obesity are risk factors for AD [550]. The finding that nutrient supplements were able to prevent genotype and diet-type effects on weight gain posed the question *Could the nutrient supplements alleviate other risk factors that were associated with AD?* Or more specifically *Could nutrient supplements alleviate features of AD, such as cognitive deficits or β-amyloid pathology?* These were already within the aims of the current thesis. However, the surprising finding that the nutrient supplements could also alter *risk factors* for AD indicated that they may alter *some* aspects of the disease process.

The aims of the study were addressed using two separate cohorts of mice. The first cohort of mice consisted of normal mice and Amy mice that were each fed either the

AIN93-M diet or the Oz-AIN diet. These studies aimed to demonstrate the potentially detrimental effects of the sub-optimal Oz-AIN diet and genotype on physiology (Chapter 2), β-amyloid neuropathology (Chapter 4) and behavioural deficits (Chapters 5 and 7) that are associated with AD.

The second cohort of mice consisted of three groups of Amy mice that were fed the AIN93-M diet, the Oz-AIN diet or the Oz-AIN Supp diet. A group of normal mice that were fed the Oz-AIN diet served as a control. These studies aimed to demonstrate the potentially protective effect of nutrient supplements against diet-type and genotype induced changes in physiology (Chapter 3), β-amyloid neuropathology (Chapter 4) and behavioural deficits (Chapters 6 and 8) that are associated with AD.

Studies that used the first cohort of mice ran for 18 months. However, there were difficulties managing over-grooming after 15 months. Therefore, the studies that used the second cohort of mice only ran for 15 months. While this was not an original part of the study design, it provided the opportunity to compare β-amyloid neuropathology in mice at two ages that are the equivalent of 10 human years apart, long after β-amyloid neuropathology had been established [132].

10.2. Beta-amyloid pathology

10.2.1. Low power microscopy

Low power microscopy indicated that diet-type did not have an effect on the amount of β-amyloid in the brains of 15 or 18 month old Amy mice. This was unexpected as others have reported (i) that high-fat diets enhance β-amyloid neuropathology in Amy mice [167, 197, 369, 387, 388, 459, 472]; and (ii) that the nutrient supplements docosahexaenoic acid and curcumin, both of which are in the Oz-AIN Supp diet, reduce β-amyloid neuropathology [86, 214, 217, 219].

Park *et al.* demonstrated that a high-fat diet induced hypercholesterolemia and induced β-amyloid neuropathology and microglial activation in normal mice, and that these events were enhanced in AD mice [472]. The differences in mouse model and β-amyloid models used may explain the discrepancies in results of Park *et al.* and the current study. Park *et al.* investigated the effects of diet using ApoE deficient mice. ApoE is an apoplipoprotein that plays an important role in lipid metabolism, transport and clearance from the brain [552]. Therefore, it would be expected that the mice used by Park *et al.* would have altered lipid metabolism compared to the Amy mice used in the current study. Furthermore, Park *et al.* injected amyloid(20-35) directly to the cerebrum of ApoE mice, to investigate the effects of diet on β-amyloid accumulation. This may have had a profound effect on the differences in the effects of a high-fat diet on β-amyloid deposition in the brains of mice.

However, even studies that have used transgenic models of mice differ from the current study. Kinoshita's research group at the Kyoto University Graduate School of Medicine has intensively studied the effect of lifestyle (environmental enrichment, exercise, diet) on high-fat induced amyloid deposition in mice [167, 369, 459]. Recently, they have reported that feeding $APP_{SWE/IND}$ mice a high-fat diet for 20 weeks increases levels brain of β-amyloid 40 and β-amyloid 42, and that this is prevented by continual exercise [459]. Using low power microscopy, they demonstrate that a high-fat diet increased β-amyloid deposit size and number in the brains of 7-8 month old $APP_{SWE/IND}$ mice.

Li *et al.* report that the mouse model used can have profound effects on outcome of AD pathology. They demonstrated that five different transgenic mouse models had different neuropathologies and different ectopic cell cycle events, and may therefore represent different phenotypes of AD [131]. The studies carried out in preparation of

the current thesis have used a double transgenic mouse model of AD, whilst Kinoshita's research group use a single transgenic mouse model for AD. Although both mouse models involve over expression of amyloid precursor protein, they may reflect different aspects of the disease. However both mouse models develop β-amyloid deposits around 5-7 months [553, 554], which suggests that neither is a more aggressive β-amyloid model than the other.

Another potential reason for the differences in β-amyloid deposition that was observed by Kinoshita's research group and the present study may be the antibody used. The studies described in the current thesis used the 6E10 antibody to detect amyloid deposits. 6E10 is a monoclonal antibody that is directed to the N terminus of β-amyloid, and recognises the same short amino acids sequences as antibodies generated from active immunisation with β-amyloid 42 [453]. Therefore, 6E10 positive staining is interpreted as representative of the β-amyloid deposits that are similar to those observed in human AD, and is commonly used in AD research [106, 131, 453, 458, 459]. The studies by Kinoshita's research group also used 6E10, indicating that the antibody used can not account for the differences.

A third possibility to explain why Kinoshita's research group have been able to detect high-fat diet induced increases in amyloid deposition may be due to the age at which the brains were examined. They fed mice their diets from 2-3 months old to 7-8 months old. The current study fed mice their respective diets for 15 and 18 months, starting immediately after weaning. As stated earlier, both mouse models develop β-amyloid deposits around 5-7 months old. It is possible that a high-fat diet may *accelerate* β-amyloid pathology, but by 15-18 months old β-amyloid deposition in the brains of all AD mice would be equal – irrespective of diet. The result of this would be that although differences may be detected in the early stages of amyloid

deposition (at 5-7 months), differences in β-amyloid level in aged mice would be much harder to detect. This hypothesis could be confirmed by evaluating β-amyloid neuropathology of Amy mice fed the AIN93-M diet, the Oz-AIN diet or the Oz-AIN Supp diet at younger ages, and monitoring changes in β-amyloid deposition as the disease progresses.

The Oz-AIN diet is a novel diet as the P:M:S ratio has been designed to reflect the fat content in a typical Australian-type diet (1.0:2.4:2.7) [262]. Therefore, the monounsaturated fat content and the saturated fat content were relatively equal. Typical Westernised high-fat diets contain predominantly saturated fats. Using an *in vitro* model of AD, Amtul *et al*. report that saturated fats, up-stream ω-3 polyunsaturated fatty acids and arachadonic acid increase production and secretion of the two species of amyloid that are associated with AD, β-amyloid 40 and β-amyloid 42. The long chain ω-3 polyunsaturated fatty acids and monounsaturated fatty acids, on the other hand, reduced production and secretion of β-amyloid 40 and β-amyloid 42 [342]. It may therefore be plausible to suggest that while the high saturated fat content of the Oz-AIN diet had the potential to increase β-amyloid production and deposition this may have been partially counteracted by the relatively high monounsaturated fat content.

However, this does not explain why the brains of mice that were fed the Oz-AIN Supp diet had a similar number of β-amyloid deposits as mice that were fed the Oz-AIN diet. Owing to previous studies that have reported on the beneficial effects of the long chain ω-3 fatty acid docosahexaenoic acid in prevention of β-amyloid production, secretion and aggregation [86], it was anticipated that mice that were fed the Oz-AIN Supp diet would have fewer amyloid deposits than other Amy mice. This was not observed in the current study. A potential explanation of this is that the

high saturated fat content of the Oz-AIN diet led to enhanced oxidation of docosahexaenoic acid, thereby preventing its beneficial effects.

Alternately, these results could reflect a desensitisation to the beneficial effects of dietary docosahexaenoic acid after prolonged feeding. Optimal uptake of dietary docosahexaenoic acid into the brain is achieved after 2-3 months feeding [220]. However, transport of docosahexaenoic acid across the blood brain barrier has been reported to decrease after prolonged supplementation [396]. Therefore, it is possible that after feeding the Amy mice supplemented diets for 15-18 months, the potentially beneficial effects of docosahexaenoic acid on amyloid deposition may have been lost due to reduced transport across the blood brain barrier. To confirm this, assessments of cerebral docosahexaenoic acid levels and β-amyloid deposition in the brains of mice at a much younger age need to be measured.

10.2.2. Confocal microscopy.

Confocal microscopy revealed that there are three separate and distinct pathologies of β-amyloid deposition: (i) dense, intracellular deposits of β-amyloid that were associated with necrosis; (ii) large diffuse extracellular deposits of β-amyloid that contained small, intact nuclei; and (iii) small diffuse extracellular deposits that were associated with the blood brain barrier. The latter of these deposit types will be called BBB-associated deposits for the remainder of this chapter.

β-amyloid and necrosis.

Necrosis is a passive, uncontrolled form of cell death that occurs in response to cytotoxic stress such as interference with energy supply of a cell or direct damage to cellular membranes. Necrosis is characterised by cellular swelling, swollen and ruptured lysosomes, disintegrated nuclei, and ruptured cell membranes. Rupture of

the cellular membrane causes intracellular contents to spill into the extracellular space, exposing surrounding cells to toxic intracellular constituents, which induces an inflammatory response [555, 556].

Apoptosis, on the other hand, is a tightly controlled form of cell death that occurs as a natural process in development or controlled clearance of diseased or damaged cells [555, 557, 558]. For example, the regulation of neuronal loss during embryonic development is crucial for formation and stabilisation of connections within the brain, and as such results in apoptotic death of almost 70% of neurons [559].

Morphologically, apoptosis is characterised by shrinkage and condensation of cells, tightly packed organelles, chromatin condensation and DNA fragmentation. Cells break into membrane bound compartments, called apoptotic bodies, which are then engulfed by phagosomes [555, 559]. In the CNS, apoptotic neurons are phagocytosed by microgliocytes [559].

While apoptosis can be induced by either intrinsic or extrinsic mechanisms, both pathways lead to activation of the caspase cascade. The control and regulation of the intrinsic pathway is mediated through maintenance of the homeostasis between pro-apoptotic and anti-apoptotic members of the Bcl-2 family [555, 557]. The Bcl-2 family play primary roles in mitochondrial membrane stability. Damage or stress to mitochondria results in changes to mitochondrial membrane permeability, release of Cytochrome C into the cytoplasm and activation of the caspase cascade [557]. Owing to the fact that mitochondrial stress plays a large role in AD pathology, it is plausible that neuronal loss in AD is mediated through apoptosis, not necrosis.

Kudo *et al*. report that oligomeric β-amyloid induces neuronal death in a manner that is dependent on the prion protein [560], which has been demonstrated to initiate apoptosis *in vivo* [561]. Kudo *et al.* demonstrated that oligomeric β-amyloid

decreases expression of anti-apoptotic Bcl-2 and increases expression of pro-apoptotic Bin, which increases expression and activation of the pro-apoptotic Bax protein. This is consistent with reports from others that β-amyloid interacts with Bcl-2 and Bin to initiate the caspase cascade and cell death [562]. Others have reported that β-amyloid activates the caspase cascade through interactions with Death Receptor 6 [563, 564]. Collectively, these studies add support to the proposal that the mechanism by which β-amyloid induces neuronal cell death in AD is through activating intrinsic pro-apoptotic pathways.

However, in the current study, cells that were associated with high levels of intracellular β-amyloid appeared to be undergoing necrosis. They were large and diffuse, with no defined nuclei, and appeared to be 'spilling' contents into the extracellular space, all of which are consistent with necrotic morphology [555, 556].

This indicates that in the Amy mouse model, intracellular amyloid does not induce apoptosis but induces necrosis.

