2 YELLOWTAIL KINGFISH DIETARY TRIAL USING GRAPE SEED EXTRACT AS A NATURAL ANTIOXIDANT

2.1 Introduction

2.1.1 Quality of farmed fish

The quality of farmed fish and fish products is dependent on the rearing conditions and feed composition (Jensen *et al.* 1998). Feeds with a high fat content increase the growth rates of farmed fish. However, fish tissues with a high level of polyunsaturated fatty acids (PUFA) are particularly susceptible to oxidative deterioration, known as lipid peroxidation, which results from the production of damaging reactive oxygen species (ROS) (Olsen and Henderson 1997; Jensen *et al.* 1998). ROS are produced as unwanted by-products of normal metabolism, by the electron transport chain and by certain enzymes (Ritola *et al.* 2002; Hamre *et al.* 2004). ROS include the superoxide radical ($O_2^{\bullet -}$), the hydroxyl radical ($OH^{\bullet}$), the hydroperoxyl radical ($OOH^{\bullet}$), hydrogen peroxide (H_2O_2) and singlet oxygen (Hughes 2000; Wright *et al.* 2000). Iron, which is found in haemoglobin and other cellular components, catalyses the formation of the highly reactive hydroxyl radical from hydrogen peroxide in a reaction referred to as the Fenton reaction (Halliwell and Gutteridge 2003). Consequently lipid peroxidation affects storage stability and results in rancidity, which leads to undesirable flavour, texture and nutritional value changes, in fish post-mortem (Jensen *et al.* 1998). Fish dietary supplementation with antioxidants can improve the tissue lipid stability post-mortem.

The balance between prooxidative and antioxidative factors in fresh fish is maintained by numerous enzymatic and non-enzymatic defence systems. However, the enzymatic systems become inactive post-mortem, whereas the non-enzymatic systems (antioxidants) remain active, at least until they are exhausted (oxidized). The fish quality after processing or during storage relies on the level of antioxidants in the fish tissue to delay the onset of lipid peroxidation. Lipid peroxidation is the major

contributor to the chemical spoilage of fatty fish post-mortem (Petillo *et al.* 1998). In post-mortem fish tissue, reducing compounds are lost with time and antioxidants remain in their oxidized state (Petillo *et al.* 1998). Antioxidants become inactive and lipid peroxidation proceeds due to the presence of free radicals (Petillo *et al.* 1998).

2.1.2 The use of antioxidants to preserve foods

Synthetic antioxidants have been used to prevent food deterioration and maintain nutritional value since the 1940s (Shahidi 2000). Synthetic antioxidants which have been used to retard or control lipid oxidation in foods include butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), tert-butylhydroquinone (TBHQ), ethoxyquin (1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline) and ascorbyl palmitate (Shahidi 2000; Stickney 2000).

It is common to find aquatic animal feeds containing sacrificial synthetic antioxidants like BHT, BHA and ethoxyquin to inhibit the formation of free radicals or make free radicals unreactive, which consequently reduces oxidative rancidity (Stickney 2000; Moretti *et al.* 2003; Fernandez Gimenez *et al.* 2004; Bohne *et al.* 2008). Fish meal and fish oil are commonly protected from oxidation using ethoxyquin and BHT, respectively (Hamre *et al.* 2010). BHA and BHT are used in animal feeds to a maximum level of 0.02% of the lipid content of the feed (Stickney 2000). The use of BHA and BHT in foods has now been restricted because of their potential carcinogenic effects (Shahidi 2000; Jayaprakasha *et al.* 2001; Bekhit *et al.* 2003). Ethoxyquin is approved by the European Union (EU) for use in fish feeds to a maximum concentration of 150 mg kg^{-1}, while it is not permitted in human foods because exposure of humans can have adverse effects (Stickney 2000; Bohne *et al.* 2008). Few studies have investigated the possible detrimental effects of ethoxyquin on fish health and human consumer health even though it has been used for over thirty years in salmon feeds (Bohne *et al.* 2007; Bohne *et al.* 2008). The detectable levels of these sacrificial antioxidants are reduced as they react with free radicals (Stickney 2000). The accumulation of ethoxyquin metabolites such as the major metabolite, ethoxyquin dimer (EQDM), may have greater toxicological effects on humans consuming the fish fed diets containing ethoxyquin, than ethoxyquin itself

(Bohne *et al.* 2007). Interestingly, the EU does not set a maximum residual level of ethoxyquin in fish for human consumption (Bohne *et al.* 2008).

2.1.3 The use of natural antioxidants

There are limited studies investigating the levels of synthetic antioxidants found in farmed fish flesh (Moretti *et al.* 2003). However, the carry-over effect of synthetic antioxidants to the edible portion of the fish is raising food safety concerns (Hamre *et al.* 2010). The search for natural antioxidants that provide adequate protection against oxidative stress, at economically competitive prices, is increasing. Natural antioxidants used in fish feeds will improve consumer confidence and, potentially, health. Many plant based compounds have been investigated for their antioxidant potential. Examples of these compounds include resveratrol, carnosic acid, tocols (tocopherols and tocotrienols) and catechins. Resveratrol (3,4',5-trihydroxy-*trans*-stilbene) is a phytoalexin found in foods such as grapes, red wine, peanuts, olive oil and cranberries (Wang *et al.* 2004). Herbs such as rosemary and sage contain the phenolic compounds carnosic acid, 12-methanoxycarnosic acid, carnosol and rosmanol (Richheimer *et al.* 1996; Shahidi 2000). Oils and oilseeds contain tocols which possess vitamin E activity (Shahidi 2000).

2.1.4 The use of grape seed extract (GSE) as a natural antioxidant

Polyphenols found in red wine have human health benefits including inhibiting low-density lipoprotein (LDL) oxidation and reducing the risk of heart disease and cancer (Jayaprakasha *et al.* 2001; Baydar *et al.* 2004; Yilmaz and Toledo 2004). Grapes (*Vitis vinifera*), which have been processed for grape juice or wine, produce seed and skin by-products known as pomace. This pomace is a rich source of flavonoid compounds which are antioxidants (Lau and King 2003). Grape seeds are rich in oligomeric procyanidins such as the monomeric phenolic compounds (+)-catechins, (-)-epicatechin and (-)-epicatechin-3-*O*-gallate, dimeric, trimeric and tetrameric procyanidins and proanthocyanidins, which are otherwise known as tannins (Saito *et al.* 1998; Lau and King 2003; Yilmaz and Toledo 2004a). There are between 0.3 and 0.9 g kg^{-1} of proanthocyanidins in grapes (Yilmaz and Toledo 2004). On average, 60-

70% of the extractable phenolic compounds are in the grape seeds (Luther *et al.* 2007). Grape seed extract (GSE) has undergone toxicological tests which have shown that it is not toxic in rats and it is recommended both as a human nutritional supplement and for use as a food additive (Yamakoshi *et al.* 2002). Food which has had its quality improved through the use of natural antioxidants may also pass on the antioxidant health benefits to the human consumer. Tarac Technologies (Nuriootpa, South Australia) have developed Vin*life*®, a water-soluble grape skin and seed extract made from 100% Australian grapes (www.tarac.com.au). Vin*life*® is standardized to ensure that the antioxidant activity of the polyphenols is consistent in each batch (Appendix A).

We will now focus on the biological effect of GSE as an antioxidant in both *in vivo* and *in vitro* studies on the protection of tissues against free radical attack and lipid peroxidation. Flavonoids found in GSE are chain-breaking inhibitors of the peroxidation process which can scavenge free radicals and chelate metal ions (Morel *et al.* 1993; Tebib *et al.* 1997; Yilmaz and Toledo 2004). The antioxidant activity of GSE on lipid peroxidation has been examined in various *in vitro* systems. Jayaprakasha *et al.* (2001) used the thiocyanate method to examine the antioxidant effect of GSE in preventing the peroxidation of 18:2n-6 (linoleic acid, LA). An extract of GSE prepared in a 17:3 ethyl acetate:water mixture exhibited 86% inhibition of LA peroxidation after a 100 h incubation (Jayaprakasha *et al.* 2001). The study by Luther *et al.* (2007) went further to investigate the inhibitory effect of GSE on lipid peroxidation in fish oil. The fish oil oxidation rate was accelerated by exposure to a temperature of 80°C and an air flow of 7 L h^{-1} for 4.5 h (Luther *et al.* 2007). Lipid peroxidation of total PUFA, including 18:3n-3 (α-linolenic acid, ALA), LA, 20:5n-3 (eicosapentaenoic acid, EPA) and 20:4n-6 (arachidonic acid, AA) was suppressed when the fish oil contained 16.7 mg of GSE (Luther *et al.* 2007). However, GSE did not suppress lipid peroxidation of 22:6n-3 (docosahexaenoic acid, DHA) in the fish oil (Luther *et al.* 2007). Morel *et al.* (1993) showed that catechin inhibited lipid peroxidation and had iron-chelating activity in a rat iron-loaded hepatocyte culture.

An antioxidant is most effective when it is incorporated into cellular membranes within close proximity to the unsaturated lipids, which are abundant in cellular

membranes (Lau and King 2003). Therefore, dietary supplementation of an antioxidant is favourable rather than post-mortem addition to flesh (Lau and King 2003). Tebib *et al.* (1997) assessed *in vivo* lipid peroxidation of tissues in rats fed a high cholesterol and vitamin E deficient diet supplemented with grape seed tannins. The polymeric grape seed tannins reduced lipid peroxidation in plasma and heart, liver and intestinal mucosa tissues as effectively as vitamin E (Tebib *et al.* 1997). Grape seed proanthocyanidin extract (0.27 g kg^{-1} d^{-1}) was shown to inhibit oxidation of LDL and therefore have anti-atherosclerotic activity in rabbits after an 8 week feeding trial (Yamakoshi *et al.* 1999). However, GSE (2.59% or 5.18% of diet (w/w)) was found to be detrimental to the growth of chickens, even when methionine was added to the feed to counteract the digestibility problem caused by tannins (Lau and King 2003). Tannins have been reported to cause digestibility problems in ducks, chickens and rats which have lead to reduced growth (Elkin *et al.* 1990; Lau and King 2003).

2.1.5 Antioxidants in aquaculture

Lipid and haemoglobin oxidation are the major contributors to the chemical spoilage of fatty fish post-mortem (Petillo *et al.* 1998; Sohn *et al.* 2007). Bleeding of fish is done to reduce the haemoglobin in the flesh (Sohn *et al.* 2007). However, these authors showed that bleeding of yellowtail (*Seriola quinqueradiata*) does not reduce the haemoglobin concentration enough to prevent lipid oxidation. Antioxidants may be required to achieve oxidative stability of yellowtail flesh during storage (Sohn *et al.* 2007). In recent years both the aquaculture industry and the consumers of aquaculture products have shown more interest in the influence of natural dietary antioxidants on the flesh quality of farmed fish.

The influence of dietary natural antioxidants on flesh quality has been studied in several marine fish of commercial importance in European aquaculture. The antioxidant effects of various dietary vitamin E concentrations on fillet lipid peroxidation have been studied extensively in species such as sea bass (*Dicentrarchus labrax*) (Gatta *et al.* 2000), Atlantic halibut (*Hippoglossus hippoglossus* L.) (Ruff *et al.* 2002; Tocher *et al.* 2002), turbot (*Scophthalmus maximus*) (Tocher *et al.* 2002; Ruff *et al.* 2003), sea bream (*Sparus aurata* L.)

(Mourente *et al.* 2002; Tocher *et al.* 2002), rainbow trout (*Oncorhynchus mykiss*) (Jensen *et al.* 1998; Chaiyapechara *et al.* 2003) and Atlantic salmon (*Salmo salar* L.) (Scaife *et al.* 2000; Hamre *et al.* 2004). Although vitamin E is considered a natural antioxidant, synthetically derived vitamin E is most commonly added to fish feed (Chen *et al.* 2008). The vitamin E concentration in the flesh of rainbow trout and sea bass correlates with the concentration supplied in the diet (Jensen *et al.* 1998; Gatta *et al.* 2000). A vitamin E saturation point in the flesh of sea bass, Atlantic halibut and turbot occurs after 6-9 weeks of feeding diets containing high concentrations of vitamin E (Gatta *et al.* 2000; Ruff *et al.* 2002; Ruff *et al.* 2003). Results suggest that vitamin E supplementation prior to slaughter reduces lipid peroxidation in fresh fish fillets in refrigeration and frozen storage (Ruff *et al.* 2002; Ruff *et al.* 2003). However, when vitamin E and C are used as a combined dietary supplement, there is not additional protection against lipid peroxidation over and above the protection provided by vitamin E alone (Ruff *et al.* 2003).

Fish and fish products are refrigerated or frozen during transport and retail display. Very few dietary antioxidant trials have simulated the effect of storage to investigate the oxidation of lipids and deterioration of flesh quality post-mortem. The studies which have examined the effect of ice or refrigerated storage over periods up to 12 days have all used feeds supplemented with vitamin E (Gatta *et al.* 2000; Ruff *et al.* 2002; Chaiyapechara *et al.* 2003; Ruff *et al.* 2003). The effect of cold storage, with simulated retail display conditions, has been examined in four fish species (Gatta *et al.* 2000; Ruff *et al.* 2002; Chaiyapechara *et al.* 2003; Ruff *et al.* 2003). Whole Atlantic halibut which were fed diets supplemented with 189 or 613 mg kg^{-1} vitamin E for up to 24 weeks were stored on and covered with ice in a commercial display cabinet under fluorescent illumination for 9 days (Ruff *et al.* 2002). Lipid oxidation was lower in the fillets of fish fed with the higher vitamin E concentration at 2, 4 and 7 days of storage (Ruff *et al.* 2002). However, even after 9 days of storage the lipid oxidation was low with <0.6 mg malonaldehyde (MDA) kg^{-1} fillet (Ruff *et al.* 2002). Whole sea bass which were fed diets supplemented with 139, 254, 493 or 942 mg kg^{-1} vitamin E for 87 days were stored covered in ice in boxes which were placed at 1°C for 12 days (Gatta *et al.* 2000). Lipid oxidation was higher in the fillets of fish fed with the lowest vitamin E concentration at 1, 3, 6, 9 and 12 days of storage (Gatta *et al.* 2000). Interestingly, in all of the fillets the lipid oxidation did not increase over

the 12 days of storage, regardless of the vitamin E concentration (Gatta *et al.* 2000). Whole turbot which were fed diets supplemented with combinations of vitamin E and vitamin C at 500/100, 1000/100, 100/1000 and 100/100 mg kg^{-1}, respectively, for up to 15 weeks were stored on and covered with ice in a commercial display cabinet under fluorescent illumination for 9 days (Ruff *et al.* 2003). Lipid oxidation was higher in the fillets of fish fed the combinations of vitamin E and C containing lower vitamin E (100/1000 and 100/100 mg kg^{-1}) from 2 days of storage (Ruff *et al.* 2003). Fillets from rainbow trout which were fed diets supplemented with 300 or 1500 mg kg^{-1} vitamin E and 15 or 30 g lipid 100 g^{-1} feed for 10 weeks were stored at 4°C for 7 days (Chaiyapechara *et al.* 2003). Lipid oxidation was lower in fillets of fish fed with the higher vitamin E concentration (Chaiyapechara *et al.* 2003). However, the high lipid diets had more lipid oxidation, regardless of the concentration of vitamin E present (Chaiyapechara *et al.* 2003).

Supplementation of fish feed with vitamins is expensive and the feed manufacturing process partially destroys α-tocopheryl acetate, the synthetic form of vitamin E, which results in a loss of at least 33% (Chaiyapechara *et al.* 2003). Therefore, to account for the loss more vitamin E needs to be added prior to extrusion pelleting, which adds to the cost. The economic burden, together with the previous studies on vitamin E highlights the potential for other economically viable natural antioxidants to protect fish flesh from lipid oxidation during storage under retail conditions. GSE may become an economically viable natural antioxidant as it is a by-product of the production of a high value product, namely wine.

The aim of the work presented within this chapter was to investigate the effect of GSE as a natural antioxidant substitute for the synthetic antioxidant ethoxyquin in the feed of yellowtail kingfish (YTK, *Seriola lalandi*), an important aquaculture species in southern Australia. Hamre *et al.* (2010) showed that ethoxyquin could be substituted with natural antioxidants in fish feeds effectively. We examined the edible portion of the fish, the fillet. The activity of GSE as an antioxidant was assessed by studying the lipid oxidation and fatty acid compositional changes in both fresh and refrigeration stored fillets. To simulate retail storage of fish fillets they were stored for 4 days at 4°C in a glass door refrigerator where fillets were exposed to fluorescent illumination.

2.2 Methods

2.2.1 Materials

All reagents used were obtained from Sigma-Aldrich®, Australia unless otherwise stated.

2.2.2 Experimental diets

The experimental diets were produced at the Australasian Experimental Stockfeed Extrusion Centre (AESEC), Roseworthy campus, The University of Adelaide, SA, Australia. The feeds were formulated with fish meal, wheat gluten and fish oil. The fish oil was obtained from Skretting Australia (Cambridge, Tasmania, Aust.) and the custom mineral/vitamin premix was obtained from Lienert Australia Pty. Ltd. (Roseworthy, SA, Aust.) (Table 2.1). Sixty g kg^{-1} of fish oil was added to the formulation at the time of extrusion using a Wenger X-85 extruder. A UAS vacuum infusion system (UAS Canada Inc., BC, Canada) was used to enrich the feeds with 150 mg kg^{-1} ethoxyquin and/or 250 or 1250 mg kg^{-1} Vin*life*® (Tarac Technologies), with a further 30 g kg^{-1} of fish oil. The pellet size for all diets was 3 mm. The final formulation and proximate composition of the experimental diets are given in Table 2.2. The ethoxyquin and GSE content in the four diets can be seen in Table 2.2. To avoid lipid peroxidation all diets were stored at -20°C until feeding.