Although all neurons in the Amy mouse brain produced β-amyloid, there were some neurons that stained brighter for β-amyloid than others under confocal microscopy. Of these neurons, some had intact nuclei and were surrounded by diffuse β-amyloid staining. These neurons were potentially the source of β-amyloid that formed the large diffuse deposits. This is consistent with reports from others that neurons secrete intracellular β-amyloid into the extracellular space [565, 566]. Some neurons did not secrete β-amyloid, leading to a build up of intracellular β-amyloid. These neurons appeared to undergo necrosis.

Intracellular accumulation of β-amyloid occurs in the early stages of AD [519]. In a normal, healthy neuron clearance of β-amyloid occurs by proteolysis in late endosomes and lysosomes [19, 567]. However, high-levels of intracellular β-amyloid

can overwhelm the proteolytic machinery in endosomes and lysosomes, resulting in β-amyloid accumulation [19]. This may cause lysosomes to swell and rupture, releasing hydrolytic contents into the cytoplasm resulting in necrosis [568]. This provides a potential mechanism for how the build up of intracellular amyloid led to necrosis in Amy mice.

β-amyloid at the Blood Brain Barrier.

The blood brain barrier is a specialized network of vascular endothelial cells, astrocyte end foot processes and pericytes [177, 569, 570]. The main role of the blood brain barrier is to regulate the movement of molecules and cells into and out of the brain, therefore protecting the brain from potentially neurotoxic substances within the blood stream [177, 433, 570]. Beta-amyloid can be transported across the blood brain barrier by receptor mediated transcytosis via one of two receptors. The receptor for advanced glycation end products (RAGE) facilitates β-amyloid transport into the brain, and low-density lipoprotein receptor-related protein-1 (LRP-1) facilitates β-amyloid clearance out of the brain [177, 571].

In AD brains, RAGE and LRP-1 distribution changes so that there is an increase in RAGE along the blood brain barrier, and a decrease in LRP-1 [177, 571]. The consequence of this is that β-amyloid clearance is reduced, while movement into the brain is enhanced, enabling a build up of cerebral β-amyloid.

Sutcliffe *et al*. report that inhibition of synthesis of β-amyloid in the liver of transgenic mice lowered peripheral and cerebral β-amyloid levels [428]. This suggests that intra-cerebral β-amyloid deposits may be due to β-amyloid invasion across the blood brain barrier from the periphery [428]. This may be a result of increased RAGE along the blood brain barrier or increased blood brain barrier permeability or both.

Consistent with this, Donahue *et al.* confirmed that the distribution of LRP-1 and RAGE in AD brains differs from normal brains. However, they did not detect differences in co-localisation between β-amyloid and either LRP-1 or RAGE in AD brains compared normal brains [571]. This suggests that although there are differences in blood brain barrier permeability in the brains of AD patients relative to controls, they do not interfere with β-amyloid transport into or out of the brain.

The presence of the BBB-associated deposits had not been anticipated, and the study had not been designed to investigate their origin. Chapter 4 presented three potential origins for the BBB-associated deposits:

i) The BBB-associated deposits may be representative of β-amyloid clearance, whereby β-amyloid is transported across the blood brain barrier by LRP-1, and then transported to the liver where it is broken down [424, 426].
ii) The BBB-associated deposits may be representative of invasion of β-amyloid across the blood brain barrier by RAGE, which may a consequence of a dysfunctional blood brain barrier [427].
iii) The BBB-associated deposits may be representative of amyloid precursor protein, and reflect amyloid precursor protein that has been dislodged from the neuronal membrane by docosahexaenoic acid interactions with lipid rafts.

The Oz-AIN diet did not affect the number of any of the β-amyloid deposit types in Amy mouse brains. However, nutrient supplements did alter the ratio of necrosis-associated deposits: diffuse deposits: BBB-associated β-amyloid deposits in the brains of Amy mice. This was possibly due to an increase in the number of BBB-associated deposits, because these deposits were more frequent in the brains of Amy mice that were fed the Oz-AIN Supp diet compared to Amy mice that were fed the

Oz-AIN diet or the AIN93-M diet. This indicates that a component of the Oz-AIN Supp diet altered movement of β-amyloid across the blood brain barrier.

Hyperhomocysteinemia induces oxidative stress and impairs blood brain barrier permeability [572]. Kalani *et al*. demonstrated that these events can be prevented in mice by dietary supplementation with folic acid [572]. Similarly, curcumin has been demonstrated to maintain blood brain barrier integrity in rodent models of stroke [573]. These studies do not necessarily indicate whether the BBB-associated β-amyloid was representative of invasion or clearance of β-amyloid across the blood brain barrier [572]. However, they do suggest that folate and curcumin in the Oz-AIN Supp diet had the potential to maintain blood brain barrier integrity, therefore potentially preventing unwanted movement of amyloid into the cerebral parenchyma.

Dietary docosahexaenoic acid has beneficial effects on blood brain barrier function through enhanced cerebrovascular volume and reduced plasma β-amyloid levels in AD mice and AD patients [86, 450]. Increased ω-3 polyunsaturated fatty acid intake is associated with decreased plasma β-amyloid 40 and β-amyloid 42 in the brains of aged, cognitive healthy adults [450]. This suggests that docosahexaenoic acid supplements enhance clearance of β-amyloid across the blood brain barrier, and that the BBB-associated deposits were representative of β-amyloid clearance. However,

Diets that are high in saturated fats enhance mitochondrial dysfunction and oxidative stress in the blood brain barrier [449]. Although the Oz-AIN Supp diet contained high levels of anti-oxidants, they may not have been able to prevent the levels of oxidative damage induced by a high-fat diet. Furthermore, docosahexaenoic acid and eicosapentaenoic acid are susceptible to oxidation in conditions of high-oxidative stress, and undergo rapid oxidation upon uptake by the brain [224]. Together, the saturated fats and the oxidised fatty acids may increased oxidative stress at the blood

brain barrier, leading to increased permeability and easier movement of β-amyloid into the brain [177].

I have shown here that the β-amyloid pathology in the Amy mouse model includes high levels of necrosis that may be a result of excessive accumulation of β-amyloid. The studies into the effects of diet on β-amyloid neuropathology in Amy mice have revealed that there were no detrimental effects of the Oz-AIN diet on β-amyloid deposition in the Amy mouse brains. However, the Oz-AIN Supp diet increased the number of BBB-associated β-amyloid deposits, potentially as a result of increased oxidative damage and invasion of β-amyloid from the peripheral blood stream.

10.3. Behavioural deficits.

10.3.1. The potentially detrimental effects of the Oz-AIN diet on behavioural deficits in normal and Amy mice.

Spatial learning, spatial memory and olfactory dysfunction have all been reported in human AD patients and mouse models of AD [82, 83, 107, 108, 120, 121]. The studies that investigated the effect of the Oz-AIN diet on spatial learning, spatial memory and olfactory abilities used the first cohort of mice. These studies suggested that each of these behavioural deficits were independently affected by genotype, diet-type and age, irrespective of changes in either of the other two behaviours. The findings from tests investigating the effects of the Oz-AIN diet on behavioural abilities of mice from the first cohort are outlined in Summary One, on page 616.

Summary One: The effects of genotype and the sub-optimal Oz-AIN diet on the behavioural deficits in Amy mice.

Spatial learning (Chapter 5)

12 months: No diet-type or genotype effects were detected on overall improvements. However, the Amy mice fed the Oz-AIN diet were the only mice that made significant improvements throughout the training period, suggesting that the Oz-AIN diet *conserved spatial learning* in 12 month old Amy mice.

15 months: Irrespective of diet or genotype, mice did not demonstrate intact spatial learning abilities.

18 months: Genotype and diet-type had an effect on spatial learning. Amy mice had poorer spatial learning abilities than normal mice. The Oz-AIN diet may *conserve spatial learning*, as normal and Amy mice fed the Oz-AIN diet made greater improvements than genotype matched mice.

Spatial memory (Chapter 5)

12 months: Genotype and diet-type had a beneficial effect on spatial memory. The Amy mice fed the Oz-AIN diet had better spatial memory than normal mice fed the Oz-AIN diet or Amy mice fed the AIN93-M diet.

15 months: Diet-type and genotype had a detrimental effect on spatial memory in Amy mice. Amy mice that were fed the Oz-AIN diet failed to demonstrate spatial memory at 15 months.

18 months: There were no significant genotype or diet-type effects detected. However, only mice fed the Oz-AIN diet demonstrated intact spatial memory

Olfaction (Chapter 7)

6 months: Genotype effects were dependent on diet-type. Amy mice fed the Oz-AIN diet had poorer olfactory abilities than normal mice fed the Oz-AIN diet. Normal and Amy mice fed the AIN93-M diet has similar olfactory abilities.

12 months: Genotype effects were dependent on diet-type. Amy mice fed the Oz-AIN diet had poorer olfactory abilities than normal mice fed the Oz-AIN diet. Normal and Amy mice fed the AIN93-M diet has similar olfactory abilities.

Olfactory decline occurs earlier than memory impairments and β-amyloid plaque deposition in AD patients and in rodent models of AD [107, 505, 506, 574]. High-fat diets increase β-amyloid levels in AD-type mice [197] and elevated levels of β-amyloid impair olfactory circuitry [575].

Mouse olfactory sensory neurons each express specific olfactory receptors, from a selection of over 1000 olfactory receptor genes [575, 576]. Each olfactory receptor determines the neurones response to specific odors and is also important for axonal guidance to the olfactory bulb [575, 576]. Neurons from the olfactory bulb send spatially structured signals to olfactory cortical areas such as the piriform cortex, which send further projections to the entorhinal cortex and hippocampus [505]. Impairments to these olfactory circuits, either through aberrant olfactory neuron projection, synaptic loss, or damage to receptors impairs olfactory function and perception.

Cao *at el.* report that β-amyloid impairs axon projection and connectivity in pre-synaptic olfactory neurons in mice [575]. They demonstrate that either over expression of APP_{SWE} in AD- transgenic mice, or over expression of β-amyloid 40 or β-amyloid 42 induced by intranasal delivery of a viral vector, impaired olfactory sensory neuron axon projection [575]. This interference with neuron targeting was observed prior to the onset of β-amyloid plaques, and therefore reported as being independent of β-amyloid deposition [575]. Consistent with this, Wesson *et al.* report that AD-mice have impaired the olfactory circuits and hyperactive responses, long before behavioural impairments are observed [505]. However, Wesson *et al.* report that these deficits are observed at the same age that β-amyloid deposits form in the olfactory bulbs, suggesting that plaques may play a role in impaired olfactory functioning [505]. It is possible that the high-fat content of the Oz-AIN diet elevated β-amyloid levels in the olfactory bulbs of 12 month old Amy mice, disrupting

olfactory networks and subsequently impairing olfactory functioning. However, measurements of β-amyloid levels in the bulbs of Amy mice fed the Oz-AIN diet would be required to confirm this.

Alternately, changes in food preference and not olfactory decline, may account for these results. Tucker *et al.* reported that mice are less inclined to interact with high-fat food rewards compared to other food rewards when they were fed a high-fat diet [121], which may explain why mice that were fed the Oz-AIN diet took longer to locate the buried chocolate pellet than mice that were fed the AIN93-M diet.

The beneficial effects of the Oz-AIN diet on spatial learning and spatial memory may be related to the high-fat content of the Oz-AIN diet and the relationship between dietary fats and astrocyte function. Along with their anti-inflammatory capabilities, astrocytes also play crucial roles in anti-oxidant defence systems [437, 577-579]. AD brains contain high levels of activated astrocytes that have increased expression of antioxidant proteins such as peroxiredoxin-6 [437]. Furthermore, antioxidant mechanisms provided by astrocytes do not decline with age, highlighting their importance in the aging brain [578].

However, high-fat diet induced metabolic changes in mice can alter astrocyte activity [580]. Patil *et al.* reported that saturated fatty acid metabolism by astrocytes increases β-amyloid production, tau hyperphosphorylation and impaired glucose uptake by neurons *in vitro* [581]. This suggests that in the context of a high-fat diet astrocytes not only lose their anti-oxidant defence mechanisms, but they also promote β-amyloid neuropathology.

The inflammatory markers tumor necrosis factor-α and interferon-γ increase β-amyloid production and synthesis in primary mouse astrocytes [582]. Owing to the

fact that astrocytes greatly outnumber neurons in the CNS, Zhao *et al.* suggest that astrocytes may be a primary source of β-amyloid in AD [582].

The studies described above are somewhat limited, as they have been conducted *in vitro.* While this may offer some insight into the interactions between cells in well controlled media, responses of cells to specific stimuli may be completely different *in vivo*. Consistent with this, *in vivo* studies have reported that high-fat diets alleviate neurological deficits that are caused by dysfunctional astrocyte lipid metabolism [486], rather than have the detrimental effects that the *in vitro* studies suggest.

It is possible that a high-fat diet may be beneficial to other functions of astrocytes as well. Potentially, the high-fat content of the Oz-AIN diet enhanced astrocytic antioxidant capabilities. This may have prevented or alleviated oxidative stress levels in the brains of Amy mice, therefore preventing neuronal loss. If this was the case, the differences in astrocyte populations in the brains of Amy mice that were fed the AIN93-M diet or the Oz-AIN diet would have been detected in the studies of the neuropathology of AD brains. However, there did not appear to be any remarkable differences in astrocyte activation in the brains of Amy mice that were fed the AIN93-M diet or the Oz-AIN diet, and therefore no further quantification was carried out.

The protective capabilities of the Oz-AIN diet on spatial learning and memory were lost at 15 months of age, and returned again at 18 months of age, suggesting that diet-type affects spatial memory differently at different ages of life. Similar to this Boitard *et al.* report that a high-fat diet impairs memory when fed to 3 week old mice, but not 12 week old mice [484]. The differing effects of a high-fat diet on spatial memory at different ages may explain the discrepancies between the findings reported here that the Oz-AIN diet had a beneficial effect on spatial memory, and

reports from others that high-fat diets impair spatial memory in AD-type mice [82, 472, 484].

Collectively, these studies suggest that the Oz-AIN diet has different effects on AD-associated behavioural deficits, and that these effects differ at different stages of life. The Oz-AIN diet had a detrimental effect on olfactory ability, but conserved spatial memory abilities in 12 and 18 month old mice.

10.3.2. The potentially beneficial effect of nutrient supplements on behavioural deficits in normal and Amy mice.

The potential beneficial effect of nutrient supplements on olfactory function, spatial learning and spatial memory in Amy mice was assessed at 6, 12 and 15 months. These studies indicated that the potential benefits of nutrient supplementation on behavioural deficits associated with AD were independently affected by diet-type and age, irrespective of changes in either of the other two behaviours. A summary of findings from tests investigating the potentially beneficial effects of nutrient supplements on behavioural abilities of mice from the first cohort are outlined in Summary Two, on page 621.

Summary Two: The potentially beneficial effect of nutrient supplements on behavioural deficits in Amy mice.

Spatial learning (Chapter 6)

6 months: All mice demonstrated intact spatial learning abilities. There were no effects of genotype, diet-type or nutrient supplements.

12 months: Diet-type and genotype had a beneficial effect on spatial learning abilities of Amy mice fed the Oz-AIN diet. These beneficial effects were lost when mice were fed nutrient supplements. Amy mice fed the Oz-AIN Supp diet made smaller improvements than Amy mice fed the Oz-AIN diet.

15 months: Diet-type and genotype had a detrimental effect on spatial learning abilities of 15 month old Amy mice. This was prevented with nutrient supplements. Amy mice fed the Oz-AIN Supp diet made improvements in latency that were greater than Amy mice fed the Oz-AIN diet, and the same as Amy mice fed the AIN93-M diet or normal mice that were fed the Oz-AIN diet.

Spatial memory (Chapter 6)

12 months: All mice demonstrated intact spatial memory. There were no effects of diet-type, genotype or nutrient supplements.

15 months: Diet-type had an effect on spatial memory. Amy mice fed the AIN93-M diet failed to demonstrate spatial memory, whilst Amy mice fed the Oz-AIN diet or the Oz-AIN Supp diet had intact spatial memory. This suggests that while nutrient supplements do not affect spatial memory, *diets that were high in total fat had a protective effect*.

There were no genotype effects on spatial memory.

Olfaction (Chapter 8)

6 months: Diet-type had a detrimental effect on olfactory abilities of Amy mice fed the Oz-AIN diet. This was prevented with nutrient supplements. Amy mice fed the Oz-AIN Supp diet had similar olfactory abilities to Amy mice fed the AIN93-M diet. There were no genotype effects on olfactory abilities.

15 months: Diet-type had a detrimental effect on olfactory abilities of Amy mice fed the Oz-AIN diet. Diet-type effects were prevented by nutrient supplements.

There were no genotype effects on olfactory abilities.

As suggested earlier in this chapter, the poor performance of Amy mice that were fed the Oz-AIN diet in the Buried Chocolate Test may have been due to decreased motivation to locate the chocolate due to the high-fat content of the Oz-AIN diet. Although the Oz-AIN Supp diet is also high in fat, it also contains fish oil, which undoubtedly smells different to chocolate. Therefore, potentially the mice that were fed the Oz-AIN Supp diet considered the chocolate to be novel, and were more motivated to locate the chocolate than Amy mice that were fed the Oz-AIN diet.

It is also possible that the nutrient supplements were able to prevent the detrimental effects of β-amyloid in the olfactory bulbs of Amy mice, conserving olfactory abilities. As discussed earlier in this chapter, the high-fat content of the Oz-AIN diet may have enhanced β-amyloid production and deposition in the olfactory bulbs, impairing olfactory circuitry in Amy mice [107, 505, 506, 574]. Docosahexaenoic acid reduces β-amyloid synthesis [583] potentially through reduced expression of presenilin-1 and γ-secretase [584]. While presenilin-1 and γ-secretase levels have not been measured in the brains of Amy mice used in the current study, this provides a potential mechanism to explain the beneficial effects of the Oz-AIN Supp diet against olfactory dysfunction. Docosahexaenoic acid in the Oz-AIN Supp diet may have prevented excessive accumulation and secretion of β-amyloid in the olfactory bulbs of Amy mice by decreasing expression of presenilin-1 and γ-secretase. Additionally, docosahexaenoic acid increases expression of the anti-apoptotic members of the Bcl-2 family. This may have counteracted β-amyloid-induced increase in expression of pro-apoptotic members of the Bcl-2 family [585]. This is another potential mechanism through which the nutrient supplements in the Oz-AIN Supp diet may have conserved olfactory abilities of Amy mice.

Nutrient supplements did not have a beneficial effect on spatial learning at 12 months of age. Although all 12 month old Amy mice demonstrated intact spatial learning

abilities, the mice that made the greatest improvements were Amy mice that had been fed the sub-optimal Oz-AIN diet. The mice that received the Oz-AIN Supp diet made the smallest improvements, suggesting that nutrient supplements may *impair* spatial learning abilities of 12 month old mice that were fed a high-fat diet. This was an unexpected finding as the nutrient supplements in the Oz-AIN Supp diet improve rodent spatial learning abilities in other AD models [86, 202, 204, 328, 330, 333, 334, 348, 465, 488, 489, 546, 586].

Wiesmann *et al.* report that feeding mice a diet that has been supplemented with a nutrients required for optimal membrane synthesis, including docosahexaenoic acid, eicosapentaenoic acid, folate and vitamins B6, B9 and B12, improves learning strategies of AD-type mice [488]. Although mice that were fed either the supplemented diet or a control diet both learned the location of the platform, they utilised different search strategies. The AD-type mice that were fed the supplemented diet utilised a 'chaining' search strategy, whereby they circled the pool at the same distance from the pools edge as the submerged platform [488]. This is a more organised search strategy than the random strategies utilised by mice that were fed rodent chow [488].

It is possible that diet may have affected the spatial learning strategies of the 12 month old Amy mice that were used in the current study in a similar way. Potentially, the Amy mice that were fed the Oz-AIN diet utilized a random search strategy on the first day of training, and this led to increased latencies and distances travelled before locating the platform, compared to other mice. As a result of this, Amy mice that were fed the Oz-AIN diet may have had more 'room for improvement' before they were able to reach the platform with similar latencies as mice that were fed optimal diets. In contrast to this, the Amy mice that were fed the Oz-AIN Supp diet may have employed more organised search strategies, such as

chaining. The consequence of this may have been that they found the platform much faster on the first day of training, and hence had less 'room for improvement'. Without the data of actual paths travelled, this is speculative. However, it does provide a potential explanation for why Amy mice that were fed the Oz-AIN Supp diet appeared to make smaller improvements in the latency and distance travelled to a submerged platform over five training days.

The results of the current study suggest that learning and memory may be independently affected by different dietary components in 15 month old Amy mice. Amy mice that were fed the optimal AIN93-M diet or the Oz-AIN Supp diet demonstrated intact spatial learning abilities at 15 months, whilst mice that were fed the sub-optimal, high-fat Oz-AIN diet failed to demonstrate spatial learning. As the Oz-AIN diet and the Oz-AIN Supp diet are both high in total fat content, this suggests that total fat content does not influence learning abilities of 15 month old mice. This contrasts findings from others that have reported that high-fat diets impair spatial learning abilities of mice [167, 459, 472]. However, the high-fat diets used in such studies often contain optimal levels of essential vitamins and minerals, which may have been the reason for the reported differences.

The nutrient supplements docosahexaenoic acid and curcumin have well established beneficial effects on spatial learning abilities [329, 390, 488, 587], suggesting that they may have played a role in the beneficial effects of diet on spatial learning abilities of Amy mice. However, the AIN93-M diet, which also conserved spatial learning in mice, did not contain curcumin or docosahexaenoic acid, indicating that other dietary factors may also be involved.

While the evidence for B vitamin supplementation is not strong, B vitamin deficiency impairs spatial learning abilities in aged mice [333, 496]. Chen *et al.* report that folate supplements potentiated the beneficial effects of memantine, a

pharmaceutical treatment for AD, in an AD mouse model [330]. This suggests that folate works synergistically with other treatments for AD to reduce behavioural or neuro-pathological deficits. The Oz-AIN Supp diet and the AIN93-M diet contained optimal levels of folate, and this may have had promoted the beneficial effects of other neuro-protective dietary elements of each of these diets.

As stated above, the poor learning abilities of the 15 month old Amy mice that were fed the Oz-AIN diet were not likely to be attributed to the total fat content, because the Amy mice that were fed the Oz-AIN Supp diet, which was also high in fat, demonstrated spatial learning. Therefore, it is likely that other elements of the Oz-AIN diet played a role in the detrimental effects of diet on spatial learning. Young & Kirkland report that there is an inverse relationship between dietary niacin intake and spatial learning abilities [588]. The Oz-AIN diet contained 152.13% of the amount of niacin that is recommended for rodents. It is possible that these high niacin levels may have contributed to the impaired learning abilities of Amy mice.

Although total fat content did not appear to have an effect on *spatial learning,* it may have had an effect on *spatial memory*. Somewhat paradoxically, the high-fat diets may have improved spatial memory abilities of 15 month old mice.