Table 2.1 Custom mineral/vitamin premix formulation

The formulation of the custom mineral/vitamin premix obtained from Lienert Australia Pty. Ltd. which was used in the experimental diets.

Custom mineral/vitamin premix components	Final feed concentration
Fat-soluble vitamins	
Vitamin A, IU kg^{-1}	3000
Vitamin D, IU kg^{-1}	750
Vitamin E, mg kg^{-1}	100
Vitamin K, mg kg^{-1}	7
Water-soluble vitamins, mg kg^{-1}	
Riboflavin	20
Pantothenic acid	30
Niacin	65
Vitamin B12	0.03
Biotin	0.3
Folate	4
Thiamin	10
Vitamin B6	15
Vitamin C	100
myo-Inositol	200
Minerals, mg kg^{-1}	
Magnesium (as magnesium sulphate)	5
Iron (as iron sulphate)	40
Zinc (as zinc sulphate)	30
Manganese (as manganese sulphate)	20
Copper (as copper sulphate)	3
Iodine (as potassium iodide)	1.1
Selenium (as sodium selenite)	0
Cobalt (as cobalt sulphate)	1

Table 2.2 Diet formulations

The four diet formulations, including ethoxyquin and GSE concentrations, and the proximate composition.

	Diet			
	1	2	3	4
Ingredients (g kg^{-1})				
Fish meal	600	600	600	600
Wheat gluten	200	200	200	200
Fish oil	90	90	90	90
Lecithin	10	10	10	10
Sulphur	10	10	10	10
Choline chloride	2	2	2	2
Custom Mineral/Vitamin Premix	2	2	2	2
Water additional approx	100	100	100	100
Ethoxyquin	0.15	0.15	0.15	0
Grape seed extract	0	0.25	1.25	0.25
Proximate composition				
Dry Matter (% of wet weight)	91.84	92.97	93.34	92.79
Crude Protein (% of dry weight)	53.75	54.38	54.38	55.00
Crude Fat (% of wet weight)	16.01	17.38	17.11	16.16
Crude Ash (% of wet weight)	16.23	16.24	16.15	16.60

2.2.3 Experimental fish and dietary trial conditions

The experiment was performed at the South Australian Research and Development Institute (SARDI) Aquatic Sciences facilities (West Beach, SA, Australia). Juvenile yellowtail kingfish (YTK, *Seriola lalandi*) were obtained from the Clean Seas Tuna Ltd. hatchery at Arno Bay, South Australia and were maintained in a 5 kilolitre (kL) tank. They were fed a standard commercial YTK diet prior to the trial commencing. The dietary trial was conducted in twelve 1 kL tanks, with each tank piped with filtered re-circulated seawater from Gulf Saint Vincent. Compressed air was provided via a diffuser to every tank for oxygenation of the water. Netting was secured over the tops of the tanks. The tank layout and experimental diets fed to the fish in each tank can be seen in Figure 2.1.

Ten fish were randomly selected for each trial tank and the weight of each fish was recorded. The fish were stocked in the experimental tanks at an initial mean weight per tank of 778 g. Spare fish were kept in a separate 1 kL tank and maintained on the standard commercial YTK diet, to replace any trial fish which died during the trial. The fish were fed the standard commercial YTK diet for 1 day in the experimental tanks to allow them to acclimatize to the experimental conditions. Thereafter, the fish were fed once daily to satiation, six days a week, for 8 weeks. Each diet was fed to triplicate tanks as shown in Figure 2.1.

The diet trial was performed at a mean water temperature of 16.4 ± 1.4°C with exposure to a natural light cycle. The water quality parameters, including ammonium concentration, temperature, pH, dissolved oxygen concentration and salinity were measured every two days for the duration of the trial. Mr. Arron Strawbridge (SARDI Aquatic Sciences facilities) measured these parameters. The seawater had a salinity of 37.2 g L^{-1} and a pH of 7.73-8.17 throughout the trial. The oxygen level was 96.8 ± 2.7%.

The outdoor area where the tanks were positioned was covered by a large roof. Tanks 34, 35 and 36 were on the northern end of the rows of tanks and received more sunlight than the other tanks. Therefore, these tanks had more algal growth than the other tanks due to their position. All tanks were cleaned at least once a week to

remove any algal growth on the sides of the tank and excess food and waste products were siphoned from the bottom of the tank.

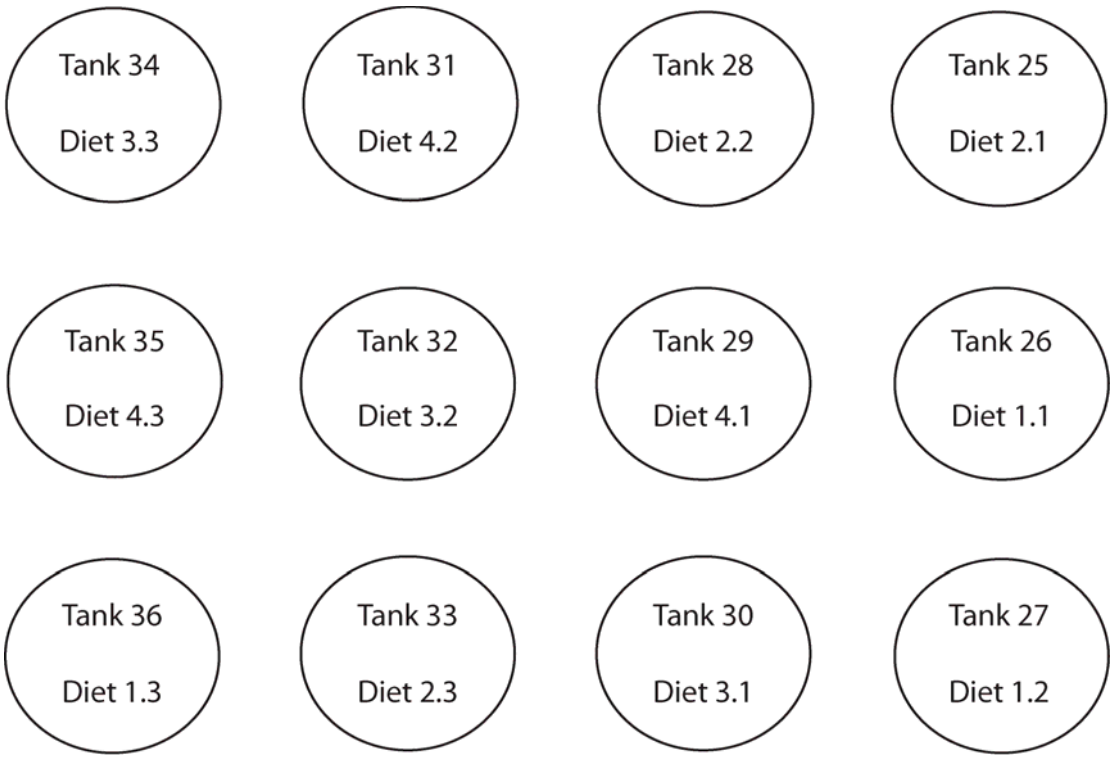

Figure 2.1 Tank layout

The layout of the 12 tanks used in the study and the diet fed to the fish in each of these tank. The diets were fed in triplicate tanks e.g. diet 1 was fed as diet 1.1, 1.2 and 1.3.

2.2.4 Sampling of fish

An initial harvest of ten fish was performed at the start of the trial to obtain baseline measurements. These fish will be referred to as the baseline fish. The ten fish were randomly selected from the 5 kL tank with a net and were euthanased in ice water. The weights of the fish were recorded. To aid in bleeding the fish, an incision was made under the gills and the tail was removed. Fish were filleted and dissected to obtain both fillets and the liver, which were immediately placed on ice. Samples were transported to Flinders University and stored at -80°C within 2 h of commencing the harvest.

At the end of the 8-week experimental period, all of the fish were slaughtered. There was a 24 h starvation period prior to slaughter to avoid interference from the gut contents. The fish were removed from the tanks with a net and were euthanased in ice water as quickly as possible to avoid any stress to the fish. The fish were weighed and their length recorded. Seven fish from each tank were chosen to be filleted and dissected to obtain both fillets and the liver, which were immediately placed on ice. The liver was weighed immediately. The remaining three fish from each tank were kept whole for proximate analysis or pathology testing. Samples were transported to Flinders University on ice within 4 h of commencing the harvest and stored in one of two ways. The right fillet from each fish was used in a shelf life experiment to simulate retail display conditions; these fillets were stored in a glass door refrigerator at 4°C for 4 days, then at -80°C until analysis. The left fillet and liver from each fish were stored at -80°C immediately to represent fresh tissue. Whole fish for proximate analysis or pathology testing were stored on ice during transport to the respective locations for analysis.

Core samples (8 mm in diameter and approximately 300 mg in weight) were obtained from the same location on the fillet each time for fatty acid extraction and thiobarbituric acid reactive substances (TBARS) analysis. The skin was removed from each sample. Figure 2.2 depicts the sampling locations on the fillet.

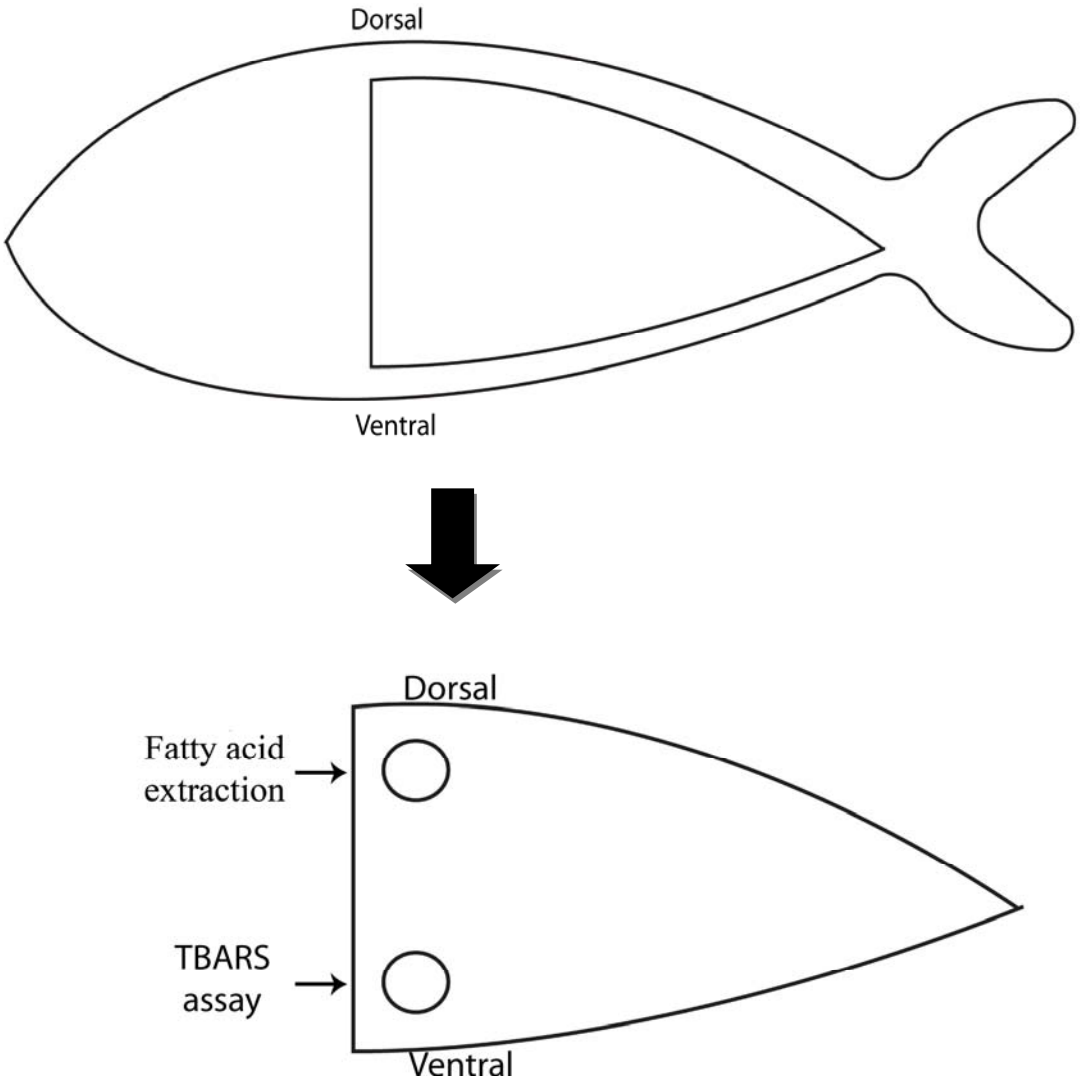

Figure 2.2 Fillet sampling locations

The location on the fillet used for obtaining samples for fatty acid extraction and the TBARS assay.

2.2.5 Survival and growth measurements

Various survival and growth measurements were calculated using the following equations.

IBW (g), initial body weight
WG (%), percentage weight gain = 100 x ((final body weight (FBW) – IBW)/IBW)
TL (cm), total fish length, distance from the snout to the tail
SGR (% gain day^{-1}), specific growth rate = 100 x ((ln FBW – ln IBW)/56 days)
FCR, feed conversion ratio = dry feed intake (g) / wet WG (g)
HSI (%), hepatosomatic index = 100 x (liver weight) x $(body\ weight)^{-1}$
Survival (%) = 100 x ((initial fish number – dead fish number)/initial fish number)

2.2.6 Proximate composition analysis

Proximate analysis was used to determine the composition of the four feeds and two randomly selected fish from each tank, at the end of the trial. Proximate analysis was used to determine moisture, protein, lipid and ash (Stickney 2000). The dry matter content allowed composition to be compared without taking into account the effects caused by differences in moisture content (Stickney 2000). The crude protein content was determined by oxidising the organic matter and all forms of nitrogen were reduced to ammonium sulphate (Stickney 2000). Based on the assumption that protein contains 16% (w/w) nitrogen, the total nitrogen content was multiplied by the empirically derived conversion factor 6.25 to calculate crude protein content (Stickney 2000). Normalisation to the dry weight of the sample was done to obtain the final protein content (Stickney 2000). The crude fat content was determined by extracting lipids with organic solvents (Stickney 2000). The ash content was determined by combusting the sample at 600°C, which oxidised all organic material and left the inorganic content (Stickney 2000).

The proximate analyses were carried out by SARDI Pig and Poultry Production Institute, Nutrition Research Laboratory, Roseworthy Campus, The University of Adelaide, Australia. The feeds were analysed for dry matter, crude ash and crude fat

and data are reported on an ‘as received’ basis, while feed for crude protein determination was laboratory oven-dried prior to analysis and reported on a ‘dry matter’ basis. The fish were freeze-dried prior to analysis of dry matter, crude protein, crude fat, crude ash and moisture, and were reported on a ‘dry matter’ basis.

2.2.7 Pathology testing

Pathology testing was performed on six fish from the trial, each selected from a different tank, and on four fish maintained on the standard commercial YTK diet in a separate tank. The six fish from the trial were chosen based on weight and appearance. Three fish with low body weight (48.5 ± 1.4 g), which had an appearance of looking unhealthy and three fish with high body weight (156.6 ± 21.3 g), which had an appearance of looking healthy, were chosen (Table 2.3). Testing was conducted to ensure disease was not responsible for the observed variation in the size and appearance of the fish. Macroscopy, microscopy and gross pathology analysis were carried out by Gribbles Vetlab, 33 Flemington St, Glenside, SA, Australia.

Table 2.3 The weight and length of the six fish from the trial chosen for pathology testing.

	Diet 1		Diet 2	Diet 3		Diet 4
	Tank 27	Tank 36	Tank 28	Tank 32	Tank 34	Tank 29
Weight (g)	47.4	150.4	180.3	139	48.1	50.1
Length (cm)	18.3	24.2	25	23.3	18.8	19.3

2.2.8 Fatty acid analysis

2.2.8.1 Extraction of total lipid from tissues

The core sample taken from the fillet (Figure 2.2) was allowed to thaw at room temperature. A glass plate was cleaned with saline and methanol to ensure no fatty acid residues remained. Using a scalpel and tweezers, the tissue was cut into small pieces on the glass plate. Approximately 300 mg of fillet was used, with the exact weight recorded. Two mL of cold 0.9% (w/v) saline was added to the tissue and it was vortex mixed. The sample was homogenised using a T25 digital ULTRA-TURRAX® disperser (IKA®-Group). Total lipid was extracted from the samples with chloroform:methanol (2:1 by volume) basically according to the method of Folch *et al.* (1957). All solvents contained 0.005% (w/v) BHA as an antioxidant, unless otherwise stated. Three mL of methanol was added, vortex mixed and allowed to stand for 5 min at room temperature. Six mL of chloroform was added, vortex mixed and allowed to stand for a further 10 min at room temperature. The aqueous and organic phases were separated by centrifugation at 1,500 *g* for 10 min. The lower organic phase containing the lipid was recovered and transferred into a weighed glass scintillation vial for evaporation to dryness under a stream of nitrogen. The lipid weight was determined by re-weighing the scintillation vials after the sample had been dried under nitrogen. The lipid was resuspended in 5 mL of 1% (v/v) sulphuric acid in methanol. Total lipid was methylated at 70°C for 3 h, shaking every 30 min. Fatty acid methyl esters (FAME) were extracted into 750 µl of distilled H_2O and 2 mL of heptane. After mixing well and allowing the layers to separate, the top heptane layer was transferred to a gas chromatography vial containing anhydrous sodium sulphate. The samples were concentrated under nitrogen if necessary to a final volume of 500 µl and analysed by gas chromatography (GC).