Valladolid-Acebes *et al*. report that high-fat diet (45% kcal) feeding for 2 months impaired performance in hippocampal dependent object-location tasks, but increased hippocampal neuronal spine density in 5 and 8 week old mice [82]. Impaired performance in hippocampal-based tasks is often associated with lower spine densities [589, 590], whilst increased spine density has been associated with improved performance in hippocampal dependent tasks [591]. In an attempt to explain their potentially conflicting results, Valladolid-Acebes *et al*. suggest that the increased hippocampal spine densities may be a compensatory response to the potentially detrimental effects of a high-fat diet.

Von Bohlen und Halbach *et al.* report that although adult (6-7 months) and aged (20–22 months) did not differ in performance throughout the acquisition period of the Morris Water Maze, aged mice demonstrated significantly impaired spatial memory in the Test trial. This impaired spatial memory was associated with a decrease in hippocampal spine density [592]. This provides a potential explanation for how the high-fat diets improved spatial memory, but not spatial learning in 15 month Amy mice. If decreased spine density is a natural phenomenon associated with aging that is normally associated with impaired spatial memory, but not spatial learning; and if a high-fat diet increases spine density in the hippocampus, then it is possible that the benefits of the increased spine density in response to a high-fat diet may not be apparent until later in life, when spine density decreases in the brains of normal aged controls. This may explain the discrepancies between the current study an studies that have demonstrated that a high-fat diet has detrimental effects in young mice [82, 459, 484, 592].

Although 15 month old Amy mice that were fed the Oz-AIN diet failed to demonstrate spatial learning, they demonstrated spatial memory. This may at first appear counter-intuitive. *If mice failed to LEARN then how could they REMEMBER?*

The measure of learning was the degree of improved latency and distance travelled before reaching the platform within a two minute trial. The measure of memory was the percentage of time that mice spent in the Test Quadrant compared to the Opposite Quadrant whilst searching for the platform. It is entirely possible that Amy mice failed to improve latency or distance because they could not recall specifically where the platform was, but recognised it once they were there. This would explain why, on the last day of training, Amy mice that were fed the Oz-AIN diet spent more time in the Test Quadrant than the Opposite Quadrant whilst searching for the platform.

10.4. Relationship between behaviour and pathology

There did not appear to be any relationship between behavioural deficits and β-amyloid neuropathology in Amy mice.

Comparison of β-amyloid neuropathology and behavioural deficits of 15 month old Amy mice.

<table>
<tr><th></th><th>Low power microscopy</th><th>Confocal microscopy</th><th>Spatial learning</th><th>Spatial memory</th><th>Olfaction</th></tr>
<tr><td>AIN93-M diet</td><td rowspan="3">Same levels of β-amyloid throughout the brain</td><td rowspan="2">Greater % of necrosis-associated deposits, with negligible % of BBB-associated deposits.</td><td>Learned location of platform by Day 4.</td><td>Failed to demonstrate spatial memory</td><td>Found chocolate with fastest latency</td></tr>
<tr><td>Oz-AIN diet</td><td>Failed to learn location of platform</td><td rowspan="2">Demonstrated spatial memory</td><td rowspan="2">Took longer to locate the chocolate</td></tr>
<tr><td>Oz-AIN Supp diet</td><td>Equalized distribution of deposits, due increased % of BBB-associated deposits.</td><td>Learned location of platform by Day 2.</td></tr>
</table>

At 15 months of age the Amy mice that were fed the Oz-AIN diet demonstrated severe spatial learning deficits. Low-power microscopy revealed β-amyloid deposits throughout the brains at the same age. The finding that normal mice did not have β-amyloid deposits and demonstrated spatial memory may, at first, suggest that there is a relationship between β-amyloid deposition and spatial memory deficits in Amy mice. This is consistent with research from others who report correlations between spatial learning abilities and β-amyloid levels in Amy mouse brains [374]. However,

performance of Amy mice that were fed the AIN93-M diet or the Oz-AIN Supp diet suggests otherwise. The Amy mice that were fed the AIN93-M diet or the Oz-AIN Supp diet both had spatial learning abilities that were similar to those of normal mice, despite having the same number of β-amyloid deposits as Amy mice that were fed the Oz-AIN diet. This suggests that there was no relationship between β-amyloid deposits and behaviour. This is consistent with recent findings from human studies that have reported that the degree of cognitive decline does not correlate well with β-amyloid deposition, but correlates better with neurodegeneration is indicated by neuronal integrity, hippocampal volume and cortical thickness [593].

The only real difference between Amy mouse brains was that the 15 month old Amy mice that were fed the Oz-AIN Supp diet had a greater percentage of BBB-associated β-amyloid than age-matched Amy mice that had been fed either the AIN93-M diet or the Oz-AIN diet. If there was a relationship between β-amyloid and any of the behavioural deficits observed in AD that were tested in this project, then it would be expected that the Amy mice that were fed the Oz-AIN Supp diet differed from other Amy mice when performing that specific task.

However, no such differences occurred for any of the behaviours tested. The Amy mice that were fed the Oz-AIN Supp diet demonstrated similar spatial memory abilities and similar olfactory skills as Amy mice that were fed the Oz-AIN diet; and displayed intact spatial learning abilities, which was similar to the Amy mice that were fed the AIN93-M diet. This adds support to the conclusion that there was no relationship between β-amyloid neuropathology and behavioural deficits in Amy mice.

There were trends to suggest that the 18 month old Amy mice that were fed the AIN93-M diet may have had more β-amyloid deposits than Amy mice that were fed the Oz-AIN diet. The Amy mice that were fed the AIN93-M diet also failed to

demonstrate intact spatial memory in the MWM, suggesting that there may have been a relationship between the number of β-amyloid deposits and spatial memory in aged Amy mice. However, normal mice that were fed the AIN93-M diet, that did not generate β-amyloid deposits, also failed to demonstrate intact spatial memory. This indicates that the loss of spatial memory skills in aged mice was more likely to be associated with diet rather than amyloid.

Comparison of β-amyloid neuropathology and behavioural deficits of 18 month old Amy mice.

<table>
<tr><th></th><th>Low power microscopy</th><th>Confocal microscopy</th><th>Spatial learning</th><th>Spatial memory</th><th>Olfaction</th></tr>
<tr><td>AIN93-M diet</td><td rowspan="2">Non-significant trends for mice AIN93-M to have more deposits.</td><td rowspan="2">Greater % of necrosis-associated deposits, with negligible % of BBB-associated deposits.</td><td rowspan="2">Failed to demonstrate spatial learning.</td><td>Failed to demonstrate spatial memory.</td><td rowspan="2">Not assessed at 18 mo.</td></tr>
<tr><td>Oz-AIN diet</td><td>Demonstrated intact spatial memory.</td></tr>
</table>

10.5. Telomere length.

Despite the growing awareness that telomere attrition correlates with AD neuropathology [241, 248, 594], there is a limited amount of literature on telomere length in the brains of AD-type mice [233]. Therefore, a small exploratory study was also conducted to investigate telomere length in the brains of 15 and 18 month old Amy mice and this was presented in Chapter 9.

It is reported herein that while telomere length was shorter in the brains of normal mice that were fed the Oz-AIN diet at 18 months compared to 15 months, the opposite occurred in the brains of Amy mice. The brains of 18 month old Amy mice

that were fed the Oz-AIN diet were longer than those of 15 month old Amy mice. This suggested that there are genotype effects on telomere length in the brains of Amy mice, and that telomere length increases with age in Amy mice fed the Oz-AIN diet.

Owing to the inability of DNA polymerases to synthesize to the 3' end of the DNA sequence, telomeres shorten with every cellular division [228, 231, 237]. Consequently, telomere length decreases with age in proliferative tissues such as such as white blood cells, liver, heart, fat and skin [240, 534].

The same dynamics occur in the CNS. Telomere length in proliferative cells, such as microglia cells decreases with age [537, 538]. Flanary *et al.* measured telomere length in microglia from normal 3 and 30 month old rats. They report that there was a sub-set of microglia that contained very long telomeres, which did not exist in 30 month old rats. This suggested that the microglia within this subset had undergone rapid proliferation and telomere shortening and entered senescence well before other microglia [538]. In accordance with this, in the current project the brains of 18 month old mice had shorter telomeres than those of 15 month old normal mice.

The current study has not evaluated whether the changes in telomere length were attributed to changes in microglial population numbers. However, microglia are one of the major cell types capable of replication in the CNS, and make up more than 10% of glial cells in the brain [116]. This indicates that the age-related decrease in telomere length in the brains of normal mice may have been due to changes in microglia population. Potentially, there was a decrease in number of microglia with long telomeres in 18 month old normal mice compared to 15 month old mice. However, this needs to be confirmed with further studies.

The current study also found that telomere length in the brains of 18 month old Amy mice was longer than that of 15 month old mice, suggesting that telomere length actually increases with length in the brains of aging Amy mice. This contradicts reports from others that telomere attrition occurs in human AD brains and in rodent models, which is likely to be attributed to changes in microglial population [537, 538].

However, there are a few studies that support the current findings [233, 241]. Thomas *et al.* report that human AD brains have longer telomeres than age matched normal brains [241]. Roylan *et al.* report that longer telomeres enhanced behavioural deficits and β-amyloid pathology in AD-type mice. In order to demonstrate this, Roylan *et al.* crossed AD-type mice with telomerase knockout mice. Telomerase is required to maintain telomere length, and telomerase knockout mice have short telomeres. Roylan *et al.* report that the double transgenic mice had shorter telomeres and did not have the behavioural deficits or β-amyloid pathology that was observed in the AD-type mice that were able to maintain telomere length [233]. This suggests that telomere shortening is required to maintain cognitive functioning and prevent β-amyloid neuropathology.

Eitan *et al*. investigated telomere length in the brains of aging purkinje neurons in the cerebellum [595]. They report that although telomerase protein expression does not change with age, telomerease activity increases dramatically in the brains of aged mice. Eitan *et al*. propose that telomerase is necessary to maintain DNA stability in neurons, and therefore prevent apoptosis. This may not necessarily mean that telomerase activity is increased in the brains of aging Amy mice. However, coupled with the reports from Roylan *et al.*, it does provide a potential mechanism for how the brains of 18 month old Amy mice had longer telomeres than 15 month old Amy

mice. Potentially, increased β-amyloid levels increased expression of telomerase and upregulated telomerase activity, which may have resulted in increased telomere length. Before this conclusion can be made, telomerase activity in the brains of 15 and 18 month old mice needs to be measured.

In the current study, there were no clear correlations between telomere length and behavioural deficits or β-amyloid neuropathology. At 15 months of age, the Amy mice that were fed the Oz-AIN diet had significant spatial learning deficits but superior spatial memory compared to Amy mice that were fed the AIN93-M diet, there were no differences in telomere length. This suggests that at 15 months of age, behavioural deficits do not predict telomere length in Amy mouse brains.

In contrast to this, the 18 month old Amy mice that were fed the Oz-AIN diet had significantly longer telomeres than Amy mice that were fed the AIN93-M diet, and also performed better in the spatial learning and spatial memory tasks, suggesting that at 18 months of age, there may be a relationship between telomere length and behavioural abilities of Amy mice. This contradicts the reports from Roylan *et al.* that telomere shortening prevented behavioural deficits. However, Roylan *et al.* assessed cognitive abilities of AD-type mice at 12 months, whereas the current comparisons are made between 18 month old mice. This suggests that if there is a relationship between telomere length and behavioural deficits, that it is age and model dependent.