2.2.8.2 Extraction of total lipid from feeds

The feeds, which had previously been stored at -80°C until analysis, were ground into a fine powder in a scintillation vial. Approximately 1 g of ground feed was used, with the exact weight recorded. Total lipid was extracted basically according to the method in section 2.2.8.1. Exceptions to the method included transferring the lower

organic phase to a weighed glass scintillation vial, then adding another 6 mL of chloroform to each sample to extract all of the fatty acids. The samples were allowed to stand for 5 min before re-centrifugation under the same conditions as previously stated. The lipids were evaporated to dryness under a stream of nitrogen and then the scintillation vials were re-weighed to determine the lipid weight. Ten mg of lipid from each feed was transferred to a new 20 mL scintillation vial for methylation.

2.2.8.3 Extraction of lipid classes from tissues

The tissue samples were prepared for extraction as outlined in section 2.2.8.1. The cholesterol ester, triacylglycerol, free fatty acid, sterol and phospholipid lipid classes were extracted from the samples using chloroform:methanol as outlined for total lipid extraction in section 2.2.8.1. After drying the lipid under nitrogen, it was resuspended in 150 µl chloroform:methanol (9:1 by volume) and spotted onto 0.3 mm Silica Gel (60H) thin layer chromatography (TLC) plates (Merck, Darmstadt, Germany). Lipid classes were separated by TLC using a mobile phase of petroleum spirit:diethyl ether:glacial acetic acid (180:30:2 by volume). The plate was sprayed with fluorescein 5-isothiocyanate in methanol before visualization under ultraviolet light. Each lipid class was scraped into a 5 mL glass vial containing 2 mL of 1% (v/v) sulphuric acid in methanol. The fatty acids were methylated at 70°C for 3 h, shaking every 30 min. FAME were extracted into 250 µl of distilled H_2O and 500 µL of heptane. After mixing well and allowing the layers to separate, the top heptane layer was transferred to a gas chromatography vial containing anhydrous sodium sulphate. The samples were concentrated under nitrogen to a final volume of 100 µl, transferred into a gas chromatography insert and analysed by gas chromatography.

2.2.8.4 Analysis of FAME by gas chromatography

FAME were analysed using a Hewlett-Packard 6890 gas chromatograph (Hewlett Packard, Palo Alto, CA, USA) fitted with a flame ionisation detector and a BPX-70 50 m capillary column coated with 70% cyanopropyl polysilphenylene-siloxane (0.25 mm film thickness and 0.32 mm internal diameter; SGE, Australia). The carrier gas was helium at a flow rate of 2.0 mL min^{-1} and the split-ratio was 20:1. The injection port temperature was 250°C and the detector temperature was 300°C. The

initial column temperature was 140°C and it was increased to 220°C at a rate of 4°C min^{-1} and held at 220°C for up to 3 min. The identity of each fatty acid peak in the chromatogram was ascertained by comparing its retention time to an authentic lipid standard (Nu-Chek Prep, Inc., MN, USA). The chromatogram of the authentic lipid standard can be seen in Figure 2.3. FAME were quantified using GC Chemstation software (Agilent Technologies, Australia). The amount of each fatty acid was expressed as a percentage of the total amount of all fatty acids, by expressing the peak area for an individual fatty acid as a percentage of the total peak area for all fatty acids. Alternatively, each fatty acid was quantified by comparing all of the peaks in the profile with the peak size of a C17:0 internal standard. The limit of detection was 0.05% total fatty acids. A peak which eluted after 18:2n-6 could not be identified using the authentic lipid standard. Roxanne Portolesi has previously analysed this peak using GC-mass spectrometry (MS) and identified it as 3-t-butyl-4-hydroxyanisole (BHA) (Portolesi 2007). BHA had been added to all solvents during the extraction process to prevent lipid oxidation.

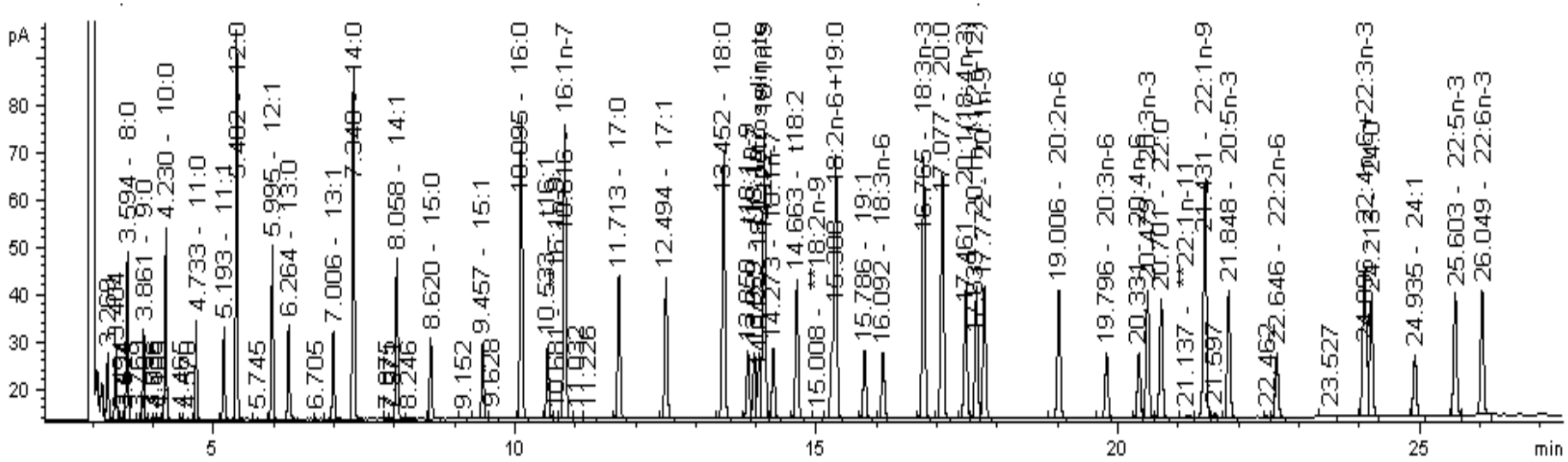

Figure 2.3 The gas chromatogram of the authentic lipid standard (NuChek Prep, MN, USA). The retention time for each fatty acid peak in the standard was used to identify the peaks obtained for the samples.

2.2.9 Lipid quality parameters

Lipid quality parameters were calculated from the fatty acid composition data using the following equations.

The unsaturation index represents the number of double bonds available for peroxidation (Mourente *et al.* 2002; Tocher *et al.* 2002).
Unsaturation index of feed or fillet = percentage of each fatty acid x number of double bonds in that fatty acid.

The flesh lipid quality (FLQ) index represents the relationship between the main n-3 LCPUFA (EPA and DHA) and the total lipids in the muscle (Senso *et al.* 2007).
FLQ = 100 x ((percentage of EPA + percentage of DHA)/percentage of total fatty acids).

The index of atherogenicity (IA) represents the relationship between saturated fatty acids which are pro-atherogenic and unsaturated fatty acids which are anti-atherogenic (Senso *et al.* 2007).
IA = (12:0 + (4 x 14:0) + 16:0)/(MUFA + n-6 PUFA + n-3 PUFA)

The index of thrombogenicity (IT) represents the relationship between saturated fatty acids which are pro-thrombogenic and MUFA, n-6 PUFA and n-3 PUFA which are anti- thrombogenic (Senso *et al.* 2007).
IT = (14:0 + 16:0 + 18:0)/((0.5 x MUFA) + (0.5 x n-6 PUFA) + (3 x n-3 PUFA) + (n-3 PUFA/n-6 PUFA))

2.2.10 Measurement of thiobarbituric acid reactive substances (TBARS)

The thiobarbituric acid reactive substances (TBARS) assay measures aldehydes which are secondary end products of lipid peroxidation (Tocher *et al.* 2002). The measurement of TBARS was carried out using a method adapted from Burk *et al.* (1980). The core sample taken from the fillet (Figure 2.2) was approximately 300 mg. Two hundred mg of this tissue was homogenised, using a small hand held

homogeniser in 15 mL of 20% (v/v) trichloroacetic acid (TCA) containing 0.5 mL of 1% (w/v) BHT in ethanol. Thirty mL of 50 mM (w/v) thiobarbituric acid (TBA) was added and the reagents were mixed and heated at 100°C for 10 min. The tubes were allowed to cool before being centrifuged at 2,000 *g* for 10 min at 4°C to remove protein precipitates. The supernatant was assayed at 532 nm in duplicate. The absorbance was recorded against a blank at the same wavelength. A MDA standard curve was constructed and the TBARS value for the samples was expressed as nmol MDA per gram of fillet.

2.2.11 Statistical analysis

Experimental data were analysed statistically using the PASW Statistics 17.0.2 for Windows (SPSS, Chicago, IL, USA) software package. The data were checked for normality and homogeneity of the variances by the Levene test which tests the assumption that the group variances are homogenous. The effects of dietary treatment on growth performance, feed conversion efficiency and proximate composition were analysed by one-way analysis of variance (ANOVA). The effects of diet and storage on the fillet fatty acid composition, fat content, unsaturation index, IA, IT, FLQ index and TBARS values were determined by two-way ANOVA. When a significant effect was found by one- or two-way ANOVA, means were compared by the Tukey's or Games-Howell *post hoc* tests. The Tukey's test was used when the homogeneity of variance assumption was not violated in the ANOVA analysis (the Levene test of equality of error variances was not significant at the 0.05 level for all analyses). The Tukey's *post hoc* test found the difference between the means of all of the groups which was then compared to a critical value to see if the difference was significant. The critical value was the honestly significantly difference (HSD) which is the point when a mean difference becomes honestly significantly different. The Games-Howell *post hoc* test was used when the homogeneity of variance assumption was violated in the ANOVA analysis (the Levene test of equality of error variances was significant at the 0.05 level for all analyses). Differences were reported as statistically significant when $P<0.05$.

2.3 Results

2.3.1 Analysis of YTK experimental diets

The four experimental diets were formulated to be identical except for the variation in the concentrations of ethoxyquin and GSE. As expected the proximate compositions of the four diets were all very similar. Across the four diets the average values ± SD for dry matter was 92.7 ± 0.6% of wet weight, crude protein was 54.4 ± 0.5% of dry weight, crude fat was 16.7 ± 0.7% of wet weight and ash was 16.3 ± 0.2% of wet weight. The feed fat content determined by fatty acid analysis in our laboratory and by proximate analysis at SARDI were very similar (Table 2.4).

The total saturated, monounsaturated, n-9, n-7, n-6 and n-3 fatty acids were found to be consistent across the four diets (Table 2.5). Considering the important n-3 fatty acids, the diets contained approximately 1% ALA and 10% of both EPA and DHA. With respect to the important n-6 fatty acids, the diets contained approximately 6.6% LA and 1% AA. The unsaturation index was consistent across all of the feeds at approximately 188.5.

Table 2.4 The fat content of the experimental diets determined by proximate analysis at SARDI Pig and Poultry Production Institute, as outlined in section 2.2.6, or by fatty acid analysis in our laboratory, as outlined in section 2.2.8.2.

Diet	Fat (% of wet weight)	
	Proximate analysis	Fatty acid analysis
1	16.0	17.2
2	17.4	17.7
3	17.1	18.0
4	16.2	19.4

Table 2.5 The fatty acid composition (% of total fatty acids) and unsaturation index of the experimental diets.

Fatty Acid	Composition (% of total fatty acids)			
	Diet 1	Diet 2	Diet 3	Diet 4
14:0	5.4	5.5	5.6	5.6
16:0 (palmitic acid)	17.2	17.3	17.3	17.4
18:0 (stearic acid)	4.2	4.2	4.2	4.2
Total saturated fatty acids (Sats)	30.6	30.4	30.5	30.4
16:1n-7 (palmitoleic acid)	7.0	7.1	7.1	7.1
18:1n-9 (oleic acid)	17.5	17.6	17.3	17.6
18:1n-7	3.2	3.3	3.3	3.3
22:1n-11	2.3	2.3	2.4	2.4
Total monounsaturated fatty acids (MUFA)	34.8	35.0	34.8	35.2
Total n-9	22.3	22.2	22.1	22.3
Total n-7	10.2	10.4	10.4	10.4
18:2n-6 (LA)	6.8	6.7	6.5	6.6
20:4n-6 (AA)	1.0	1.0	1.0	1.0
22:4n-6	0.1	0.1	0.1	0.1
22:5n-6	0.7	0.7	0.6	0.6
Total n-6	9.3	9.1	8.9	8.9
18:3n-3 (ALA)	1.0	1.0	1.0	1.0
18:4n-3 (SDA)	1.9	1.9	2.0	1.9
20:5n-3 (EPA)	10.1	10.1	10.2	10.1
22:5n-3 (DPA)	1.5	1.5	1.5	1.4
22:6n-3 (DHA)	10.0	10.0	10.1	9.9
Total n-3	24.8	24.8	25.1	24.7
n-3/n-6	2.7	2.7	2.8	2.8
LA/ALA	6.5	6.7	6.2	6.6
EPA/AA	10.4	10.6	10.8	10.6
EPA/ALA	9.7	10.1	9.8	10.1
DHA/EPA	1.0	1.0	1.0	1.0
Unsaturation index	188.8	188.3	189.1	187.7

2.3.2 Growth performance and feed utilization efficiency

The growth performance and feed utilization efficiency of the fish fed the four different diets was compared. The data obtained at the end of the 8 week feeding trial are given in Table 2.6. Mortality was very low during the experiment with survival ranging from 96.7 to 100%. The one mortality occurred at the end of the trial, so the fish was not replaced. Fish weight gain ranged from 56.6 to 79.6%, which is a broad range indicating substantial variation. The variation occurred not only between diets, which may have been expected, but also within tanks of fish fed the same diet (Figure 2.4). The weight gain of the fish fed diet 1 containing no GSE was just as variable as the weight gain of the fish fed the diets containing GSE. Thus, the variation in weight gain could not be attributed to the inclusion of GSE in the diets. Due to the large fish-to-fish variability, there were no significant differences in any of the growth performance or feeding efficiency parameters between any of the different diets. Although there were no significant differences for SGR or FCR, the lowest SGR value was 0.8% gain day^{-1} which corresponded with the highest FCR value of 21.4. This was for fish consuming diet 3 which had the highest GSE content. The whole-body dry matter, ash, fat, protein and moisture content of fish was not affected by the dietary treatments (Table 2.7).

Table 2.6 Growth performance and feed utilisation efficiency of the fish fed the experimental diets for 8 weeks. Each diet was fed to 3 replicate tanks and each tank contained 10 fish. The data are the mean ± SE (n=3). There were no significant differences between the diets for any of the parameters measured.

	Diet 1	Diet 2	Diet 3	Diet 4
IBW (g)	77.8 ± 0.6	78.0 ± 0.8	76.1 ± 0.3	79.5 ± 0.1
TL (cm)	22.8 ± 0.5	23.1 ± 0.1	22.4 ± 0.6	22.3 ± 0.4
WG (%)	65.8 ± 11.2	79.6 ± 11.2	56.6 ± 18.8	64.9 ± 7.7
SGR (% gain day^{-1})	0.9 ± 0.1	1.0 ± 0.1	0.8 ± 0.2	0.9 ± 0.1
FCR	18.0 ± 2.0	15.6 ± 1.1	21.4 ± 5.6	16.8 ± 0.4
HSI (%)	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.7 ± 0.1
Survival (%)	100	96.7	100	100

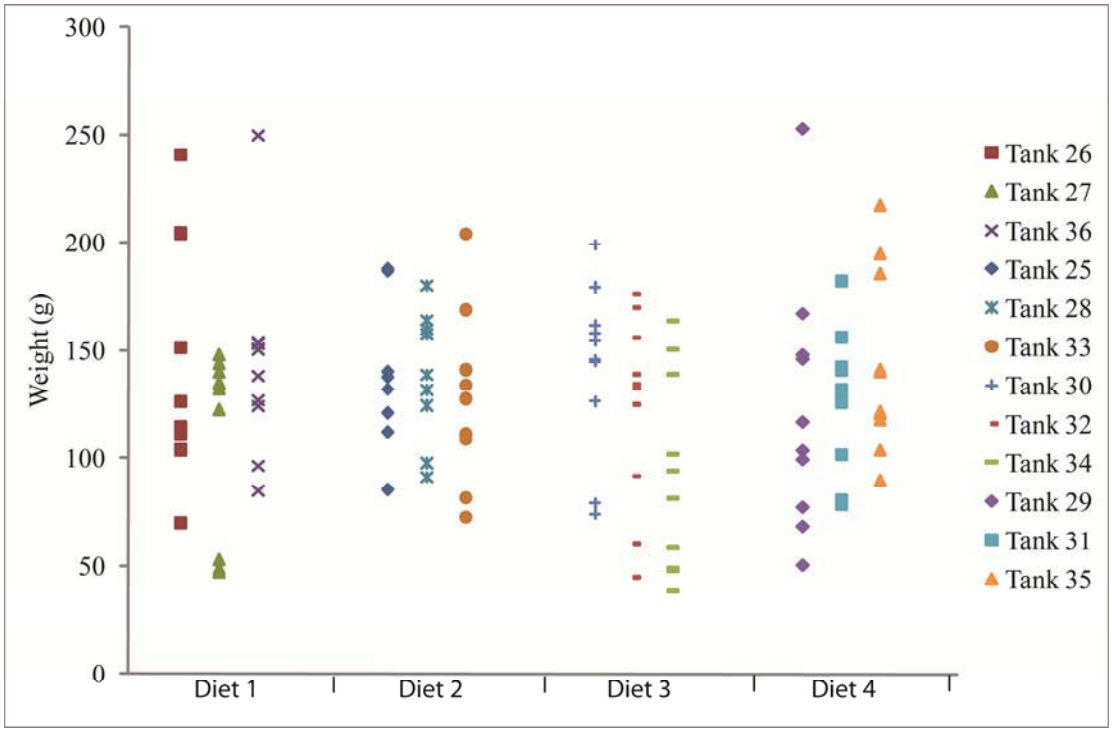

Figure 2.4 The variation in fish weight between diets and also within tanks of fish fed the same diet. The weights of the fish were recorded at the end of the 8 week feeding trial. Each diet was fed to 3 replicate tanks and each tank contained 10 fish.