There may have been links between telomere length and β-amyloid pathology. The 15 month old Amy mice that were fed the Oz-AIN diet had similar β-amyloid neuropathology to Amy mice that were fed the AIN93-M diet, but there was no significant differences in telomere length between Amy mice that were fed different diets. However, at 18 months of age, when Amy mice that were fed the Oz-AIN diet

had significantly longer telomeres than those fed the AIN93-M diet, there were no differences in pathology.

Collectively, these results suggest that while behaviour, β-amyloid pathology, and telomere length are all influenced by age, genotype and diet, they do not have any significant effect on each other.

10.6. Conclusion.

In conclusion, this project has demonstrated the effects of an Australian-type rodent diet alone, or in combination with nutrient supplements, on the behavioural deficits and β-amyloid neuropathology in an Amy mouse model.

A novel finding of this project was that the β-amyloid pathology in this Amy mouse model includes high levels of necrosis that may be a result of excessive accumulation of β-amyloid. Diet-type did not appear to have an effect on the occurrence of these necrosis associated deposits. The Oz-AIN diet did not appear to have an effect on β-amyloid deposition in the brains of Amy mice. This may suggest that diet does not have a detrimental effect on AD neuro-pathology. However, the Oz-AIN Supp diet increased the number of β-amyloid deposits that were associated with the blood-brain barrier. This was potentially as a result of increased oxidative damage and invasion of β-amyloid from the peripheral blood stream. Further studies could confirm this by examining the oxidative stress effects of the Oz-AIN Supp diet in closer detail. While these studies have shown that diet does influence β-amyloid neuro-pathology, they suggest that a combination of nutrient supplements may have detrimental effects, not beneficial effects, and promote β-amyloid deposition in Amy mouse brains.

Behavioural deficits in AD are independently affected by diet. Olfactory and spatial learning deficits were accelerated by the sub-optimal, high-fat Oz-AIN diet, and this was prevented with nutrient supplements. However, spatial memory appeared to be conserved by high total fat content. This suggests that dietary fats play different roles in the brain. Potentially, this may be mediated through changes in neuronal spine density and improved signalling in old age, however further studies need to be conducted to confirm this.

The Oz-AIN diet induced rapid weight gain and obesity in Amy mice. This weight gain was alleviated by supplementing the Oz-AIN diet with polyphenolic compounds, B vitamins and polyunsaturated fatty acids, in the Oz-AIN Supp diet. As weight gain and obesity are both risk factors for AD, these results suggest that diet may not just alter features AD but may also affect other aspects of the disease process.

Collectively, these studies have found that the Oz-AIN diet had a detrimental effect on physical attributes of Amy mice, but has protective effects against some of the behavioural and pathological characteristics of AD. On the other hand, nutrient supplements had a beneficial effect on physical attributes and spatial learning abilities of 15 month old Amy mice, but may promote β-amyloid deposition.

APPENDIX I

Confirming Genotype of mice using PCR and gel electrophoresis.

1.1. Tissue collection and storage.

Mouse tail tips (0.8 cm – 1.0 cm) were collected by Flinders University Animal House Staff and placed into labelled 1.5 mL eppendorf tubes. Tail tips were then transported to CSIRO in a polystyrene box containing ice. DNA for genotyping was extracted immediately and stored at -4° C.

1.2. DNA isolation from mouse tail tips.

DNA was extracted from tail tips using the DNeasy Blood and Tissue Kit (69506, © QIAGEN) as per the protocol outlined in the DNeasy® Blood and Tissue Handbook (© QIAGEN, 2006).

1.2.1. Procedure

A rocking incubator (Orbital mixer incubator, Ratek instruments, Australia) was pre-heated to 56°C.

Tail tips were cut into 0.5 cm pieces and placed in a 1.5 mL microcentrifuge tube that had been labelled with the corresponding mouse number.

180 µL Buffer ATL (Tissue Lysis Buffer, 19076, QIAGEN) and 20 µL proteinase K (19131, QIAGEN) were added to each of the labelled microcentrifuge tubes containing brain tissue. To ensure that buffer ATL, proteinase K and tail tips were thoroughly mixed, samples were vortexed for 30 sec each at room temperature using a Townson Tru-Mix vortex (Townsom Tru-Mix, Townson & Mercer Pty. Ltd.).

Samples were placed in the rocking incubator (Orbital mixer incubator, Ratek instruments, Australia) and incubated at 56°C, until tail tips were completely lysed (3-5 hours). To aid lysis, tissues were vortexed (Townson Tru-Mix, Townson & Mercer Pty. Ltd.) mid-way through incubation.

Buffer AL (Lysis Buffer, 19075, QIAGEN) and ethanol (E7023, Sigma-Aldrich) were combined 1:1, as per Table 2 to make the Buffer AL+ethanol mix. 400µL of the Buffer AL+ethanol mix was added to each sample, which was then immediately vortexed (Townson Tru-Mix, Townson & Mercer Pty. Ltd.) for 30 sec.

Table 1. Sample calculations for the amount of Buffer AL and ethanol that are required for 24 samples.

Reagent	**Amount per microcentrifuge tube**		**Number of samples**	**Amount added to master mix**
Buffer AL	200 µL	x	24	4,800 µL
Ethanol (96%)	200 µL	x	24	4,800 µL
TOTAL	**400 µL**	x	24	**9,600 µL**

Samples were transferred to a DNeasy spin column (QIAGEN, Australia), placed in a 2 mL collection tube (19201, QIAGEN, Australia) and centrifuged at 8,000 rpm for 1 min at room temperature in a microcentrifuge (Eppendorf centrifuge 5415R, Eppendorf). The flow-through solution and collection tube were discarded, and the DNeasy spin column was placed in a new 2 mL collection tube.

500 µL Buffer AW1 (Wash Buffer (1), QIAGEN) (in 96% ethanol) was added to each spin column that contained sample. Samples were centrifuged at 8,000 rpm for 1 min at room temperature in a microcentrifuge. The flow-through solution and collection tube were discarded, and the DNeasy spin column was placed in a new 2 mL collection tube.

500µL Buffer AW2 (Wash Buffer (2), QIAGEN) (in 96% ethanol) was added to each spin column that contained sample. Samples were centrifuged at 14,000 rpm for 3 min at room temperature in a microcentrifuge to dry the DNeasy membrane. The flow-through solution and collection tube were discarded, and the DNeasy spin column was placed in a new 2 mL collection tube.

200 µL Buffer AE (Elution Buffer, 19077, QIAGEN) was added carefully and directly onto the spin column membrane in each spin column that contained sample. Samples incubated at room temperature for 1 minute. Samples were centrifuged at 8,000 rpm for 1 min at room temperature in a microcentrifuge.

To ensure maximum DNA yield, this step was repeated. The flow-through was collected and re-added to the spin column. Samples were then centrifuged at 8,000 rpm for 1 min at room temperature in a microcentrifuge.

Samples were transferred to labelled 1.5 mL eppendorf tubes and stored at 4°C.

1.3. DNA quantification in purified DNA samples

The RT-qPCR to amplify DNA for genotyping requires 40ng DNA per well. The concentration of DNA in each sample was measured using a nanodrop® spectrophotometer (ND1000, NanoDrop Technologies) and then diluted to 10 ng/µl with ultra-pure water (UPW).

1.3.1. Protocol

A nanodrop® spectrophotometer (ND1000, NanoDrop Technologies, USA) that was connected to a laptop running NanoDrop 1000 software (version 3.6.0, Thermo Fisher Scientific, USA) was used to measure the amount of DNA in the purified DNA samples. All samples and reagents were measured using Gilson Pipetman® pipettes (USA) and plugged sterile pipette tips (10µL).

Prior to use, the nanodrop® spectrophotometer was cleaned by dropping 2µL UPW onto the platform and wiped with an anti-static tissue. To form a baseline reading, 2µL of Buffer AE (Elution Buffer, 19077, QIAGEN), which the DNA samples had been made in was transferred onto the nanodrop® spectrophotometer platform. The baseline measurement was set by selecting “blank” in the main screen of the nanoDrop 1000 software.

1 µL of DNA sample was added to the nanodrop® spectrophotometer platform. The nanoDrop 1000 software provided a measurement of DNA purity and amount after selecting “measure” on the main screen of the nanoDrop 1000 software. The 260/280 reading is a measure of DNA purity. Samples that are pure have a 260/280 reading between 1.7 and 1.9. The NanoDrop 1000 software also provides a measurement of DNA content (ng/ µL).

The nanodrop® spectrophotometer platform was cleaned with 2 µL UPW in between sample measurements.

DNA samples were then adjusted to 5 ng/ µL in UPW. DNA content was re-measured using the nanodrop® spectrophotometer to ensure accuracy of dilutions.

DNA samples were stored at 4°C until use for RT-qPCR.

1.4. RT-qPCR

1.4.1. PCR master mix

All reagents for the PCR (excluding DNA) were combined to make a master mix (Table 2) in the biohazard hood. Enough PCR master mix was for samples and to allow for 4% pipetting error.

When making the PCR master mix, the SYBR 1 mastermix (#4367396 Applied Biosystems), which contains AmpliTaq Gold DNA polymerase, dNTPs, SYBR I Green Dye, opitimised buffers and passive reference dye (ROX) was added to the mixture slowly, as it contains detergent and may therefore be prone to bubble if handled roughly. Master-mix was made up immediately before each PCR and used fresh.

Table 2. Sample calculations for reagents in the PCR master mix and amount of DNA

	Amount	**x**	**number of samples + 5% (error)**	**TOTAL**
UPW	6.5 µL	x	33 samples	214.5 µL
SYBR 1 master mix	12.5 µL	x	33 samples	412.5 µL
Primer (2 µmol)	2.5 µL	x	33 samples	82.5 µL
Q solution	2.5 µL	x	33 samples	82.5 µL
TOTAL	24.0 uL			792.0 µL
DNA	2.0 µl			

24µL of PCR master mix was added to each well on a PCR strip in a BioHazard fume hood. PCR strips were then removed from the hood and 2µL of DNA sample was added to each well.

PCR strips were then placed into a PCR machine (AN07918M PCR Machine) attached to an ipac pocket PC, and run under the following cycling conditions:

Table 3. PCR cycling conditions for DNA amplification.

Step	Temperature	Time	Comment
1	95 °C	15 min	Initial polymerase activation
2	94 °C	30 sec	38 cycles
3	69 °C	1 min	
4	72 °C	1 min	
5	72 °C	2 min	1 cycle
6	4 °C		

1.5. Gel analysis

1.5.1. Gel preparation

The gel was prepared by mixing 4 g agar in 200 mL of 0.5xTAE buffer, and then adding 4 µL gel red to the mixture.

The agar was dissolved in a microwave on low for 2 minutes (min), and then allowed to stand until the solution had cooled but was still liquid.

Once cooled, the solution was poured into a 20 well comb gel tray, with 2 x 20 well combs placed an equal distance apart, to allow for 19 samples to be run in duplicate on each well (Figure 1). When gel had set, gel combs were gently removed, with care taken not to break any of the wells. The gel was placed into a gel tank that contains 5 x TAE buffer and connected to a BioRad Power Pac (Power Pac Basic, BioRad).

2 µL DNA Ladder (Geneworks,) and 4 µL 6 x loading dye (Geneworks) were combined. 3µL of the DNA ladder mixture was added to the first well of each row.

4 µL loading dye was added to each DNA sample before adding 15 µL of each sample to the remaining 19 wells on each row, so that each sample is run in duplicate (Figure 1).

The power pac was then set to 80 mV, and the gel left to run for 2 hours.

After 2 hours, the gel was carefully removed from the gel tank, and placed in a BioRad Universal Hood (Segrate, Italy) connected to a computer running Quantity One Software (BioRad).