Table 2.7 Proximate analysis of the fish fed the experimental diets for 8 weeks. Each diet was fed to 3 replicate tanks and each tank contained 10 fish. One fish from each tank was sampled for proximate analysis. The data are the mean ± SE (n=3). There were no significant differences between the diets for any of the parameters measured.

Diet	Dry Matter	Crude Ash	Crude Fat	Crude Protein	Moisture
			(% of dry weight)		
1	96.0 ± 0.2	12.5 ± 0.4	13.4 ± 1.3	70.4 ± 1.3	74.8 ± 0.2
2	96.0 ± 0.2	11.0 ± 0.3	16.6 ± 1.3	67.5 ± 1.0	73.2 ± 0.5
3	96.1 ± 0.3	14.9 ± 3.6	11.3 ± 4.9	71.0 ± 2.9	76.0 ± 2.5
4	95.8 ± 0.2	12.7 ± 0.7	12.6 ± 1.4	70.0 ± 1.0	74.9 ± 0.6

2.3.3 Pathology testing

A large variation in the size and appearance of the fish in the trial was observed and pathology testing was done to ensure disease was not responsible. The six fish fed the trial diets were found to have no food in their gastro-intestinal tracts while the fish being maintained on the standard commercial YTK diet in a separate tank had full stomachs. There were no lesions on the heart, liver, kidney, spleen, stomach, small intestine including pancreas, gills or body wall/skin tissues examined which suggested that viral, bacterial or parasitic infection was not present in any of the fish. The larger fish had mild fat vacuolation of the hepatocytes which is considered physiological and suggests better or more adequate nutrition. Three of the fish from the trial (one small, two large) and two fish maintained on the commercial YTK diet had an undershot jaw, while the other fish maintained on the commercial YTK diet had a parrot mouth. Therefore, pathology testing did not find any evidence of infectious disease in any of the fish examined but there were some jaw malformations which may have affected feeding efficiency.

2.3.4 The effect of dietary GSE supplementation on fillet quality

2.3.4.1 Fillet fat content

The fat content of fillets from the fish fed the experimental diets was compared with the fat content of fillets from the baseline fish harvested prior to the commencement of the feeding trial. The fat content of the ten baseline fish varied between 0.6 and 1.4% of wet weight, with a mean value of 0.9% of wet weight (Figure 2.5). After 8 weeks of feeding with the experimental diets there was no significant difference in the fillet fat content compared with the baseline fish (Figure 2.5). A trend emerged in the fish fed diet 3 (1250 and 150 mg GSE and ethoxyquin kg^{-1} feed, respectively) and diet 4 (250 mg GSE kg^{-1} feed) such that fish weight was positively correlated with fillet fat content (Figure 2.6). However, this trend was not observed in the fish fed diet 1 (150 mg ethoxyquin kg^{-1} feed) and diet 2 (250 and 150 mg GSE and ethoxyquin kg^{-1} feed, respectively) (Figure 2.6). Fish weighing approximately 150 g displayed a variable fat content when they were fed diets 1, 2 and 3 (Figure 2.6). There was no significant difference between the total fat content of the fresh fillets and the fillets stored at 4°C for 4 days, for any of the dietary treatments (Figure 2.7).

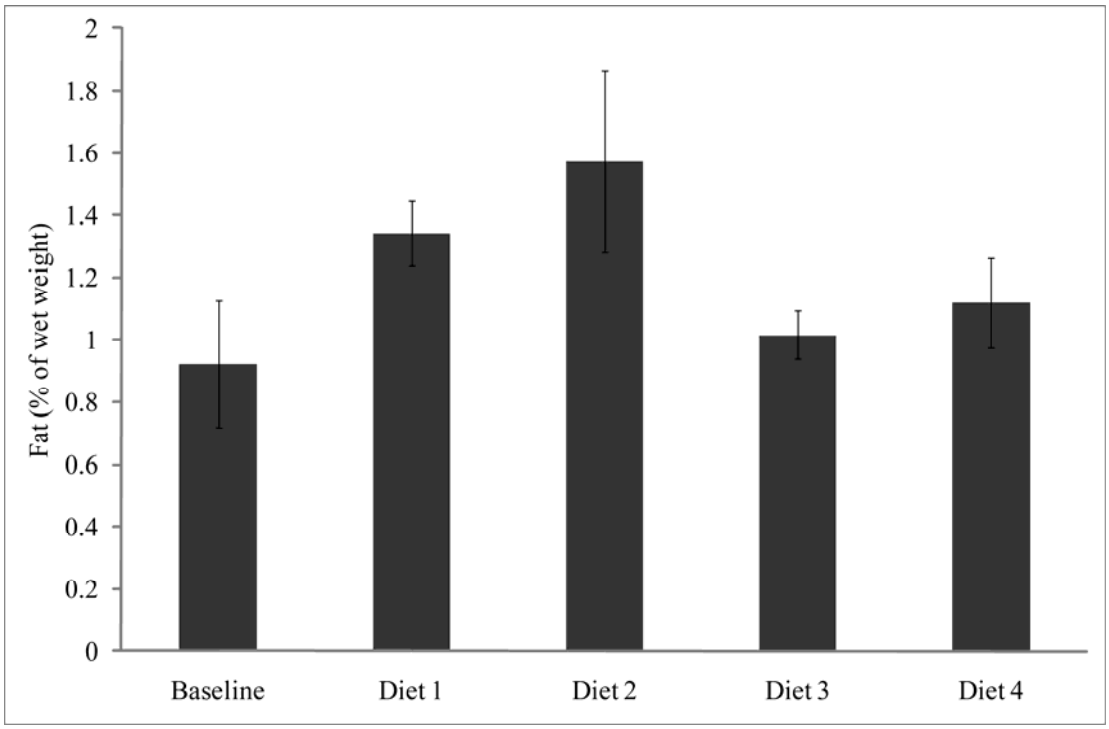

Figure 2.5 Fat content of the fillets stored immediately after harvest at -80°C. There were 10 baseline fish. The data for the baseline fish are the mean ± SD (n=10). For the fish fed the experimental diets, there were 6 fish sampled from each of the 3 replicate tanks. These data are the mean ± SE (n=3). There was no significant difference between any of the mean values.

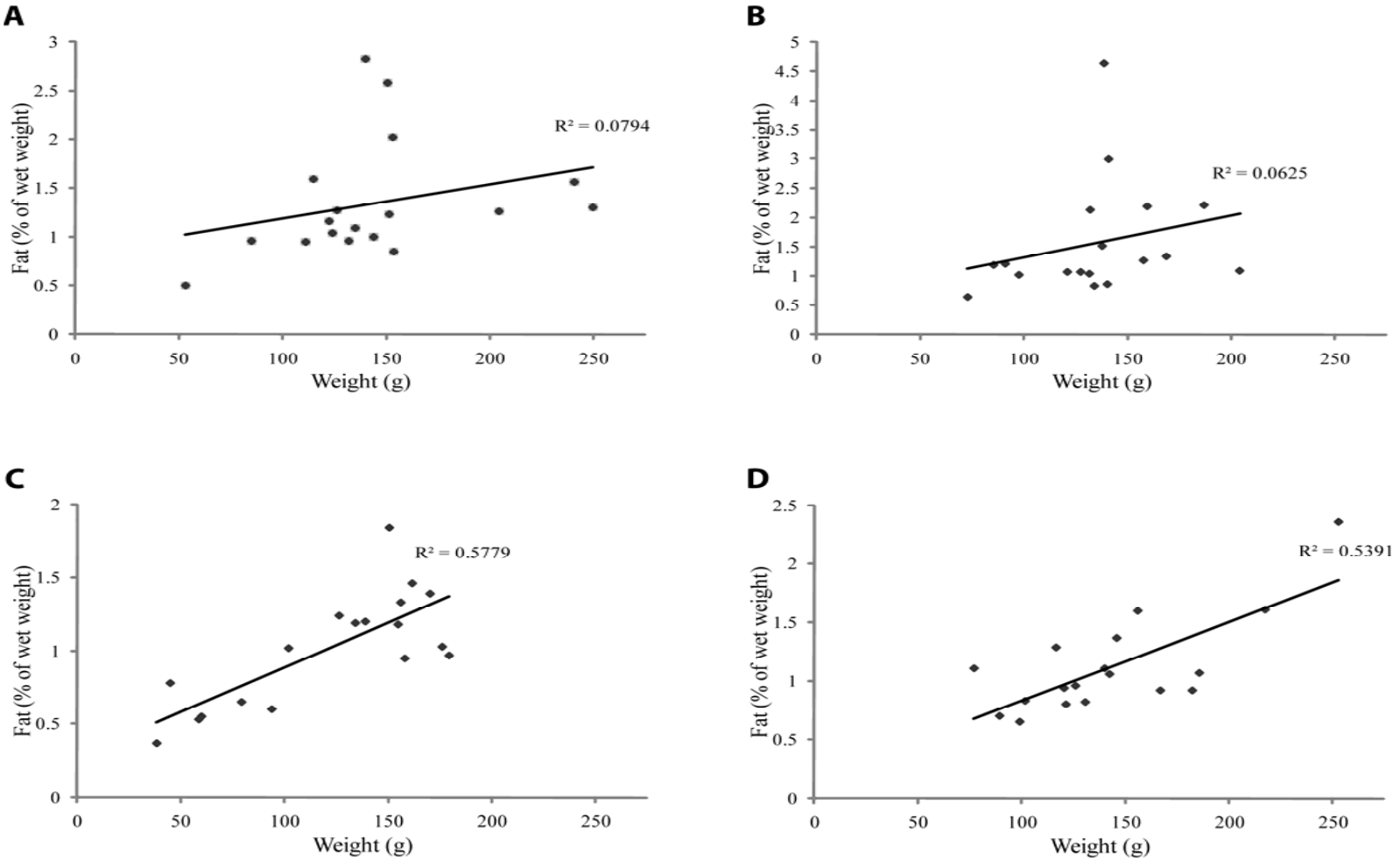

Figure 2.6 Fillet fat content correlated with fish weight. The fish were fed diet 1 (A), diet 2 (B), diet 3 (C) or diet 4 (D). Fish weight was determined at harvest and fillet fat content was determined following storage at -80°C immediately after harvest. For each diet, 6 fish were sampled from each of the 3 replicate tanks.

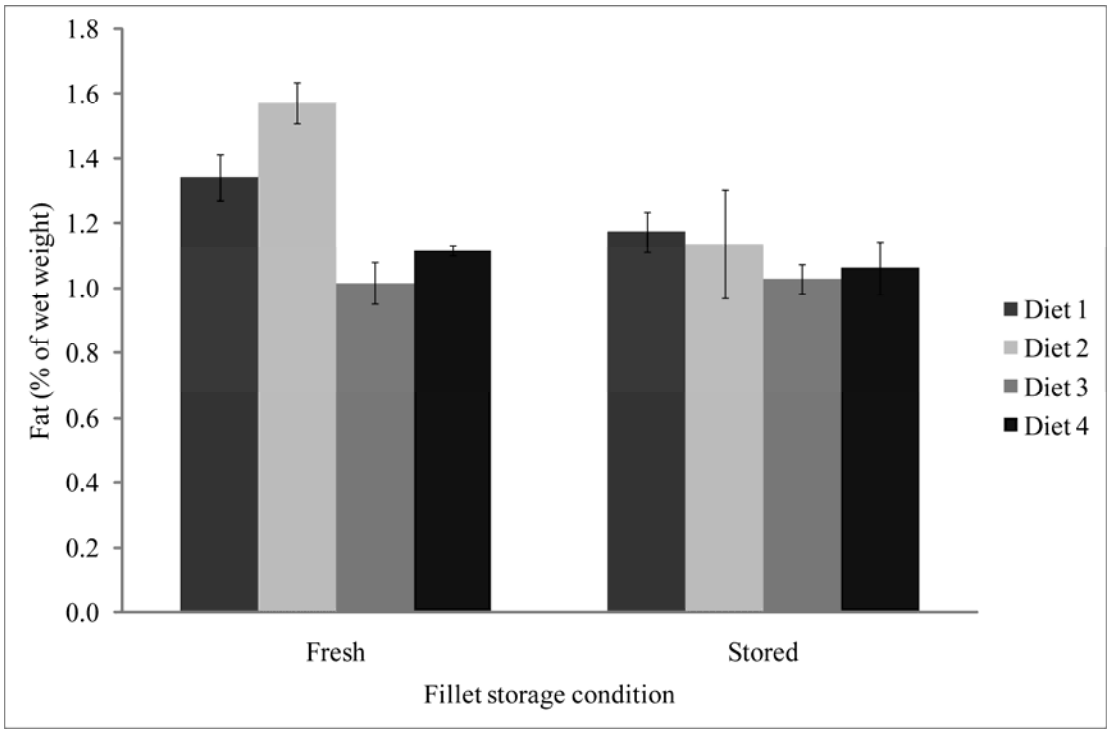

Figure 2.7 Effect of diet and storage on fillet fat content. The 'fresh' fillets were frozen at -80^{o}C immediately after harvest whereas the 'stored' fillets were stored at 4°C for 4 days and then frozen at -80^{o}C. For each of the diets, 6 fish were sampled from each of the 3 replicate tanks. The data are the mean ± SE (n=3). There were no significant differences between any of the mean values.

2.3.4.2 TBARS

The TBARS value is an indicator of lipid peroxidation. We hypothesized that GSE would inhibit the increase in TBARS value resulting from storage at 4°C for 4 days. Fish fed diets 2 and 3 received a combination of GSE and ethoxyquin whereas fish fed diet 1 received ethoxyquin alone and fish fed diet 4 received GSE alone. Storage for 4 days at 4^{o}C resulted in a significant increase in fillet TBARS value regardless of the diet (Figure 2.8). There were no significant differences between the diets immediately after harvest or after 4 days storage at 4°C (Figure 2.8). The higher concentrations of antioxidants in diets 2 and 3 did not significantly reduce the TBARS value compared to diets 1 and 4. In the fresh fillets, which were stored at -80°C immediately after harvest, the mean TBARS value across all four diets was 5.6 ± 0.5 nmol MDA g^{-1} fillet. In comparison, in the fillets which were stored at 4°C for 4 days, the mean TBARS value across all four diets was 15.0 ± 2.6 nmol MDA g^{-1} fillet, which was approximately 2.5 times higher. For the fillets stored immediately at -80^{o}C, the mean TBARS value was lower (5.6 ± 0.5 nmol MDA g^{-1} fillet) for the fish fed the experimental diets than it was for the baseline fish (7.8 ± 2.9 nmol MDA g^{-1} fillet).

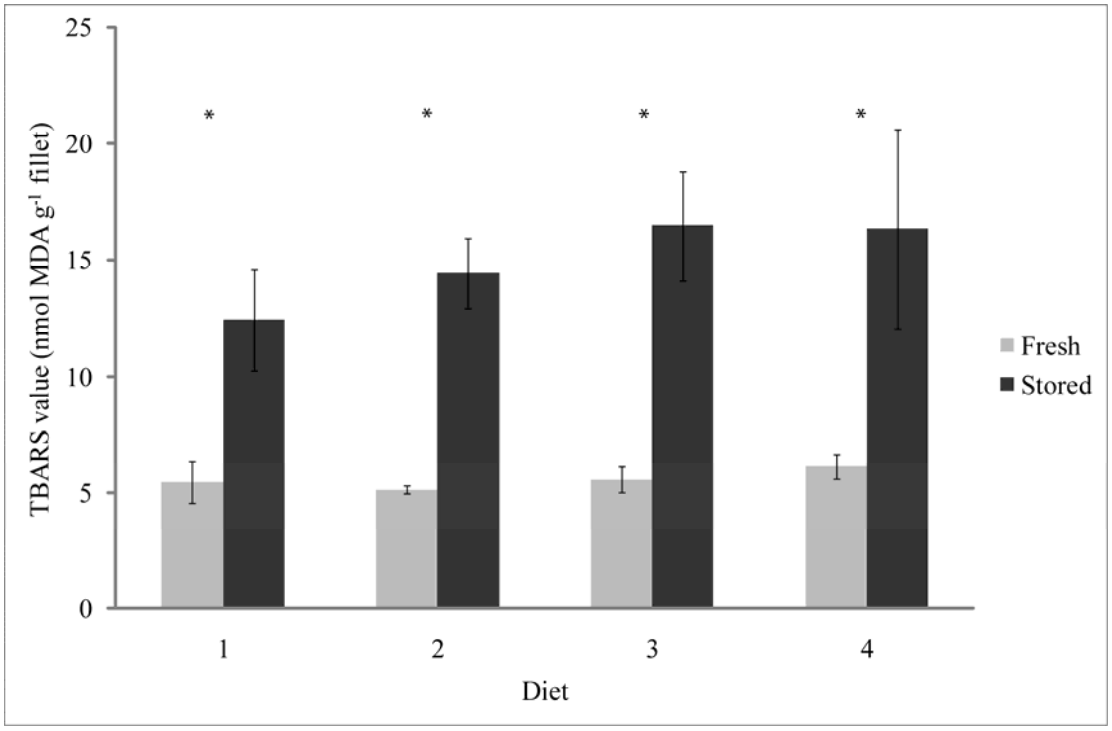

Figure 2.8 Effect of storage on TBARS value. The 'fresh' fillets were stored at -80°C immediately after harvest whereas the 'stored' fillets were stored at 4°C for 4 days and then frozen at -80°C. For each of the diets, 5 fish were sampled from each of the 3 replicate tanks. The data are the mean ± SE (n=3). Within each diet the mean values bearing an asterisk are significantly different.

2.3.4.3 Fillet fatty acid composition

Table 2.8 shows the fillet fatty acid composition, including various ratios of fatty acids, and the indices used to assess the potential for lipid peroxidation and changes in the nutritional quality of the fillets following storage at -80°C immediately after harvest. These are compared with the fatty acid composition of the fillets stored at 4°C for 4 days and then frozen at -80°C. The n-3/n-6 ratio in the fillets was expected to be constant in all of the fish, irrespective of the diet, because the dietary fatty acid source was the same. As expected, the data showed no significant difference in the n-3/n-6 ratio in the fillets frozen at -80°C immediately after harvest (Table 2.8). However, storage at 4°C led to a significant decline in the fillet n-3/n-6 ratio in the fish fed diets 1 and 4 but not in the fish fed diets 2 and 3 (Table 2.8 and Table 2.9). The accumulation of particular fatty acids can be seen using the various ratios in Table 2.8 and Table 2.9. The ratios of LA/ALA and EPA/AA in the fillets of all of the fish were at least 15.9 and 3.9, respectively (Table 2.8 and Table 2.9). The fish appear to accumulate n-3 and n-6 fatty acids differently, with a notably higher proportion of C_{18} n-6 fatty acids than C_{18} n-3 fatty acids and a higher proportion of C_{20} n-3 fatty acids than C_{20} n-6 fatty acids (Table 2.8). The ratio of DHA/EPA in the fillets of all of the fish was at least 3 (Table 2.8). The fish appear to selectively accumulate the longer chain n-3 fatty acids.

The fillet unsaturation index was used as a measure of the potential for lipid peroxidation of the fatty acids. Storage at 4°C for 4 days significantly decreased the unsaturation index from a mean across all diets of 138.9 to 136.2 (Table 2.8 and Table 2.9). The nutritional quality of the fillets was assessed using the IT and IA indices and the lipid quality of the fillets were assessed using the FLQ index. Storage at 4°C for 4 days significantly increased the IT and IA indices in the fillets from fish fed each of the diets, while significantly reducing the FLQ index. Thus, storage compromised the lipid and nutritional quality of the fish for the human consumer. There was no significant difference in the unsaturation index, IA, IT or FLQ index between diets for fillets stored under the same conditions (Table 2.8 and Table 2.9).

Table 2.8 Effect of diet on fillet fatty acid composition and various quality parameters immediately after harvest. The data for the baseline fish are the mean ± SD (n=10). For the fish fed the experimental diets, there were 6 fish sampled from each of the 3 replicate tanks. These data are the mean ± SE (n=3). Between each diet the mean values bearing an asterisk are significantly different.

Fatty acid	Composition (% of total fatty acids)				
	Baseline	Diet 1	Diet 2	Diet 3	Diet 4
14:0	0.9 ± 0.4	1.1 ± 0.1	1.2 ± 0.1	1.0 ± 0.1	0.9 ± 0.1
16:0 (palmitic acid)	16.7 ± 0.6	17.1 ± 0.1	17.0 ± 0.1	16.8 ± 0.1*	17.4 ± 0.1*
18:0 (stearic acid)	8.6 ± 0.4	8.8 ± 0.2	8.9 ± 0.2	9.6 ± 0.4	9.2 ± 0.1
Total Sats	28.3 ± 0.8	29.1 ± 0.2	29.2 ± 0.2	29.4 ± 0.3	29.6 ± 0.2
16:1n-7 (palmitoleic acid)	2.5 ± 0.6	2.5 ± 0.2	2.5 ± 0.2	2.2 ± 0.2	2.1 ± 0.1
18:1n-9 (oleic acid)	12.6 ± 1.5	10.7 ± 0.4	10.6 ± 0.5	9.9 ± 0.5	9.9 ± 0.3
18:1n-7	2.8 ± 0.1	2.9 ± 0.0	2.9 ± 0.1	2.8 ± 0.1	2.9 ± 0.0
20:1n-9	0.7 ± 0.05	1.4 ± 0.1	1.4 ± 0.1	1.2 ± 0.1	1.3 ± 0.0
Total MUFA	19.9 ± 2.3	19.4 ± 0.8	18.9 ± 0.8	18.5 ± 1.0	18.1 ± 0.5
18:2n-6 (LA)	7.2 ± 0.9	7.0 ± 0.2	7.0 ± 0.2	7.1 ± 0.2*	6.5 ± 0.1*
20:4n-6 (AA)	2.0 ± 0.2	1.2 ± 0.3	1.2 ± 0.3	1.2 ± 0.3	1.1 ± 0.3
22:4n-6	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
22:5n-6	0.6 ± 0.1	0.7 ± 0.0	0.7 ± 0.0	0.8 ± 0.0	0.8 ± 0.0
Total n-6	10.5 ± 0.7	9.5 ± 0.4	9.5 ± 0.3	9.7 ± 0.4	8.9 ± 0.3
18:3n-3 (ALA)	0.6 ± 0.1	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0
18:4n-3 (SDA)	0.0	0.0	0.0	0.0	0.0
20:5n-3 (EPA)	9.8 ± 0.3	8.9 ± 0.1	8.9 ± 0.1	9.0 ± 0.2	8.8 ± 0.1
22:5n-3 (DPA)	3.7 ± 0.3	3.7 ± 0.1	3.7 ± 0.1	3.6 ± 0.1	3.9 ± 0.1
22:6n-3 (DHA)	26.1 ± 2.6	27.4 ± 0.7	27.3 ± 0.8	28.0 ± 0.9	28.9 ± 0.6
Total n-3	40.3 ± 2.7	41.7 ± 0.8	41.6 ± 0.9	42.4 ± 0.8	43.1 ± 0.6
n-3/n-6	3.9 ± 0.5	4.6 ± 0.2	4.5 ± 0.2	4.5 ± 0.2	5.0 ± 0.2
LA/ALA	12.1 ± 1.2	18.5 ± 2.9	17.3 ± 1.6	24.9 ± 4.8	17.3 ± 0.6
EPA/AA	5.0 ± 0.4	4.1 ± 0.3	4.1 ± 0.3	3.9 ± 0.3	3.9 ± 0.1
EPA/ALA	16.7 ± 3.0	23.3 ± 3.0	22.2 ± 2.2	30.9 ± 5.5	23.8 ± 1.1
DHA/EPA	2.7 ± 0.3	3.1 ± 0.1	3.1 ± 0.1	3.2 ± 0.2	3.3 ± 0.1
Unsaturation index	144.7 ± 2.1	139.2 ± 0.8	139.2 ± 0.6	138.9 ± 0.9	138.1 ± 0.8
IA	0.29 ± 0.0	0.31 ± 0.0	0.31 ± 0.0	0.30 ± 0.0	0.30 ± 0.0
IT	0.19 ± 0.0	0.19 ± 0.0	0.19 ± 0.0	0.19 ± 0.0	0.19 ± 0.0
FLQ	35.81 ± 2.7	36.4 ± 0.7	36.8 ± 0.7	37.0 ± 0.8	37.7 ± 0.5

Table 2.9 Effect of diet on fillet fatty acid composition and various quality parameters after storage at 4°C for 4 days. For each of the diets, 6 fish were sampled from each of the 3 replicate tanks. The data are the mean ± SE (n=3). There was no significant difference between any of the mean values.

Fatty Acid	Composition (% of total fatty acids)			
	Diet 1	Diet 2	Diet 3	Diet 4
14:0	1.5 ± 0.2	1.3 ± 0.1	1.2 ± 0.1	1.4 ± 0.1
16:0 (palmitic acid)	17.5 ± 0.2	17.6 ± 0.1	17.5 ± 0.2	17.8 ± 0.2
18:0 (stearic acid)	8.7 ± 0.3	9.0 ± 0.2	9.7 ± 0.5	9.0 ± 0.2
Total Sats	29.3 ± 0.3	30.0 ± 0.3	30.2 ± 0.3	30.2 ± 0.3
16:1n-7 (palmitoleic acid)	3.1 ± 0.3	2.8 ± 0.2	2.6 ± 0.2	2.9 ± 0.2
18:1n-9 (oleic acid)	12.2 ± 0.6	11.5 ± 0.5	11.4 ± 0.7	11.8 ± 0.5
18:1n-7	3.0 ± 0.0	3.0 ± 0.1	3.0 ± 0.1	3.1 ± 0.0
20:1n-9	1.5 ± 0.1	1.4 ± 0.1	1.3 ± 0.1	1.5 ± 0.1
Total MUFA	22.1 ± 1.1	20.9 ± 0.9	20.3 ± 1.2	21.4 ± 0.9
18:2n-6 (LA)	7.5 ± 0.2	7.4 ± 0.2	7.6 ± 0.2	7.3 ± 0.2
20:4n-6 (AA)	1.3 ± 0.3	1.3 ± 0.3	1.2 ± 0.3	1.2 ± 0.3
22:4n-6	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
22:5n-6	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.0
Total n-6	10.1 ± 0.3	10.0 ± 0.3	10.2 ± 0.4	9.7 ± 0.3
18:3n-3 (ALA)	0.5 ± 0.0	0.5 ± 0.0	0.4 ± 0.0	0.5 ± 0.0
18:4n-3 (SDA)	0.0	0.0	0.0	0.0
20:5n-3 (EPA)	8.6 ± 0.2	8.7 ± 0.1	8.4 ± 0.2	8.4 ± 0.1
22:5n-3 (DPA)	3.5 ± 0.1	3.6 ± 0.1	3.5 ± 0.1	3.6 ± 0.1
22:6n-3 (DHA)	24.2 ± 1.0	25.0 ± 0.7	25.1 ± 1.0	24.9 ± 0.8
Total n-3	37.6 ± 1.0	38.7 ± 0.8	38.1 ± 1.0	38.4 ± 0.7
n-3/n-6	3.8 ± 0.2	4.0 ± 0.2	4.0 ± 0.2	4.0 ± 0.2
LA/ALA	18.0 ± 3.4	16.9 ± 1.6	24.4 ± 4.9	15.9 ± 0.6
EPA/AA	4.2 ± 0.3	4.2 ± 0.3	3.9 ± 0.3	4.0 ± 0.1
EPA/ALA	20.2 ± 3.0	20.1 ± 1.9	27.4 ± 5.7	18.8 ± 1.1
DHA/EPA	2.9 ± 0.2	2.9 ± 0.1	3.0 ± 0.2	3.0 ± 0.1
Unsaturation index	136.5 ± 0.8	137.0 ± 0.7	135.9 ± 0.8	135.3 ± 0.8
IA	0.34 ± 0.0	0.33 ± 0.0	0.32 ± 0.0	0.34 ± 0.0
IT	0.21 ± 0.0	0.21 ± 0.0	0.21 ± 0.0	0.21 ± 0.0
FLQ	32.8 ± 0.9	33.7 ± 0.7	33.5 ± 1.0	33.4 ± 0.8

We investigated the effect of storage for 4 days at 4°C on the total saturated, monounsaturated, n-9, n-7, n-6 or n-3 fatty acids in the fillet. A trend can be observed across all diets where the proportion of total saturated, monounsaturated, n-9, n-7 and n-6 fatty acids is higher in the fillets stored at 4°C for 4 days than in the fresh fillets, which were stored at -80°C immediately after harvest (Figure 2.9). Conversely, the n-3 fatty acids as a proportion of the total fatty acids was significantly higher in the fresh fillets than the fillets stored at 4°C for 4 days (Figure 2.9). The n-3 fatty acids were the largest proportion of any one series of fatty acids with fresh and stored fillet means of 42.2% and 38.4%, respectively, across all four diets (Figure 2.10). There was no significant difference in each fatty acid series between the diets when the fillets were either fresh or stored (Figure 2.10).

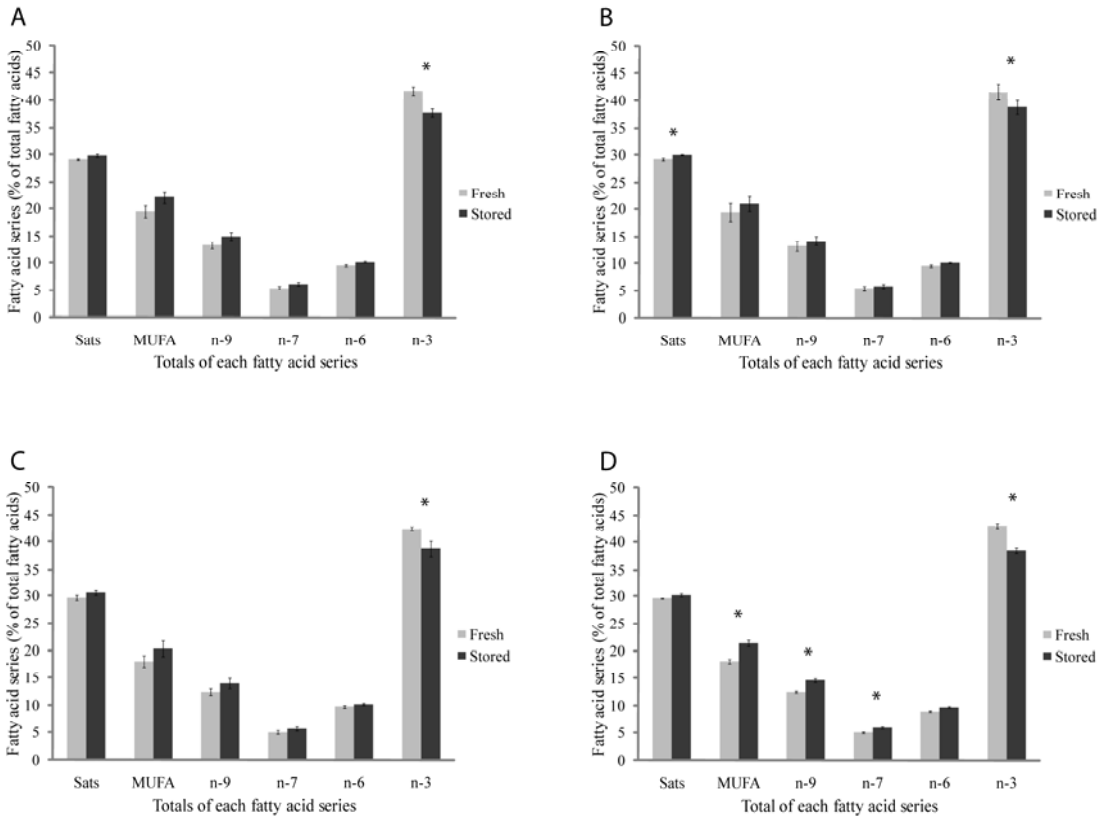

Figure 2.9 Effect of storage on each fatty acid series. The proportion of total saturated (Sats), monounsaturated (MUFA), n-9, n-7, n-6 and n-3 fatty acids was determined in the 'fresh' fillets, which were stored at -80°C immediately after harvest, or in the 'stored' fillets, which were stored at 4°C for 4 days. The fish were fed diet 1 (A), diet 2 (B), diet 3 (C) or diet 4 (D). For each of the diets, 6 fish were sampled from each of the 3 replicate tanks. The data are the mean ± SE (n=3). Within each fatty acid series the mean values bearing an asterisk are significantly different.