Quantity One software was used to adjust focus, size and position of image, before gel image was captured under UV light. Genotyping was confirmed using the resulting images. DNA from Amy mice had bands at 350 kD (Figure 1).

Appendix II

ANYmaze™ Video Tracking System Setup for the Morris Water Maze.

The Morris Water Maze is a 6 day assessment of rodent spatial learning and spatial memory. The first five days are "Training" days, and the sixth day is a "Test" and "Probe" day.

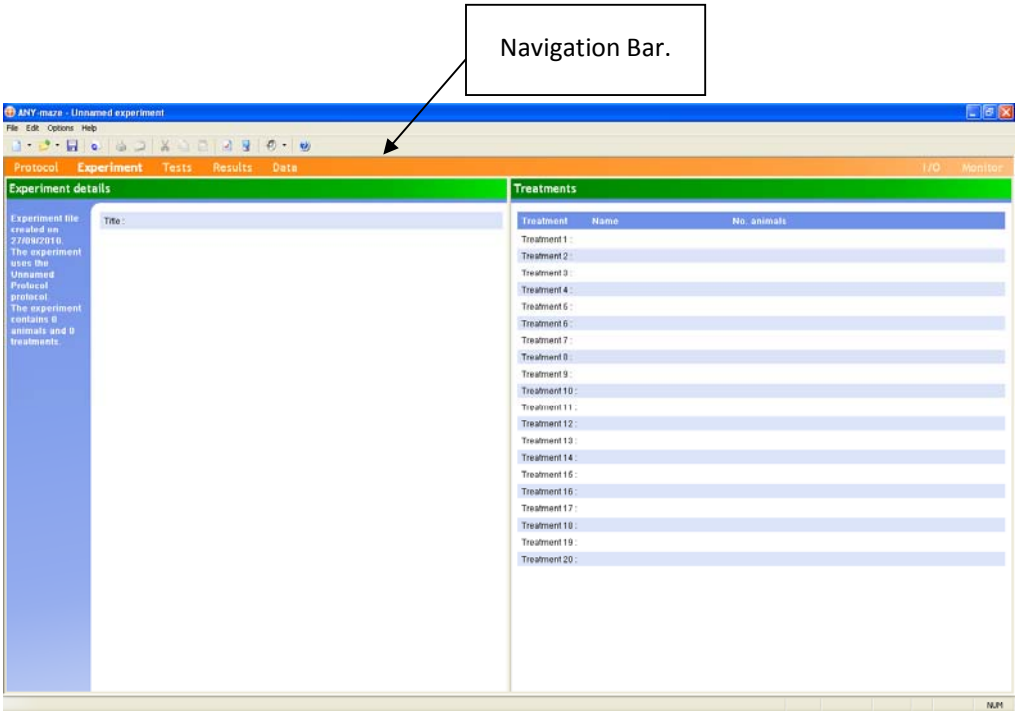
Animals are trained to find a hidden platform, which is submerged in a pool of water. The latency to finding the platform, as well as distance travelled are recorded to assess how well the mouse learns where they must get to to escape the pool.

The software used for the Morris Water Maze is ANYmaze. Before the study can begin, this apparatus needs to be set up correctly to ensure the correct parameters are measured.

ANYmaze

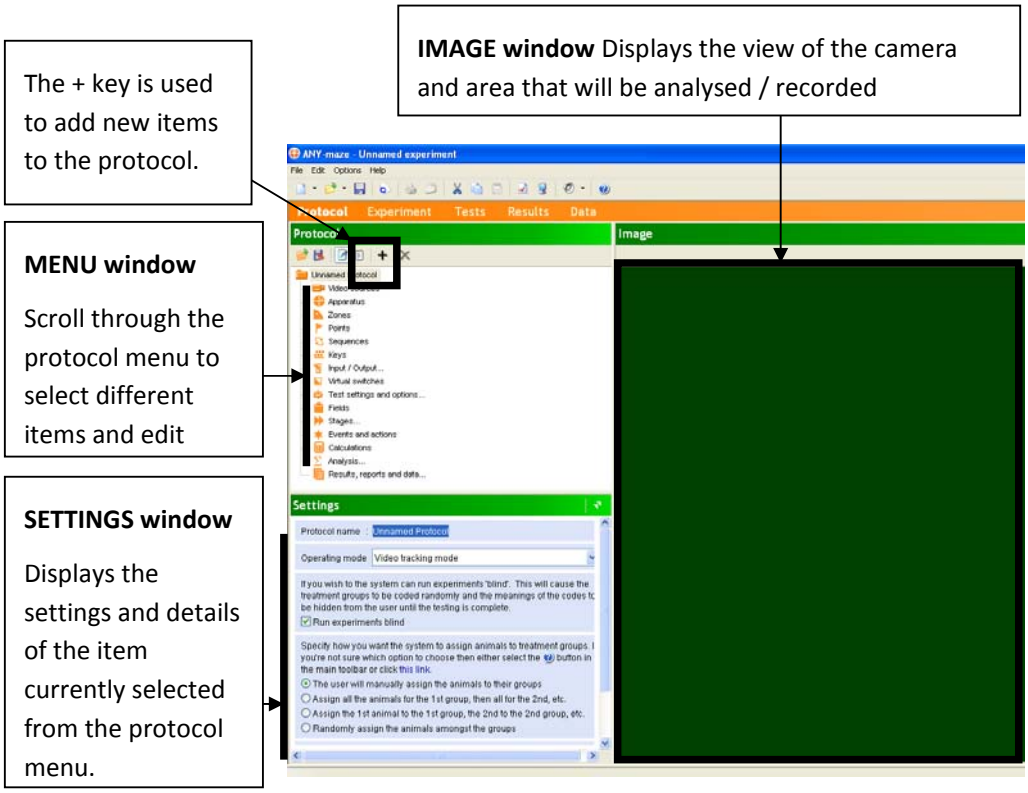
The first screen that opens in ANYmaze is a blank project.

The orange bar across the top of the screen contains five options: The five options are "PROTOCOL" "EXPERIMENT" "TEST" "RESULTS" "DATA". This bar will be used as the navigation bar to set up and run all tests.



1. SET UP PROTOCOL.

Click on “Protocol”. The following screen will appear. Select each of the relevant items from the menu to alter them.



1. Add a camera source.

a. Click the + button. A menu of available options to add to the protocol will appear. Select the top option “NEW VIDEO SOURCE”.

b. ANYmaze will automatically use an available camera. If this is not the right camera, change this in the drop down menu in Settings.

Now ANYmaze has an image to analyse. However, it needs to be told where to look for data, and what to look for. This is done in the next two steps: “Add Apparatus” and “Add Zones”.

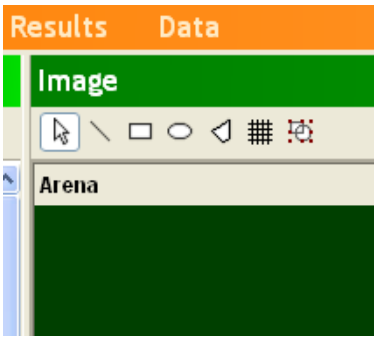
2. Add Apparatus.

a. Click the + button. Select “NEW APPARATUS” from the drop down menu.

b. Name the apparatus “Morris Water Maze”.

c. At the bottom of the Settings Window, set the ruler length (mm). The actual ruler it refers to can be seen in the Image window.

d. Use the symbols at the top of the Image window to draw the arena and virtual quadrants.



1. Daw a circle around the entire pool.
2. Draw two lines dividing the circle into 4 quadrants.
3. Draw a smaller circle in one of the quadrants (where the platform will be)
4. Select the smaller circle, and copy it by pressing “Ctrl” + “C”.
5. Press “Ctrl” + “V”.
   Another small circles will appear.
   Drag this to any of the other three quadrants.

The Arena should now look like the image below:

3. Add Zones.

Zones need to be added to let ANYmaze know what to call each part of the apparatus. The Morris Water Maze has 7 zones. To add each new zone, click the + button and select “NEW ZONE” from the drop down menu. Click on the area you are referring to, and name it appropriately by typing the name into the “Zone Name” box, in the Settings window. Select options in the Protocol window and alter them in the Settings window, as detailed in Table One.

Table 1. Zones for the Morris Water Maze.

Zone	Image	Protocol Menu	Settings
Arena		Arena Zone entry settings	Position of the zone remains the same in all tests Use the centre of the animal
Platform – Test		Arena Zone entry settings	Position of the zone remains the same in all tests Use the centre of the animal
Test Quadrant		Arena Zone entry settings	Position of the zone remains the same in all tests Use the centre of the animal
Platform – Cued		Arena Zone entry settings	Position varies within (and possibly between) animals Use the centre of the animal
	ANYmaze needs to know where the positions will be. Click the + button, select "New Zone Position"		
Platform Opposite			
Hidden Platform			
Quadrant 2 – Left		Arena Zone entry settings	Position of the zone remains the same in all tests Use the centre of the animal
Quadrant 3 – Opposite		Arena Zone entry settings	Position of the zone remains the same in all tests Use the centre of the animal
Quadrant 4 – Right		Arena Zone entry settings	Position of the zone remains the same in all tests Use the centre of the animal

4. Add Stages.

The Morris Water Maze Training period runs for 5 days. Each day there are two morning stages, and two afternoon stages. On the 6th day, there is a "Test" stage and a "Cued" stage. In the Test stage, the platform is removed from the pool, and the amount of time the animal spends in the Test quadrant and number of passes made over the Platform – Test zone assessed and used as a measure of memory retention. To assess whether or not the mouse is responding to visual cues, all visual cues are removed from the arena, and the platform is then returned to the pool in a new location. This is the "Probe" stage. In this stage, the platform has a visual cue on it (a stand that the animal can see).

Set up ACQUISITION PHASE (five day training phase).

Day 1.

1. Click the + button, select "NEW STAGE".

In the Setting window, enter the following details:

a. Stage name "DAY DATE - AM"
b. Test Duration: 120s
c. Maximum number of trials in this stage: 2
d. Group the animals and perform all trials for each group in turn. Put the animals into groups of: 3

In the Protocol menu select "Location of the Platform – Probe"

In the Setting window select "The location is irrelevant because this zone isn't used in this stage" from the drop down menu.

The mouse needs to know where the platform is before the first trial. Therefore, an accustomisation period is necessary before the first trial of the first day. This is done by selecting "Accustomisation Period" in the Protocol Window while setting up Stage 1. Tick the option "Include an accustomisation period at the start of the trials in this stage." With an accustomisation length of 30 sec (this is the amount of time the mouse will spend on the platform before training begins on the first day). Select the options to "Include customisation before the following number of trials in this stage", and type in "1" trial in the dialogue box. Select the option "wait for the user to start the test after the zccustomisation period."

2. Click the + button, select “New Stage”.

In the Setting window, enter the following details:

a. Stage name “DAY DATE - PM”
b. Test Duration: 120s
c. Maximum number of trials in this stage: 2
d. Group the animals and perform all trials for each group in turn. Put the animals into groups of: 3

In the Protocol menu select “Location of the Platform – Probe”

In the Setting window select “The location is irrelevant because this zone isn’t used in this stage” from the drop down menu.

Days 2 – 5

Repeat steps 1 and 2 for each day that the Morris Water Maze training sessions occur (omit the Accustomisation period step). On days 4 and 5, mice can be grouped into groups of 4 rather than 3 to enhance efficiency as mice will complete trials faster, but it is not necessary.

Set up TEST STAGE.

1. Click the + button, select “NEW STAGE”.

In the Setting window, enter the following details:

a. Stage name “DAY DATE - TEST”
b. Test Duration: 120s
c. Maximum number of trials in this stage: 1
d. Perform each trial for all the animals before starting the next trial

In the Protocol menu, select “Location of the Platform – Probe”

In the Setting window, select “The user will specify the location at the start of each test” from the drop down menu.