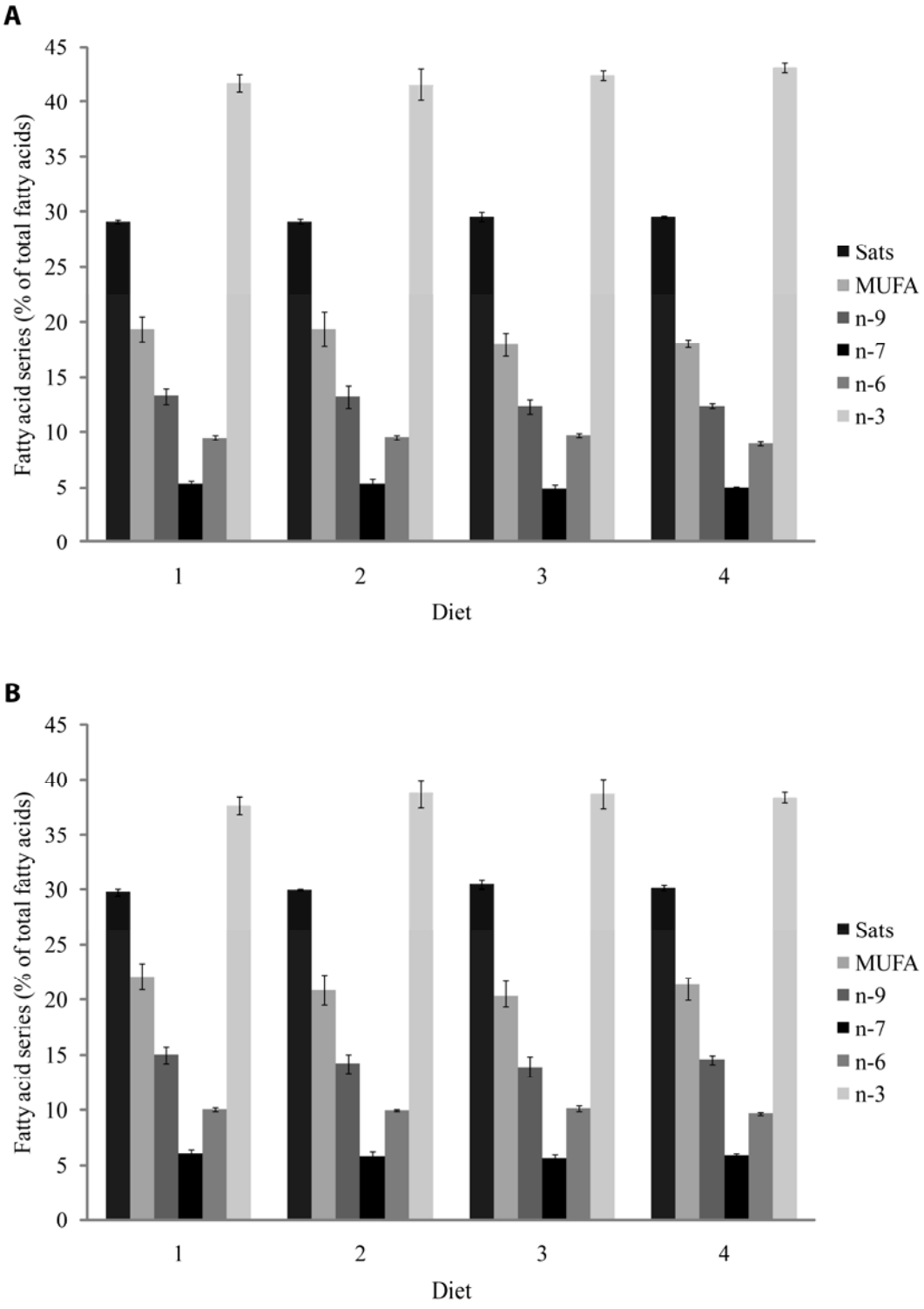

Figure 2.10 Effect of diet on each fatty acid series. The proportion of total saturated (Sats), monounsaturated (MUFA), n-9, n-7, n-6 and n-3 fatty acids in the fillets was determined following storage at -80°C immediately after harvest (A) or after storage at 4°C for 4 days (B). For each of the diets, 6 fish were sampled from each of the 3 replicate tanks. The data are the mean ± SE (n=3). There was no significant difference between any of the mean values.

A significant difference was observed in the proportion of n-3 fatty acids in the fresh as compared with the stored fillets. Therefore, these data were examined further. The key n-3 fatty acids that were investigated further were ALA, EPA, 22:5n-3 (docosapentaenoic acid, DPA) and DHA. The most interesting finding was that DHA as a proportion of the total fatty acids in the fillets stored at 4°C for 4 days was significantly lower than in the fillets immediately after harvest, irrespective of the diet (Figure 2.11). Similarly, the EPA and DPA abundance in the fillets of fish fed diet 4 and stored at 4°C for 4 days was significantly lower than the fresh fillets, which were stored at -80°C immediately after harvest (Figure 2.11). In contrast, the proportion of ALA tended to be higher in the fillets stored at 4°C for 4 days than in the fresh fillets, while this was only statistically significant in the fillets of fish fed diet 4 (Figure 2.11). Between the diets there were no significant differences in the proportion of ALA, EPA, DPA or DHA in either the fresh or stored fillets.

A trend was observed, particularly in fish fed the diets containing GSE (diets 2-4), such that fish weight was negatively correlated with the proportion of DHA in the fillet (Figure 2.12). However, the statistical strength of the relationship was typical ($R^2 \leq 0.25$), with only the fish in diet 3 bordering on having a larger than typical relationship ($R^2 \geq 0.26$) (Leech *et al.* 2008). The variation in the proportion of DHA in the fillet occurred not only between diets but also within tanks of fish fed the same diet (Figure 2.13). The proportion of DHA in the fillets of the baseline fish was also variable with values ranging from 20.3% to 28.9% of the total fatty acids (Figure 2.13). To investigate if the fatty acid composition variation, in particular DHA variation, seen in the baseline fish was due to biological variation or technical error, six samples from the same fillet were extracted. The variability due to technical error was low in the key n-3 and n-6 fatty acids in the phospholipid, free fatty acid and triacylglycerol fractions (Figure 2.14). Therefore, we propose that natural fish variation resulted in the variation in the proportion of DHA in the fillet.

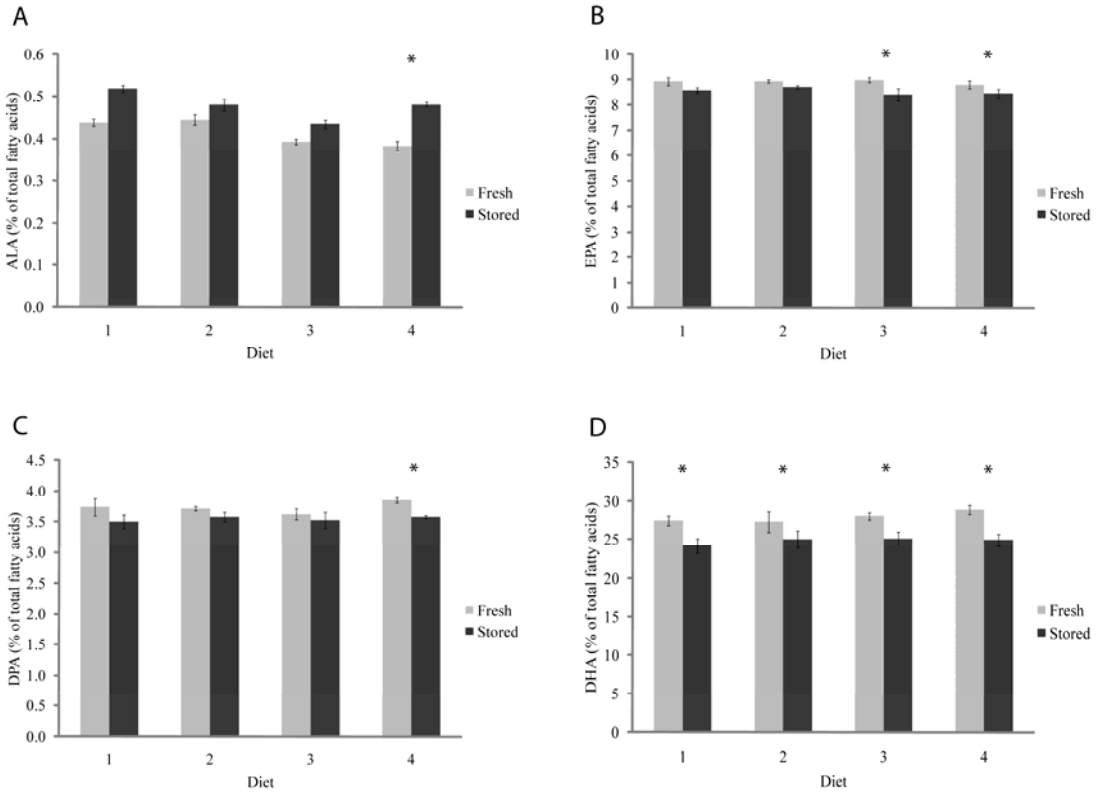

Figure 2.11 Effect of storage on ALA, EPA, DPA and DHA. The proportion of ALA (A), EPA (B), DPA (C) or DHA (D) as a percentage of total fatty acids in the fillets was determined in the 'fresh' fillets, which were stored at -80°C immediately after harvest, or in the 'stored' fillets, which were stored at 4°C for 4 days. For each of the diets, 6 fish were sampled from each of the 3 replicate tanks. The data are the mean ± SE (n=3). Within each diet the mean values bearing an asterisk are significantly different.

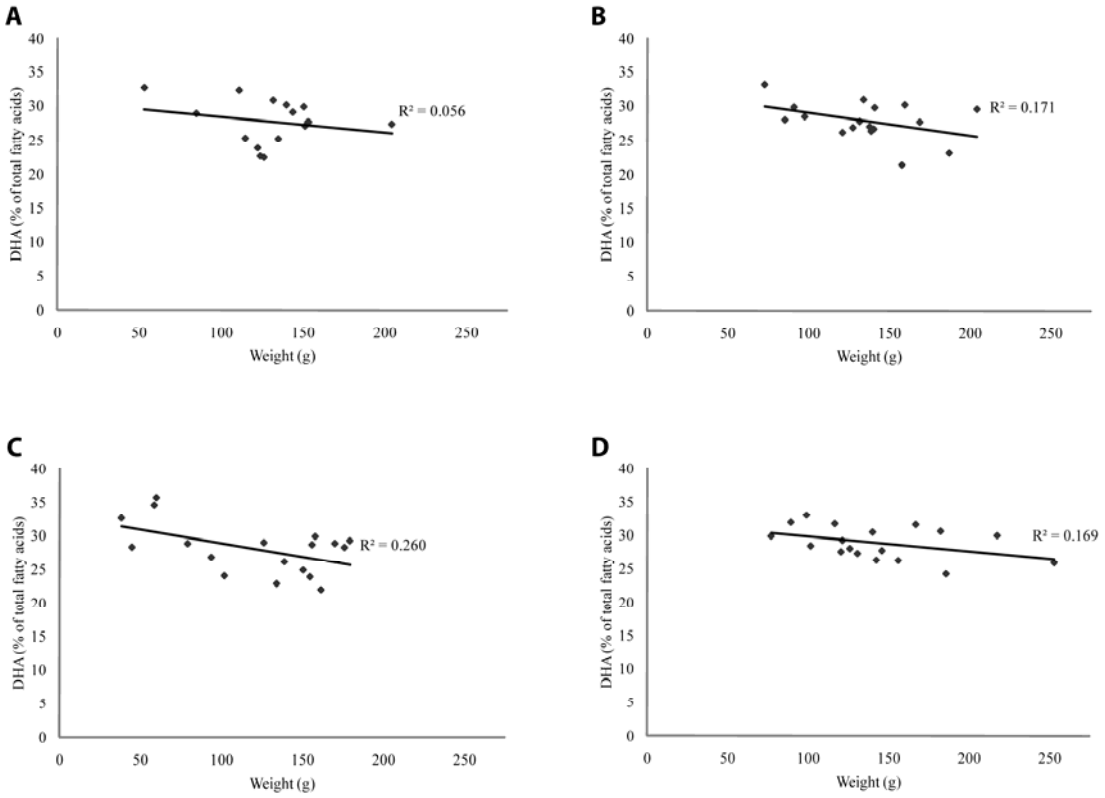

Figure 2.12 Fillet DHA content correlated with fish weight. The fish were fed diet 1 (A), diet 2 (B), diet 3 (C) or diet 4 (D). Fish weight was determined at harvest and the proportion of DHA as a percentage of total fatty acids in the fillets was determined following storage at -80°C immediately after harvest. For each diet, 6 fish were sampled from each of the 3 replicate tanks.

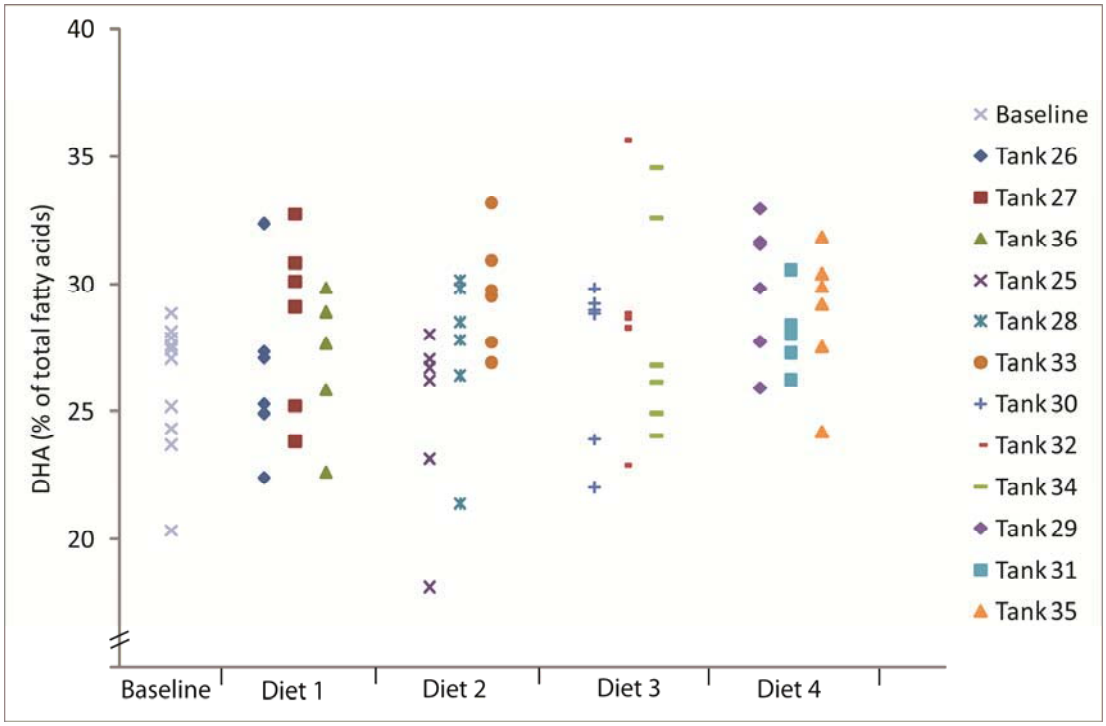

Figure 2.13 The variation in the proportion of DHA in the fillet between diets and also within tanks of fish fed the same diet. There were 10 baseline fish. Each diet was fed to 3 replicate tanks and each tank contained 10 fish.

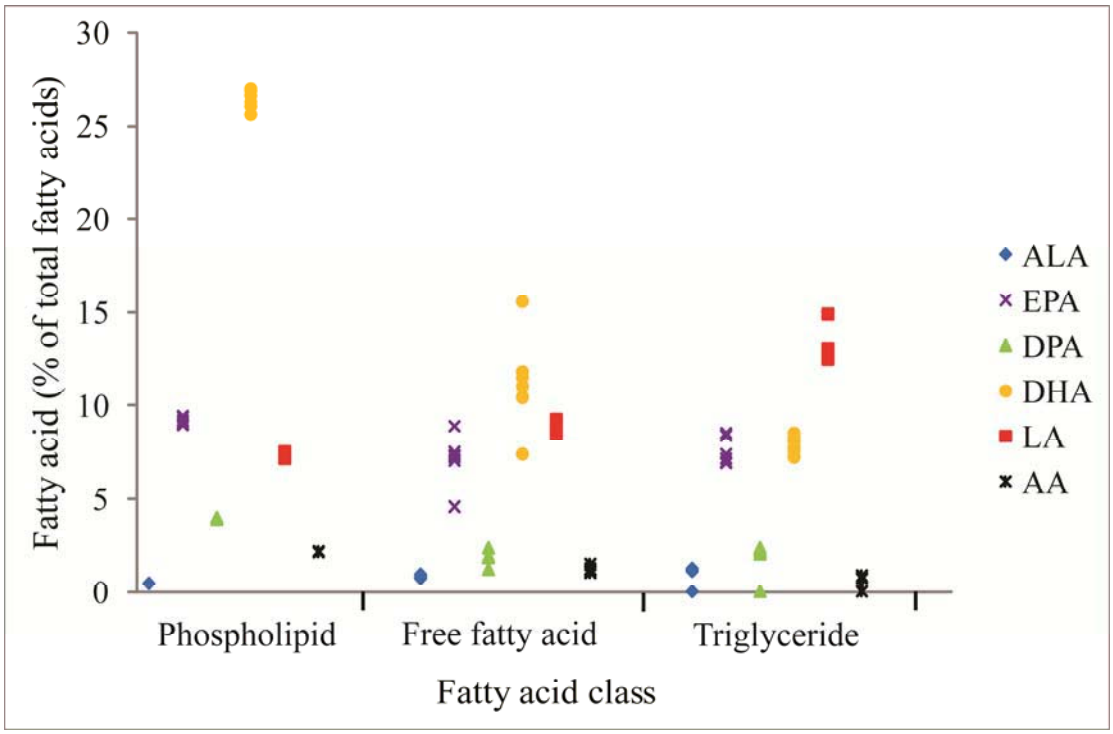

Figure 2.14 The proportion of individual fatty acids in each lipid class. The proportion of ALA, EPA, DPA, DHA, LA and AA as a percentage of the total fatty acids in the fillet within the phospholipid, free fatty acid and triacylglycerol lipid classes. One baseline fish was analysed 6 times.