Set up CUED STAGE.

1. Click the + button, select “New Stage”.

In the Setting window, enter the following details:

a. Stage name “DAY DATE - CUED”
b. Test Duration: 120s
c. Maximum number of trials in this stage: 1

d. Perform each trial for all the animals before starting the next trial

In the Protocol menu, select “Location of the Platform – Probe”

In the Setting window, select “The location is irrelevant because this zone isn’t used in this stage” from the drop down menu.

5. Set up Events and Actions.

ANYmaze needs to be told to stop the test once the animal finds the hidden platform. This is done by setting up an event (finding the platform) and executing and action (ending the test).

1. Click the + button. Select “New Event”.
    a. Name the event “Found Platform”. Click on “Help me define the trigger for this event” and follow the prompts.
    The event will be triggered if the animal remains in the Platform - Test zone for 2s
    b. Select the stages for which this event occurs: Select all stages, except for TEST STAGE and CUED STAGE.

The Cued stage relies on the mouse finding the platform in a different location, and the Test stage requires there be no platform at all. This event needs to be set up.

2. Click the + button. Select “New Event”.
    a. Name the event “Found Visual Cue”. Click on “Help me define the trigger for this event” and follow the prompts.
    The event will be triggered if the animal remains in the Platform - Probe zone for 2s
    b. Select the stages for which this event occurs: Only select CUED STAGE.

3. Click on the + button and select “New Action”.

In the settings screen, select both “Found Platform” and “Found Visual Cue”.

Click on “Test Control” and select “End the test” from the drop down menu.

6. Number of animals.

Select “Experiment” from the orange navigation bar at the top of the screen. On the right hand side of the screen, enter how many animals will be tested.

7. Running the tests.

Follow the Checklist in Table 7 to ensure that all procedures and materials are ready to start tests.

On the day of behavioural tests, open ANYmaze.

Select “Tests” on the navigation bar.

The screen should display the live video footage of the MWM arena, and trial should be set to “ready”. Start each test by selecting the green arrow (Figure 5).

Record the detail of each test in a running sheet, as per Figure 6.

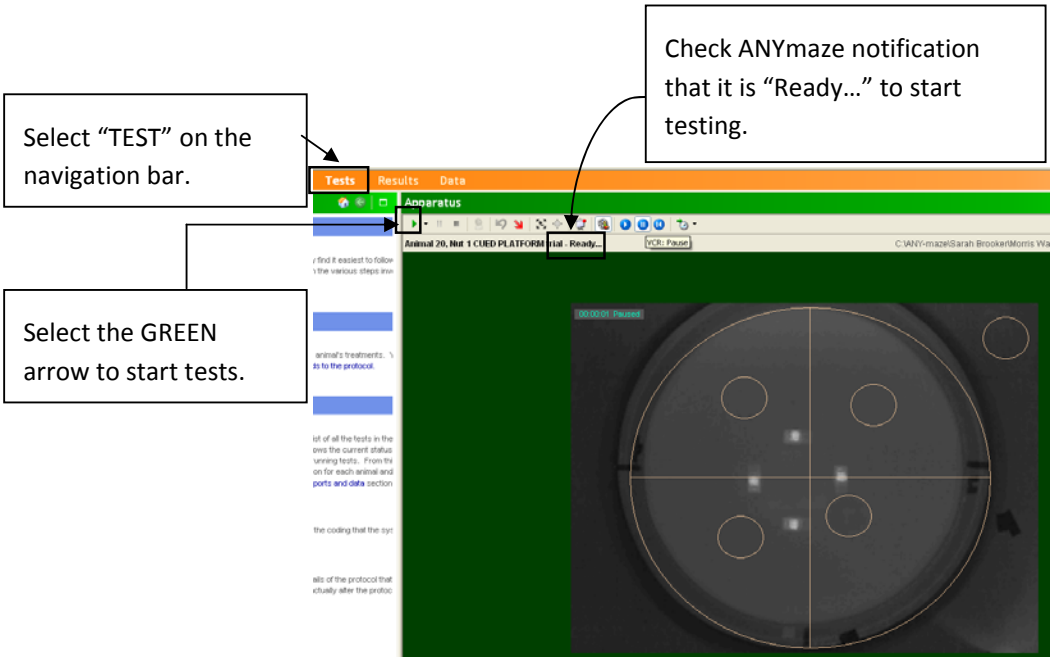


Table 2. Sample Running Sheet for the Morris Water Maze tests.

Day	Trial	Animal Number	Time of Day	Latency to Platform	Notes
Day 2	T1	# 195	7:09 AM	2:00.00	
	T1	# 235	7:14 AM	1:57.35	
	T1	# 250	7:18 AM	35.98	
	T2	# 195	7:29 AM	2:00.00	
	T2	# 235	7:24 AM	3.41	
	T2	# 250	7:28 AM	16.41	
	T1	# 211	7:32 AM	1:40.81	
	T1	# 233	7:34 AM	2:00.00	
	T1	# 239	7:39 AM	49.44	
	T2	# 211	7:42 AM	1:07.71	
	T2	# 233	7:44 AM	1:40.46	
	T2	# 239	7:49 AM	2:00.00	

8. Collecting data after testing.

Data from each stage is retrieved by selecting "DATA" from the orange menu bar across the top of the screen.

In the Measurement Options menu, under "Animal and Test information" select Animal Number, Stage, Trial Number, Test Time and Test Date. These will aid data management.

In the Measurement Options menu, under "Apparatus Measures" select Test Duration, Total Distance Travelled, and Average Speed.

From the Data inclusion menu at the bottom of the screen, under "Trial" select the Training Stages that data is required.

While ANYmaze has the capability to do statistical analysis on data, do not select those options here. All data analysis is done using Prism Software.

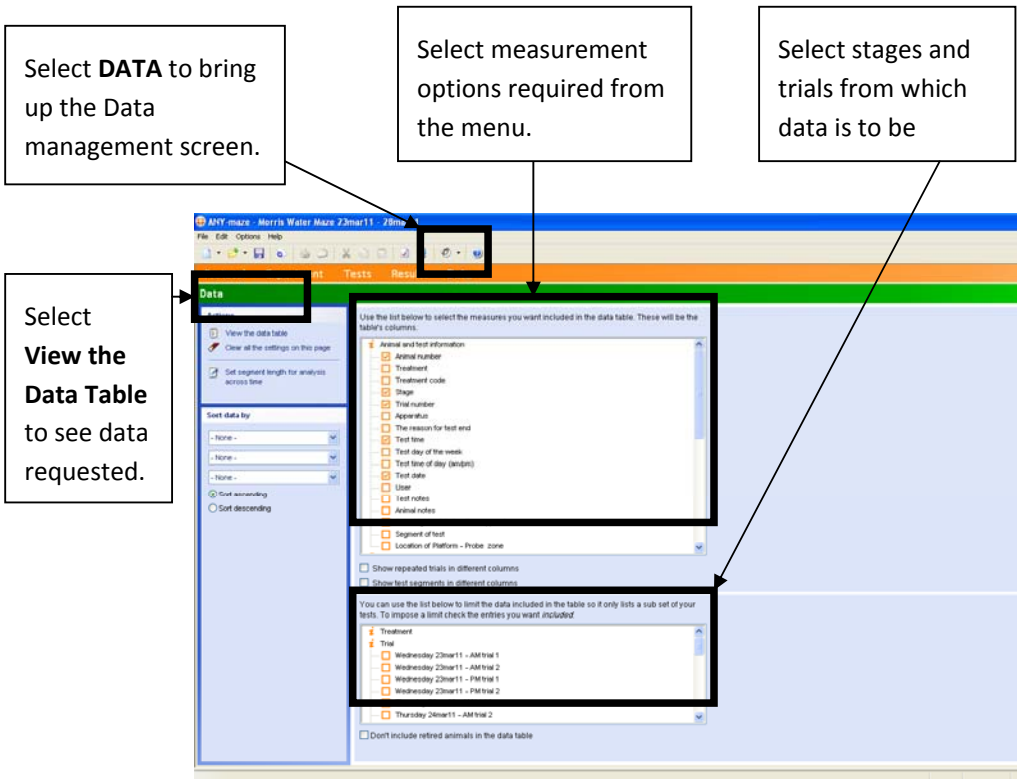


Table 3. Morris Water Maze Set Up Checklist.

Completed?	Item	Description / Action
	Log in to ANYmaze	Username: XXXXXX Password: XXXXXX
	Open File	Click "File" Scroll down to the bottom of the menu, where the list of recently opened files is. Click on the appropriate file.
	Pool	Stir water to get all the paint off the bottom. Remove any faeces
	Heat Mats	Heat 4 heat mats in the microwave for 2 min each
	Mouse cages	Once mice come out of the pool, they are placed in individual mouse cages. These need to be arranged in the fume hood.
		Arrange cages so that the ends with heat mats are touching. The mats will stay warm for longer this way.
	Once heat mats are hot, wrap them in a protective plastic bag and place them in individual mouse cages	
	Paper towel	Used to dry mice. Place paper towel in each of the mouse cages, and have spare ready to dry mice as they come out of the pool.
	Platform	Place the platform in the pool, so that it is in the Platform – Test zone.
	Running sheet	Ensure that you have a running sheet of mice to be done. (see example in Figure Five) Record: Day / Trial Mouse Number (ANYmaze) Mouse identification number Time of day Time to platform Notes

Appendix III

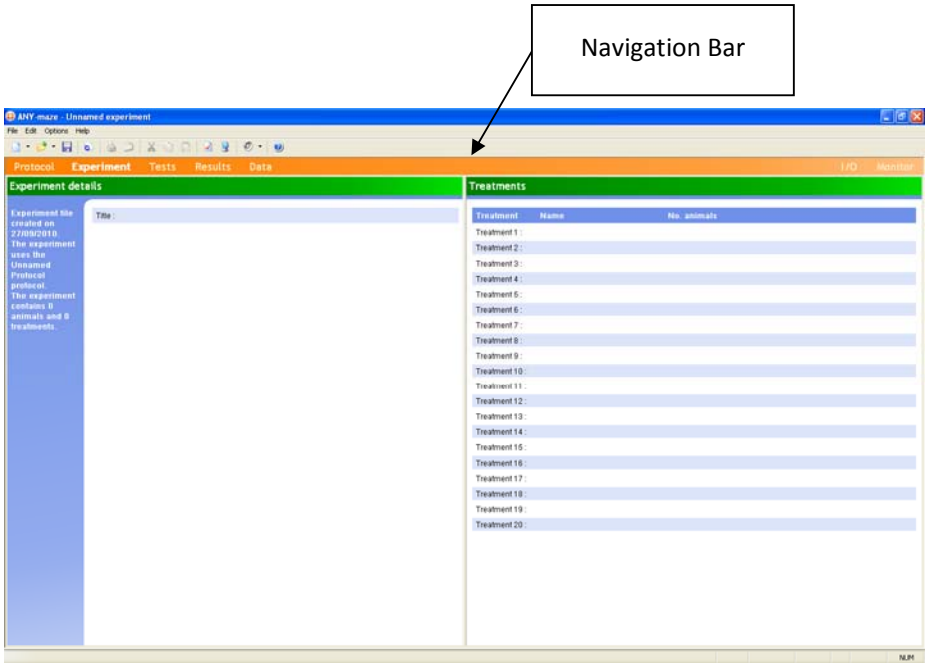
ANYmaze™ Video Tracking System Setup for the Buried Chocolate Test.