At the completion of the trial the only fatty acid series in the fillets which were significantly different from the fillets of the baseline fish, was the total saturated fatty acids in fish fed diets 3 and 4 (Figure 2.15). The proportion of the n-3 fatty acids ALA, EPA, DPA and DHA in the baseline fillets and the fillets from the fish after the trial can be seen in Figure 2.16. The proportion of ALA and EPA in the baseline fillets was significantly higher than those from fillets in any of the dietary treatments (Figure 2.16).

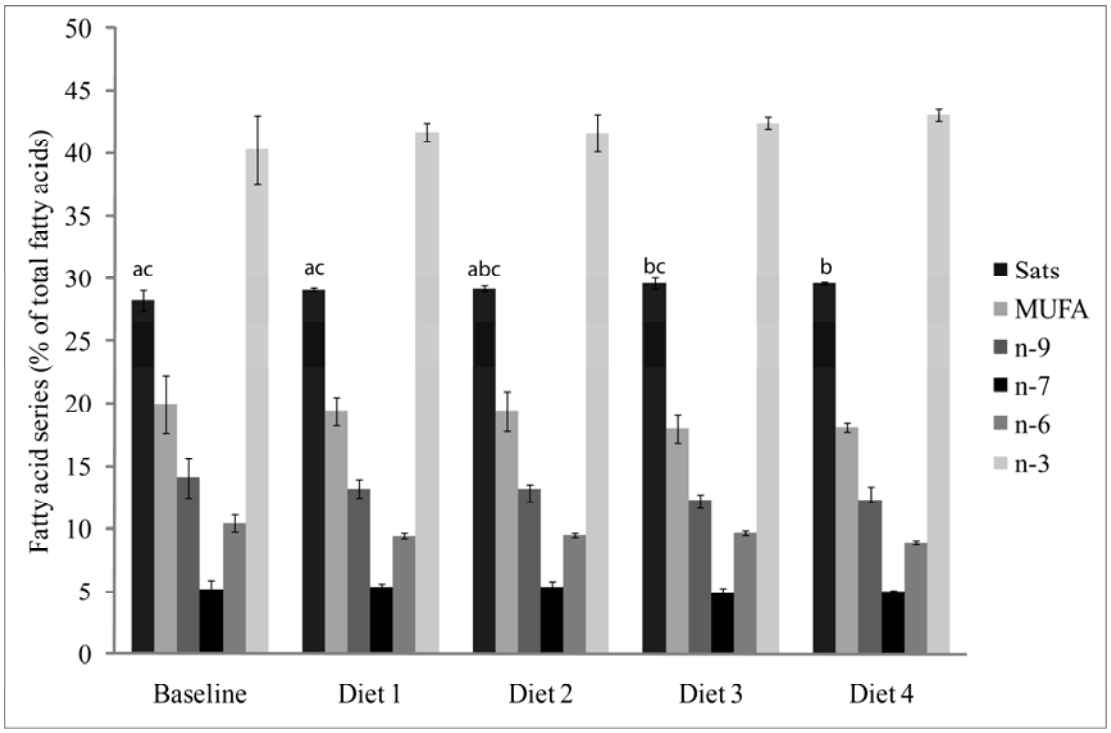

Figure 2.15 Comparison of the various fatty acid series in the fillets from the baseline fish and the fish fed the experimental diets. The proportion of total saturated (Sats), monounsaturated (MUFA), n-9, n-7, n-6 and n-3 fatty acids in the fillets was determined following storage at -80°C immediately after harvest. There were 10 baseline fish. The data for the baseline fish are the mean ± SD (n=10). For the fish fed the experimental diets, there were 6 fish sampled from each of the 3 replicate tanks. These data are the mean ± SE (n=3). The mean values for a specific fatty acid series bearing a different letter are significantly different.

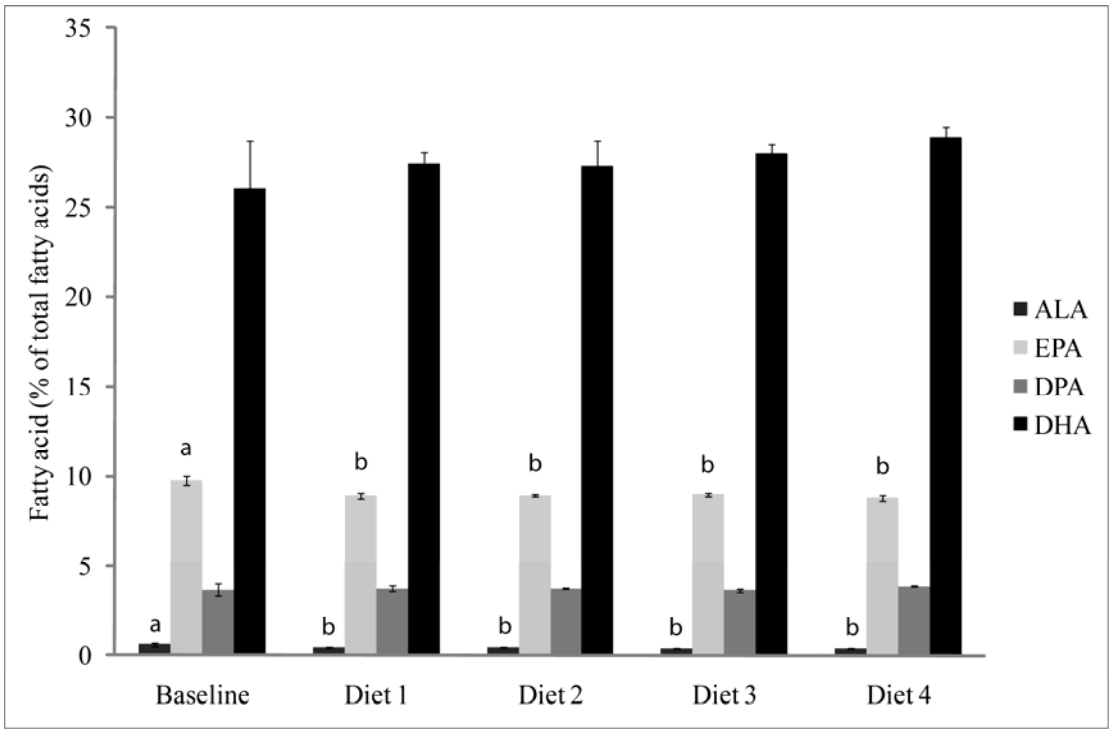

Figure 2.16 Comparison of ALA, EPA, DPA and DHA in the fillets from the baseline fish and the fish fed the experimental diets. The proportion of ALA, EPA, DPA and DHA as a percentage of total fatty acids in the fillets was determined following storage at -80°C immediately after harvest. There were 10 baseline fish. The data for the baseline fish are the mean ± SD (n=10). For the fish fed the experimental diets, there were 6 fish sampled from each of the 3 replicate tanks. These data are the mean ± SE (n=3). The mean values for a specific fatty acid bearing a different letter are significantly different.

2.3.5 Comparison of fatty acid profiles of the experimental diets and the fillets

When the fatty acid profiles of the fillets at the end of the experiment were compared with the fatty acid profiles of the experimental diets, several interesting results were observed. Firstly, there was no effect of GSE supplementation on the fillet fatty acid composition. Secondly, and more interestingly, DHA as a proportion of total fatty acids averaged 27.9% in the fillets whereas it averaged only 10% in the experimental diets. Thus there was considerable bioaccumulation of DHA. This may be due to selective uptake or it may be due to selective sparing from β-oxidation. The same result was obtained irrespective of the GSE content of the diet. A similar, though less dramatic effect was seen with DPA, the immediate precursor of DHA. DPA as a proportion of total fatty acids averaged 3.7% in the fillets whereas it averaged only 1.5% in the experimental diets. The large bioaccumulation of DHA was also reflected in a large value for the total n-3 fatty acids. The total n-3 fatty acids as a proportion of all fatty acids averaged 42.2% in the flesh as compared with only 26.7% in the experimental diets. Total and individual n-6 fatty acids were similar between the flesh and the diets but total and individual MUFA showed the inverse relationship to that seen with DHA and DPA. In other words, total MUFA as a proportion of all fatty acids averaged 35% in the diets but only 18.7% in the flesh. The main contributor to this difference was 18:1n-9 (oleic acid) and to a lesser extent palmitoleic acid. The oleic acid as a proportion of total fatty acids averaged 17.5% in the diets but only 10.3% in the flesh. Similarly, 16:1n-7 (palmitoleic acid) as a proportion of total fatty acids averaged 7.1% in the diets but only 2.3% in the flesh. Thus, overall, there was a selective exclusion or β-oxidation of palmitoleic acid and oleic acid compared with a selective uptake or sparing from β-oxidation of DPA and DHA.

2.4 Discussion

Dietary GSE supplementation had no effect on YTK survival, growth, FCR or fillet proximate composition in this trial. This is in agreement with other studies using dietary antioxidant supplementation. Dietary vitamin E supplementation in sea bass had no effect on growth rate, FCR or proximate composition (Gatta *et al.* 2000). Similarly, Ruff *et al.* (2003) showed vitamin C and E supplementation had no effect on growth rate or proximate composition in turbot. Although YTK growth and FCR were not affected by diets containing GSE, the WG and SGR of the YTK were low across all diets during the trial. WG was variable within the one tank with some fish gaining virtually no weight, while others grew rapidly. This may suggest a hierarchy whereby more rapidly growing fish competed with less rapidly growing fish for feed. The initial body weight of the fish was 77.8 g and the SGR across the four diets was 0.8 – 1.0% gain day^{-1}, which was lower than other studies with fish consuming equivalent amounts of lipid in their diets. For example, rainbow trout with an initial body weight of 110 g and a dietary lipid content of approximately 15% had a SGR of 1.86 (Chaiyapechara *et al.* 2003).

A noticeably large variation in the size and appearance of the fish, together with a poor SGR and a poor FCR lead us to conduct pathology tests at the conclusion of the trial. It has been reported that the tannins found in GSE have caused digestibility problems in ducks, chickens and rats which have lead to reduced growth (Elkin *et al.* 1990; Lau and King 2003). However, in our study the variability in fish size occurred across all diets, with or without GSE. The fish were not found to have any signs of infectious disease but jaw morphological abnormalities were common. It is common for intensive aquaculture to cause morphological abnormalities in YTK head shape, alignment of the head and body, and the shape and orientation of the upper and lower jaws (Fowler *et al.* 2003). Feeding ability can be impeded when fish have jaw and facial deformities. Formulation of fish feeds involves a balance between nutritional value and pelleting characteristics (Hardy and Barrows 2002). The pellet must remain intact in the water until the fish eats it, to ensure the fish is getting the desired nutritional requirements and to avoid water pollution (Hardy and Barrows 2002). The feeds used in this study were somewhat friable and the fish did not take to them well.

Therefore, a combination between the diet formulation and the YTK mouth deformations may have contributed to the lower than expected growth performance and FCR.

The mean initial body weight of YTK in this study was 77.8 g, which makes comparison with other dietary antioxidant supplemented fish trials difficult. Tocher *et al.* (2002) used turbot, halibut and sea bream with initial body weights of 1 g and Mourente *et al.* (2002) used gilthead sea bream with initial body weights of 1.5 g. In contrast, Gatta *et al.* (2000) used 208 g sea bass, Ruff *et al.* (2002) used 312 g Atlantic halibut and Ruff *et al.* (2003) used 347 g turbot initial body weights. Rainbow trout with a mean initial body weight of 110 g were the most comparable to the YTK size (Chaiyapechara *et al.* 2003). The rapid growth experienced early in the fish life cycle provides capacity to cause a greater effect through dietary supplementation. However, the use of commercial-size fish in dietary antioxidant supplemented trials should not be underestimated. The effect of antioxidants on fillet quality from fish which are of retail size is vastly important.

The fillet fat content and fatty acid profile in farmed fish is dependent on many factors including genetic, environmental (e.g. temperature and salinity), developmental phase, feed management and feed fatty acid composition (Senso *et al.* 2007). Farmed fish have higher lipid content than their wild counterparts (Moretti *et al.* 2003). The influence of dietary lipid content on the flesh lipid content of farmed fish is well documented (Gatta *et al.* 2000; Scaife *et al.* 2000; Grigorakis *et al.* 2002; Chaiyapechara *et al.* 2003; Moretti *et al.* 2003; Regost *et al*. 2003; Senso *et al.* 2007). Rainbow trout whole fish and fillet lipid content after feeding 15% lipid in vitamin E supplemented diets were 13.35% and 8.35%, respectively (Chaiyapechara *et al.* 2003). In the present study we have shown that YTK whole body and fillet lipid content after feeding 16.7% lipid were 13.5% and 1.2%, respectively. The whole fish lipid content in YTK was proportional to the dietary lipid content, as shown in rainbow trout. However, there was a noticeable difference in fillet lipid content between the two species. After consuming approximately the same dietary lipid content, the fillet lipid content in YTK was seven times lower than rainbow trout (Chaiyapechara *et al.* 2003). This finding is in agreement with the main lipid storage sites in these two species. The flesh of salmonid species, such as rainbow

trout, is known to be the main lipid storage site, whereas YTK store lipid in the liver (Chen *et al.* 2006; Nanton *et al.* 2007; Kolditz *et al.* 2008). However, the fillet fat content of the YTK receiving either the diet containing the high concentration of GSE (1250 mg kg^{-1}), diet 3, or GSE only, diet 4, was positively correlated with fish size, similar to reports in Atlantic halibut (Ruff *et al.* 2002). In contrast, the fillet fat content of the YTK receiving the antioxidant feeds containing ethoxyquin only or the low concentration of GSE (250 mg kg^{-1}), diets 1 and 2, respectively, was not correlated with fish size, similar to reports in gilthead sea bream (Senso *et al.* 2007).

The fatty acid composition of the YTK fillets revealed that the predominant fatty acids were 16:0 (palmitic acid) and 18:0 (stearic acid) among the saturates, oleic acid among the monounsaturates, and EPA and DHA among the polyunsaturates, each with a proportion of total fatty acids greater than 8.8%. This finding is in accordance with the predominant fatty acids in Japanese amberjack or yellowtail (*Seriola quinqueradiata*) muscle which were palmitic acid, palmitoleic acid, oleic acid, EPA and DHA, each with a proportion of total fatty acids greater than 6.8% (Sohn *et al.* 2007) (Table 2.10). YTK had a 2.8-fold higher proportion of stearic acid and 3-fold lower proportion of palmitoleic acid than the yellowtail. We proceeded to compare the fatty acid profile of the YTK in our study with values previously reported for YTK (Nichols *et al.* 1998) (Table 2.11). The two fatty acid profiles presented in this study from the baseline fish and the fish fed diet 1, where the fillets were stored at -80°C immediately after harvest, had a lower proportion of saturated fatty acids than the YTK fatty acid profile published by Nichols *et al.* (1998). The baseline fish in our study and the published YTK profile had similar proportions of MUFA, 20.9% and 19.9% respectively, and PUFA, 41.5% and 40.3%, respectively (Nichols *et al.* 1998). However, after diet 1 was fed to the fish in our study, the proportion of PUFA increased from 40.3% to 51.2%. Lower ALA, detectable 18:4n-3 (stearidonic acid, SDA) and higher palmitic acid, AA and DHA in the YTK profile published by Nichols *et al.* (1998) are an indication that the profile was from a wild-caught fish (Grigorakis *et al.* 2002). Farmed fish generally have higher levels of oleic acid and LA than wild fish because these fatty acids are not normally found in the marine food chain (Moretti *et al.* 2003). The vegetable oil, which partially replaces fish oil in farmed fish feeds, is the source of oleic acid and LA (Moretti *et al.* 2003). In this study the main dietary lipid source was 90 g kg^{-1} of fish oil which is comprised of

30-50% EPA, DPA and DHA (Whelan and Rust 2006). However, the surfactant lecithin was a component of each feed supplied at 10 g kg^{-1}. Lecithin is commonly used as an emulsifier in feeds and obtained from soybean oil, which is rich in LA and oleic acid. Noteworthy in our study, the major n-6 PUFA and monounsaturated fatty acid in the YTK fillet were LA and oleic acid, respectively.

Table 2.10 Fatty acid composition (% of total fatty acids) of ordinary muscle from yellowtail (*Seriola quinqueradiata*) (Sohn *et al.* 2007*) and the fillet from YTK fed diet 1 in this study, which was determined following storage at -80°C immediately after harvest.