The Buried Chocolate Test is a well established test of rodent olfactory abilities. A mouse's ability to uncover a buried piece of chocolate is assessed, where latency to chocolate, total distance travelled and average speed are the main outcome measures. This test is recorded using the ANY-maze™ Video Tracking System.

ANYmaze

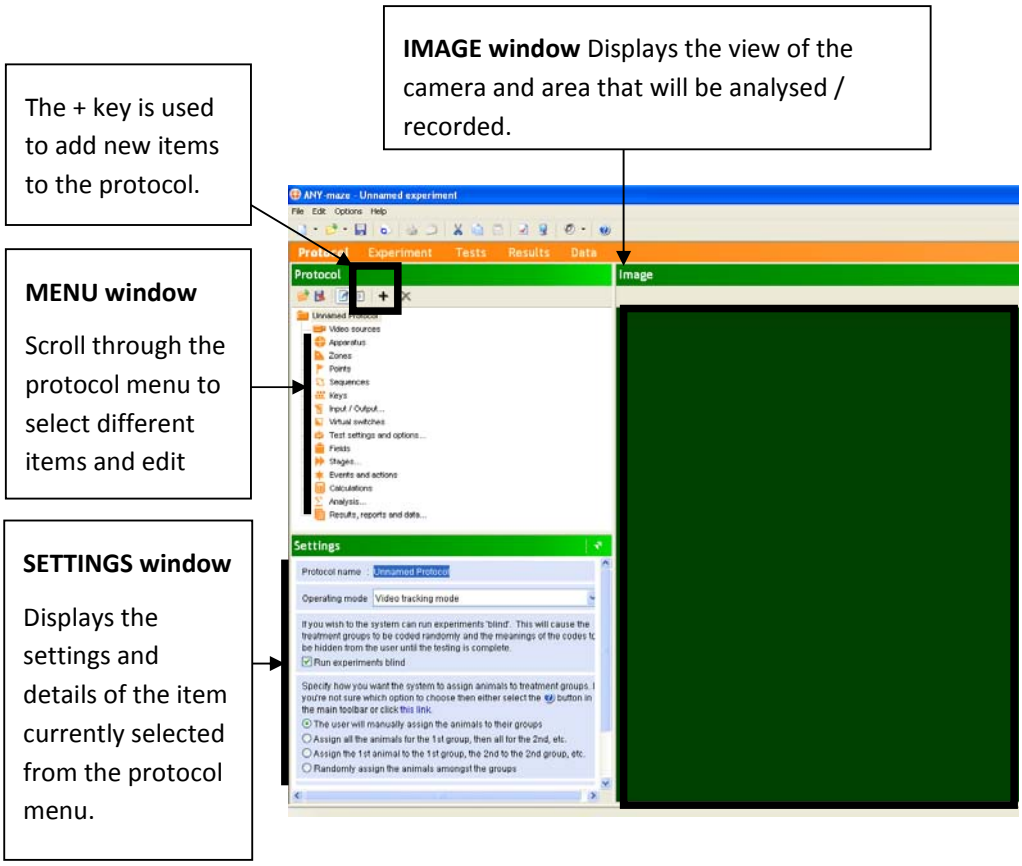
The first screen that opens in ANYmaze is a blank project.

The orange bar across the top of the screen contains five options: The five options are "PROTOCOL" "EXPERIMENT" "TEST" "RESULTS" "DATA". This bar will be used as the navigation bar to set up and run all tests.



1. SET UP PROTOCOL.

Click on “Protocol”. The following screen will appear. Select each of the relevant items from the menu to alter them



1. Add a camera source.

a. Click the + button. A menu of available options to add to the protocol will appear. Select the top option “NEW VIDEO SOURCE”.

b. ANYmaze will automatically use an available camera. If this is not the right camera, can change this in the drop down menu in Settings.

Now ANYmaze has an image to analyse. However, it needs to be told where to look for data, and what to look for. This is done in the next two steps: “Add Apparatus” and “Add Zones”.

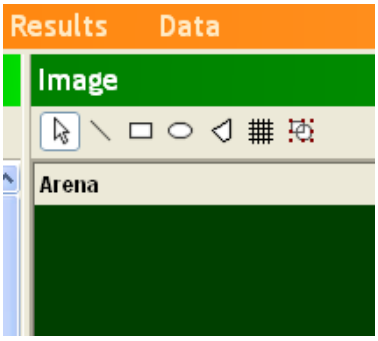
2. Add Apparatus.

a. Click the + button. Select “NEW APPARATUS”.

b. Name the apparatus “Buried Food Pellet Test”.

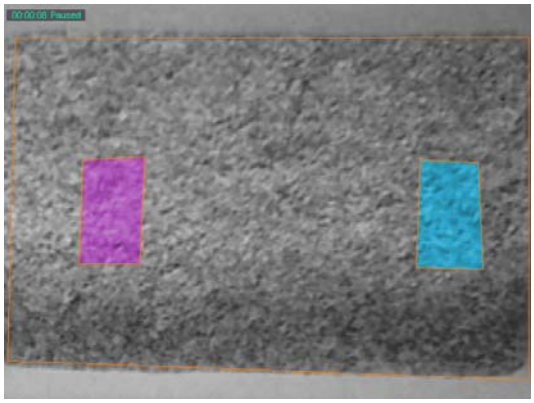
c. At the bottom of the Settings Window, type in 50 mm to set the ruler length. The actual ruler it refers to can be seen in the Image window.

d. Use the symbols at the top of the Image window to draw the arena



1. Draw a rectangle around the entire arena floor.
2. Draw a rectangle (2.5 cm x 6.0 cm) at either end of the arena so that the centre of the rectangle is approximately. 5 cm from the end of the arena.

The Arena should now look like the image below:



3. Add Zones.

Zones need to be added to let ANYmaze know what to call each part of the apparatus. The Buried Food Pellet Test arena has 2 zones: (i) the arena and (ii) the location of the hidden pellet.

Table 1: Zones for Buried Food Pellet Test.

Zone name:	Image window	Protocol Menu	Settings window
Arena		"New Zone"	Zone name: Arena Position of the zone remains the same in all tests
		Zone entry settings	Use the position of the animal's head
Hidden Pellet		"New Zone"	Zone name: Hidden Pellet Position varies within (and possibly between) animals
		Zone entry settings	Use the centre of the animal
ANYmaze needs to know where the positions will be. Click the + button, select "New Zone Position"			
Pellet Left		"New Zone Position"	Position Name: Pellet Left
Pellet Right		"New Zone Position"	Position Name: Pellet Right

3.1. Add the Arena.

To add the arena, click the + button in the Protocol menu and select “NEW ZONE” from the drop down menu.

Click on the large rectangle that was drawn in the arena when the apparatus was added (see **1.2.**). The whole arena should now be highlighted in blue.

The Settings window displays a range of options to describe this new zone. Enter the correct details to describe the Zone name and the Zone entry settings, as per Table 1.

3.2. Add the Location of the Hidden Pellet.

The location of the Pellet will change between tests. ANY-maze needs to know this. In the settings window, select “Position varies within (and possibly between) animals” from the drop down menu that describes the location of the zone. The click the + button and select “New Zone Position”, as outlined in Table 1.

4. Add stages.

The Buried Food Pellet Test consists of one test stage in one day. In the Test stage the mouse must find a buried piece of chocolate in a 3 minute period. The amount of time the mouse takes to find the chocolate, the distance travelled and the average speed of mice must all be recorded.

Set up TEST STAGE.

1. Click the + button, select “NEW STAGE”.

   In the Setting window, enter the following details:

   a. Stage name “DAY DATE - AM”
   b. Test Duration: 3 min
   c. Maximum number of trials in this stage: 1
   d. Perform each trial for all the animals before starting the next trial.

2. In the Protocol menu, select “Location of the Hidden Pellet zone during this stage”
3. In the Setting window, select “The user will specify the location at the start of each test”

5. Events and Actions

ANYmaze needs to be told to stop the test once the animal finds the buried chocolate. This is done by setting up an event (finding the chocolate) and executing and action (ending the test).

2. Click the + button. Select “New Event”.

    a. Name the event “Found Food”. Click on “Help me define the trigger for this event” and follow the prompts.

        The event will be triggered if the animal remains in the Hidden Pellet zone for 3s

    b. Select the stages for which this event occurs: Select the stage that the test will be run in (there should only be one there).

6. Number of animals.

Select “Experiment” from the orange navigation bar at the top of the screen. On the right hand side of the screen, enter how many animals will be tested.

7. Collecting data after testing.

Data from each stage is retrieved by selecting “DATA” from the orange menu bar across the top of the screen.

In the Measurement Options menu, under “Animal and Test information” select Animal Number, Stage, Trial Number, Test Time and Test Date. These will aid data management.

In the Measurement Options menu, under “Apparatus Measures” select Test Duration, Total Distance Travelled, and Average Speed.

From the Data inclusion menu at the bottom of the screen, under “Trial” select the Training Stages that data is required.

While ANYmaze has the capability to do statistical analysis on data, do not select those options here. All data analysis is done using Prism Software.

Select **DATA** to bring up the Data management screen.

Select measurement options required from the menu.

Select stages and trials from which data is to be

Select **View the Data Table** to see data requested.

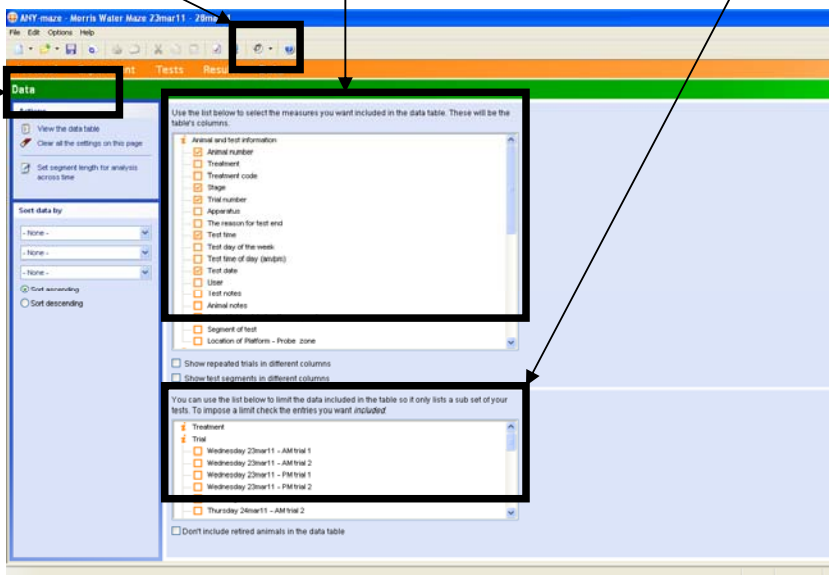


Table 2. Buried Food Pellet Test Set Up Checklist.

Completed?	Item	Description / Action
	Log in to ANYmaze	Username: XXXX Password: XXXX
	Open File	Click "File" Scroll down to the bottom of the menu, where the list of recently opened files is. Click on the appropriate file.
	Chocolate	Cut 0.8g – 1.0 g chocolate pieces in a different room on the day before the testing. On the day of testing have all the chocolate pieces required ready and in the testing room before testing commences, in an airtight container.
	Mouse cages	Have fresh mouse cages ready with plenty of food and water for mice to return to after testing finishes.
	Running sheet	Ensure that you have a running sheet of mice to be done. (see example in Figure Five) Record: Day / Trial Mouse Number (ANYmaze) Mouse identification number Time of day Time to chocolate Notes
	Stop watch	Have stopwatch ready to manually record mouse latency to chocolate.

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