Fatty Acid	Composition (% of total fatty acids)	
	Yellowtail*	YTK
14:0	5.0	1.1
15:0	0.5	0.2
16:0 (palmitic acid)	18.4	17.1
18:0 (stearic acid)	3.3	8.8
Total Sats	27.2	29.4
16:1n-7 (palmitoleic acid)	6.8	2.5
18:1n-9 (oleic acid)	18.1	10.7
18:1n-7	3.0	2.9
20:1n-11	2.3	0.6
20:1n-9	3.7	1.3
22:1n-11	4.6	0.1
22:1n-9	0.6	0.5
Total MUFA	39.0	19.4
18:2n-6 (LA)	4.7	7.0
18:3n-3 (ALA)	1.1	0.4
18:4n-3 (SDA)	1.9	0.0
20:4n-6 (AA)	0.7	1.2
20:5n-3 (EPA)	7.8	8.9
22:5n-3 (DPA)	2.3	3.7
22:6n-3 (DHA)	12.8	27.4
Total PUFA	33.7	51.2

Table 2.11 The fatty acid composition (% of total fatty acids) of YTK fillets published by Nichols *et al.* (1998)*, and the fillet values obtained in this study from the baseline fish and the fish fed diet 1, which were determined following storage at -80°C immediately after harvest (fresh).

Fatty acid	Composition (% of total fatty acids)		
	Published*	Baseline	Fresh
14:0	2.1	0.9	1.1
15:0	0.5	0.2	0.2
16:0 (palmitic acid)	24.6	16.7	17.1
17:0	0.7	0.4	0.4
18:0 (stearic acid)	8.6	8.6	8.8
20:0	0.0	0.2	0.2
22:0	0.1	0.1	0.1
24:0	0.0	0.1	0.1
Total Sats	36.6	28.3	29.1
16:1n-9	0.3	0.3	0.2
16:1n-7 (palmitoleic acid)	2.9	2.5	2.5
17:1	0.2	0.0	0.0
18:1n-9 (oleic acid)	12.6	12.6	10.7
18:1n-7	2.6	2.8	2.9
20:1(n-9+n-11)	1.0	0.7	2.0
22:1n-9	0.4	0.1	0.5
24:1n-9	0.6	0.3	0.5
Total MUFA	20.9	19.9	19.4
18:2n-6 (LA)	1.2	7.2	7.0
18:3n-3 (ALA)	0.7	0.6	0.4
18:3n-6	0.0	0.1	0.1
18:4n-3 (SDA)	0.7	0.0	0.0
20:2n-6	0.0	0.2	0.2
20:3n-6	0.1	0.2	0.2
20:4n-6 (AA)	3.2	2.0	1.2
20:5n-3 (EPA)	4.9	9.8	8.9
22:4n-6	0.2	0.2	0.1
22:5n-3 (DPA)	1.9	3.7	3.7
22:6n-3 (DHA)	28.0	26.1	27.4
Total PUFA	41.5	40.3	51.2

Freshwater fish have an n-3/n-6 ratio of 1 to 4, while marine fish tend to have a higher ratio (de Souza *et al.* 2007). The n-3/n-6 relationship is reflective of the dietary oil source (Senso *et al.* 2007). Despite the dietary lipid source in our study being fish oil, the feed n-3/n-6 ratio of 2.75 reflects the high proportion of oleic acid and to a lesser extent LA in the diets. The n-3/n-6 ratio in the YTK fresh fillets at the end of the trial was 4.5 which was comparable to the yellowtail ordinary muscle n-3/n-6 ratio of 4.6 (Sohn *et al.* 2007). In our study, the ingested EPA and DHA, found in the fish oil in the diets, was able to partially replace the n-6 fatty acids, especially AA, in the fillet (Simopoulos 2002). When comparing the proportion of fatty acids in the YTK fillet and diet, the saturated fatty acids and n-6 PUFA were found to remain the same at approximately 30% and 9.2%, respectively. The MUFA were nearly 2-fold lower in the fillet than in the feed. In contrast, the proportion of n-3 PUFA in the fillet was 1.7-fold higher than in the feed. The retention and bioaccumulation of DPA and DHA in particular is of heightened interest. Bioaccumulation of DPA and DHA from the diet into the fillet of the fish is not fully understood, yet is a very important process in enriching fish with n-3 LCPUFA. The bioaccumulation occurring in the YTK is highlighted by the ratios of EPA/ALA and DHA/EPA. The diets contained 10 times more EPA than ALA but the bioaccumulation which occurred resulted in 25 times more EPA than ALA in the fillets. Bioaccumulation resulted in 3 times more DHA than EPA in the fillet than was supplied in the diets. This trend is consistent in fish with DHA levels being 2-5 times higher than EPA (Whelan and Rust 2006). Although bioaccumulation of DHA from the feed into the fillet portion was noticeable in YTK, the DHA as a proportion of total fatty acids in the fillets was variable. Our study found large variability in the growth of the fish, even within tanks, and the proportion of DHA in the fillet, although no correlation between these two could be shown. The variation in the proportion of DHA in the fillet suggests the role of a genetic factor. YTK may have varying genetic capabilities to bioaccumulate, retain or even synthesize DHA. This could explain the variation in the proportion of DHA in the fillet within a population of YTK receiving the same supply of dietary fatty acids. In the future, fish may be genetically screened for high DHA content and subsequent selective breeding programs based on their fatty acid profile may be undertaken.

The unsaturation index has been used as a measure of the potential for lipid peroxidation by considering the total amount of oxidisable fatty acids, as well as the number of double bonds within those fatty acids (Mourente et al. 2002; Tocher et al. 2002). The YTK feed had a low unsaturation index of 188.5, compared with vitamin E supplemented diets previously fed to gilthead sea bream, with values of 290 and 345 as well as turbot and halibut with a value of 290 (Mourente *et al.* 2002; Tocher *et al.* 2002). The lower unsaturation index could potentially indicate that the oxidative stability of the YTK diets was high. There was a significant decrease in fillet unsaturation index during storage which suggests that the lipid peroxidation that was already occurring in the fillet after four days was beginning to cause a reduction in the number of double bonds available for free radical attack. The lipid and nutritional quality of fillets after storage can be assessed using the FLQ index, which compares the ratio of PUFA to the total fatty acids, the IA, which compares the ratio of PUFA to the saturated fatty acids which are highly atherogenic and IT, which compares the ratio of PUFA to the saturated fatty acids which are highly thrombogenic (Abrami *et al.* 1992; Senso *et al.* 2007). The FLQ index from fish in all of the diets significantly decreased after storage, suggesting that the PUFA were compromised by storage at 4°C for 4 days. Our data suggest that the nutritional quality of the fillets for human consumption was compromised after storage at 4°C for 4 days because the IA and IT increased in all dietary treatments, which increases the likelihood of the formation of fatty deposits in arteries and increases the tendency to form blood clots in humans (Senso *et al.* 2007). The YTK nutritional quality values are consistent with those reported by Senso *et al.* (2007) for gilthead sea bream. Gilthead sea bream were harvested at different periods of the year but the June harvest fillet fatty acid profiles were chosen as the closest seasonal comparison to the YTK harvest. Senso *et al.* (2007) reported gilthead sea bream fresh fillet IA, IT and FLQ values of 0.27, 0.17 and 30.20, respectively, which rose to 0.32, 0.20 and 33.59, respectively, after storage at 4°C for 7 days. Interestingly the FLQ index increased during storage of the gilthead sea bream fillets while the YTK FLQ index significantly decreased from 37.0 to 33.4.

The TBARS assay was used to determine the effect of dietary GSE supplementation on the oxidative stability of YTK fillets. The rate of lipid peroxidation in the fillet depends on the unsaturated fatty acid content and the presence of antioxidants (Chen

et al. 2008). In the present study, YTK fillet lipid peroxidation appeared to be reduced after dietary antioxidant supplementation with ethoxyquin and/or GSE compared to the baseline fish fillets. Apparently dietary antioxidant supplementation reduced the fillet TBARS value from a baseline value of 7.8 nmol MDA g^{-1} fillet to a mean across all dietary treatments of 5.6 nmol MDA g^{-1} fillet, although no statistical significance was found. A trend like this could potentially be explained by the lipid content of the diet and fat content of the fillet. It is widely accepted that there is more potential for fillet lipid peroxidation as the dietary lipid and fillet fat content increases. Interestingly, the mean fat content of the initial harvest fillets was 0.9% which increased to a mean of 1.2% across all dietary treatments after the dietary trial. Unexpectedly in this trial the fillet TBARS values decreased as the fillet fat content increased, although statistical significance was not found.

We investigated the effect of dietary GSE supplementation on lipid peroxidation in YTK fillets using conditions which simulated retail display storage for 4 days at 4°C. The TBARS values in the YTK fillets significantly increased during storage at 4°C for 4 days, regardless of the dietary supplementation with either ethoxyquin or GSE. Combined ethoxyquin and GSE dietary supplementation (diets 2 and 3) did not reduce the lipid peroxidation occurring in the fillet more than a single dietary supplementation with either ethoxyquin or GSE (diets 1 and 4). Diet 4 containing 250 mg kg^{-1} GSE provided the same level of oxidative stability in YTK fillets during storage as diet 1 containing 150 mg kg^{-1} ethoxyquin. This study has found that lipid peroxidation in YTK fillets is not proportional to the final concentration of dietary antioxidants, at least not when the antioxidant is GSE. High combined dietary antioxidant concentrations in diet 2 (150 and 250 mg kg^{-1} of ethoxyquin and GSE, respectively) and diet 3 (150 and 1250 mg kg^{-1} of ethoxyquin and GSE, respectively), did not provide any better protection against fillet lipid peroxidation than the lower dietary antioxidant concentrations in diet 1 (150 mg kg^{-1} of ethoxyquin) and diet 4 (250 mg kg^{-1} GSE). Tannins found in GSE may only be absorbed into the bloodstream of the fish to a certain level, which may result in most being excreted in the faeces (Yamakoshi *et al.* 1999). This could explain the apparent lack of any effect of GSE in YTK in the present study.

The TBARS value is a commonly used indicator of rancidity in fish tissue. When the TBARS value is below 0.58 μg MDA g^{-1} fillet the tissue is not considered to be rancid (Chen *et al.* 2008). Slightly rancid but acceptable tissue has TBARS values of 0.58-1.51 μg MDA g^{-1} fillet, while rancid tissue has values above 1.51 μg MDA g^{-1} fillet (Chen *et al.* 2008). The lipid peroxidation occurring in the YTK fillets during storage in the present study was higher than in other studies with sea bass, Atlantic halibut, rainbow trout and turbot (Gatta *et al.* 2000; Ruff *et al.* 2002; Chaiyapechara *et al.* 2003; Ruff *et al.* 2003). The TBARS value in fresh YTK fillets was 5.6 nmol MDA g^{-1} fillet (0.9 μg MDA g^{-1} fillet) and after storage at 4°C for 4 days this value significantly increased to 14.9 nmol MDA g^{-1} fillet (2.5 μg MDA g^{-1} fillet). In comparison, the TBARS value from turbot stored on and covered with ice for four days, with 1.4-1.8% fillet fat content, was approximately 0.9 μg MDA g^{-1} tissue for diets supplemented with 100 mg kg^{-1} of both vitamin E and vitamin C (Ruff *et al.* 2003). When diets were supplemented with 1000 mg kg^{-1} vitamin E and 100 mg kg^{-1} vitamin C the lipid peroxidation was reduced to approximately 0.1 μg MDA g^{-1} tissue (Ruff *et al.* 2003). Similarly, in Atlantic halibut with a higher fillet fat content of 9.3-12.2% the TBARS value of the fillets stored on and covered with ice for four days was approximately 0.3 μg MDA g^{-1} tissue for diets supplemented with 189 mg kg^{-1} vitamin E (Ruff *et al.* 2002). The TBARS value was reduced to approximately 0.2 μg MDA g^{-1} tissue when diets were supplemented with 613 mg kg^{-1} vitamin E (Ruff *et al.* 2002). Our study aimed to simulate retail display conditions by storing fillets at 4°C for 4 days with air flow and exposure to natural and artificial lighting. Therefore, it is not surprising that higher TBARS values were reached after 4 days than in the studies by Ruff *et al.* (2002 and 2003) where Atlantic halibut and turbot were covered with ice. The TBARS value reached after storage in our study, 2.5 μg MDA mg^{-1} fillet, was most comparable to the storage of sea bass at 1°C for 12 days (Gatta *et al.* 2000). Sea bass with a fillet fat content of 8.8-9.7% also reached a TBARS value of 2.5 μg MDA mg^{-1} muscle when diets were supplemented with 139 mg kg^{-1} vitamin E and stored at 1°C for 12 days (Gatta *et al.* 2000). However, this TBARS value was reduced to 1.1 μg MDA mg^{-1} muscle when diets were supplemented with 254-942 mg kg^{-1} vitamin E (Gatta *et al.* 2000). In contrast to these studies, we did not see a reduction in the TBARS value in the YTK fillets when the diets were supplemented with GSE and stored at 4°C for 4 days.

Although ethoxyquin or GSE were not measured in the fillet portion of the YTK, predictions about fillet content can be made based on previous studies. As much as 80-98% of the dietary ethoxyquin consumed and its metabolite, EQDM, are deposited in the muscle of Atlantic salmon within 12 weeks (Bohne *et al.* 2008). Theoretically the time required to reach a steady state level of ethoxyquin in muscle is predicted to be 13 days (Bohne *et al.* 2008). Therefore, steady state was predicted to be achieved during the duration of our study with YTK. This suggests that ethoxyquin and its metabolites were deposited in the YTK fillet and would have provided antioxidant protection of the fillet against lipid peroxidation during storage at 4°C for 4 days.

Factors which may affect the YTK fillet ethoxyquin and EQDM levels are fish size, dietary lipid content and other dietary antioxidants (Bohne *et al.* 2008). A combination of GSE and ethoxyquin were supplied in diets 2 and 3 but no additional benefits were seen in the YTK due to increased total antioxidant supplementation. We also found that GSE was no more effective at protecting the YTK fillets against lipid peroxidation or fatty acid compositional changes than ethoxyquin during storage at 4°C for 4 days. GSE and ethoxyquin provided an equivalent level of antioxidant protection which is evident by the TBARS values and fatty acid composition of fillets from fish fed diets 1 and 4. Poor digestibility, incorrect concentration or low incorporation into the fillet may have contributed to GSE not being more beneficial than ethoxyquin in providing antioxidant protection in the fillet portion of YTK. Luther *et al.* (2007) found that when determining the antioxidant activity of GSE by measuring changes in the fatty acid profile of oxidised fish oil, overall lipid peroxidation in the oil was suppressed more effectively than in selected fatty acids. The total PUFA composition was significantly higher in oxidised fish oil when it contained GSE whereas the DHA composition was reduced to the same level in oxidised fish oil with or without GSE compared to unoxidised fish oil (Luther *et al.* 2007). We did not observe GSE acting more efficiently as an antioxidant on the total PUFA or an individual PUFA. Conversely, the fish fed diets 1 and 2 had significantly lower fillet total PUFA content after storage, but the individual distribution of EPA and DPA was unchanged by the effect of storage.

The custom mineral/vitamin premix used in the diets contained 100 mg kg^{-1} of both vitamins E and C. In hindsight, these vitamins should have been excluded from the diets as their effects as antioxidants may have masked the antioxidant effect of the GSE. However, we were concerned about fish welfare related to vitamin E and C deficiencies, as these vitamins have other roles in animals, apart from the antioxidant role. Gatta *et al.* (2000) kept approximately 100 mg kg^{-1} of BHT present in all of the vitamin E supplemented diets fed to sea bass. Lipid peroxidation over 12 days of 1°C storage was higher in fish fed the lowest vitamin E concentration (139 mg kg^{-1}) but no difference was seen when fish were fed with diets containing 254, 493 or 942 mg kg^{-1} vitamin E (Gatta *et al.* 2000). Unexpectedly, lipid peroxidation did not increase in the sea bass fillet during 12 days of storage, regardless of the vitamin E concentration (Gatta *et al.* 2000). The addition of BHT to all diets may have provided extra protection against fillet lipid peroxidation, thus concealing any differences in lipid peroxidation due to vitamin E supplementation alone. Similarly, combinations of vitamin E, vitamin C, ethoxyquin and GSE in the YTK feeds may have prevented any possible protective effects of GSE from being seen.

In summary, we found three important findings for the human consumer regarding the YTK fillet DHA content. The first finding was that YTK are very capable of bioaccumulating DHA, with approximately 2.5-fold more DHA in the fillet than in the diet. Secondly, a significant decrease in the proportion of DHA in the fillet after storage was found, regardless of ethoxyquin or GSE antioxidant protection. Ethoxyquin and GSE gave the same level of protection, if any, against the oxidative degradation of DHA in the fillet during storage. The third finding was that natural fish variation resulted in a variation in the proportion of DHA in the fillet. Dietary n-3 LCPUFA, including EPA and DHA have beneficial health effects in humans, particularly in relation to neurodevelopment (Smithers *et al.* 2008), the treatment of inflammatory and autoimmune diseases (Proudman *et al.* 2008) and reduced risk of sudden cardiac death (Metcalf *et al.* 2008). Our findings suggest that consuming YTK fillets which have been stored at 4°C for 4 days will result in lower levels of DHA being ingested than what was present within the fresh fillets and the proportion of DHA in the fillet is variable within the species. The variability in the proportion of fillet DHA may be due to genetic variation. However, genetically selecting and breeding YTK with a higher proportion of fillet DHA may offset any loss of DHA

during storage of the fillets. Selecting YTK for breeding based on their fatty acid profile may be the future for increasing the human nutritional value still further.