1   GENERAL INTRODUCTION

1.1   Polyunsaturated fatty acids (PUFA)

Polyunsaturated fatty acids (PUFA) can be defined as fatty acids of 18 carbons or more in length that have two or more double bonds, which are generally interrupted by a single methylene group (Beaudoin *et al.* 2000; Tocher 2003). Long chain PUFA (LCPUFA) can be defined as PUFA of 20 carbons or more in length (Leonard *et al.* 2004). Fatty acids are defined as omega-3 (n-3) or omega-6 (n-6) depending on where the closest double bond to the methyl (omega) end of the fatty acid is located (Beaudoin *et al.* 2000). For example, n-3 fatty acids have the last double bond three carbons from the methyl end, whereas n-6 fatty acids have the last double bond six carbons from the methyl end (Whelan and Rust 2006). The essential PUFA for humans, 18:3n-3 (α-linolenic acid, ALA) and 18:2n-6 (linoleic acid, LA), must be obtained through the diet because they cannot be synthesized by humans. Humans do not have the $\Delta 12$ and $\Delta 15$ fatty acyl desaturases required for production of ALA and LA from 18:1n-9 (Ghioni *et al.* 1999). In general, humans obtain n-6 fatty acids from plant sources and n-3 fatty acids from fish and seafood. The optimal ratio of n-6:n-3 for humans is 1:1, but the Western diet currently has a ratio of 15-20:1 (Simopoulos 2002). Humans consuming a typical Western diet are not able to convert the essential dietary n-3 PUFA ALA into n-3 LCPUFA like 20:5n-3 (eicosapentaenoic acid, EPA), 22:5n-3 (docosapentaenoic acid, DPA) and 22:6n-3 (docosahexaenoic acid, DHA) because of this imbalance (Surette 2008). Omnivorous humans typically convert <1% ALA to DHA, with the remainder being oxidised (Whelan and Rust 2006). Thus, fish and seafood in general are the major sources of n-3 LCPUFA in the human diet. Of particular interest in fish are the ALA derivatives, EPA, DPA and DHA. Marine and freshwater fish muscle tissue (the edible portion) is comprised of 30-50% DPA and DHA (Whelan and Rust 2006). Dietary n-3 LCPUFA, in particular 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).

1.2 The long chain polyunsaturated fatty acid (LCPUFA) synthesis pathway

Fish can synthesize LCPUFA and/or obtain them already preformed from their diet. The efficiency of the LCPUFA synthesis pathway is dependent on the activities of the enzymes in the pathway, which in turn are dependent on the amount of preformed LCPUFA obtained through the diet (Tocher 2003). The LCPUFA synthesis pathway requires three elongation and three desaturation steps (Figure 1.1) (Sprecher 2000). The same enzymes may be involved in both the n-3 and the n-6 pathway but, in general, they have a higher affinity for the n-3 than the n-6 precursors (Sargent *et al.* 2002; Tocher 2003). In the vertebrate LCPUFA synthesis pathway, a fatty acyl Δ6desaturase converts 18:3n-3 and 18:2n-6 to 18:4n-3 and 18:3n-6, respectively, by introducing a *cis* double bond at the Δ6 position of the carbon chain (Park *et al.* 2009). Then a fatty acyl elongase, Elovl5, with malonyl-CoA as the carbon donor, adds two carbons to 18:4n-3 and 18:3n-6 to synthesize 20:4n-3 and 20:3n-6, respectively (Monroig *et al.* 2009). Next a Δ5desaturase converts 20:4n-3 to 20:5n-3 and 20:3n-6 to 20:4n-6 by introducing a *cis* double bond at the Δ5 position of the carbon chain (Park *et al.* 2009). Two consecutive elongation steps catalysed by Elovl5 and Elovl2 synthesize 24:5n-3 and 24:4n-6 from 20:5n-3 and 20:4n-6, respectively. In mammals the Elovl5 enzyme prefers $C_{18/20}$ PUFA substrates and the Elovl2 enzyme prefers $C_{20/22}$ PUFA substrates (Leonard *et al.* 2000; Inagaki *et al.* 2002; Leonard *et al.* 2002). Similarly, a recent study with Atlantic salmon suggested that there may be different elongase enzymes responsible in fish for the elongation of $C_{18/20}$ PUFA and $C_{20/22}$ PUFA (Morais *et al.* 2009). Finally, the Δ6desaturase is required once more to convert 24:5n-3 and 24:4n-6 to 24:6n-3 and 24:5n-6, respectively. The findings of Sauerwald *et al.* (1997) and de Antueno *et al.* (2001) in humans suggested that Δ6desaturation of 18:3n-3 and 18:2n-6 are in competition not only with each other, but also with 24:5n-3 and 24:4n-6. The final step in the pathway involves the movement of 24:6n-3 and 24:5n-6 from the endoplasmic reticulum (ER) to the peroxisomes for partial β-oxidation to 22:6n-3 and 22:5n-6, respectively (Sprecher and Chen 1999; de Antueno *et al.* 2001). The 24:6n-3 and 24:5n-6 are preferentially moved to the peroxisome for partial oxidation rather than the mitochondria where complete oxidation occurs (Sprecher *et al.* 1999). A

structural feature of 22:6n-3 and 22:5n-6 may signal their movement back to the ER for use as substrates in membrane lipid biosynthesis rather than complete oxidation (Sprecher *et al.* 1999).
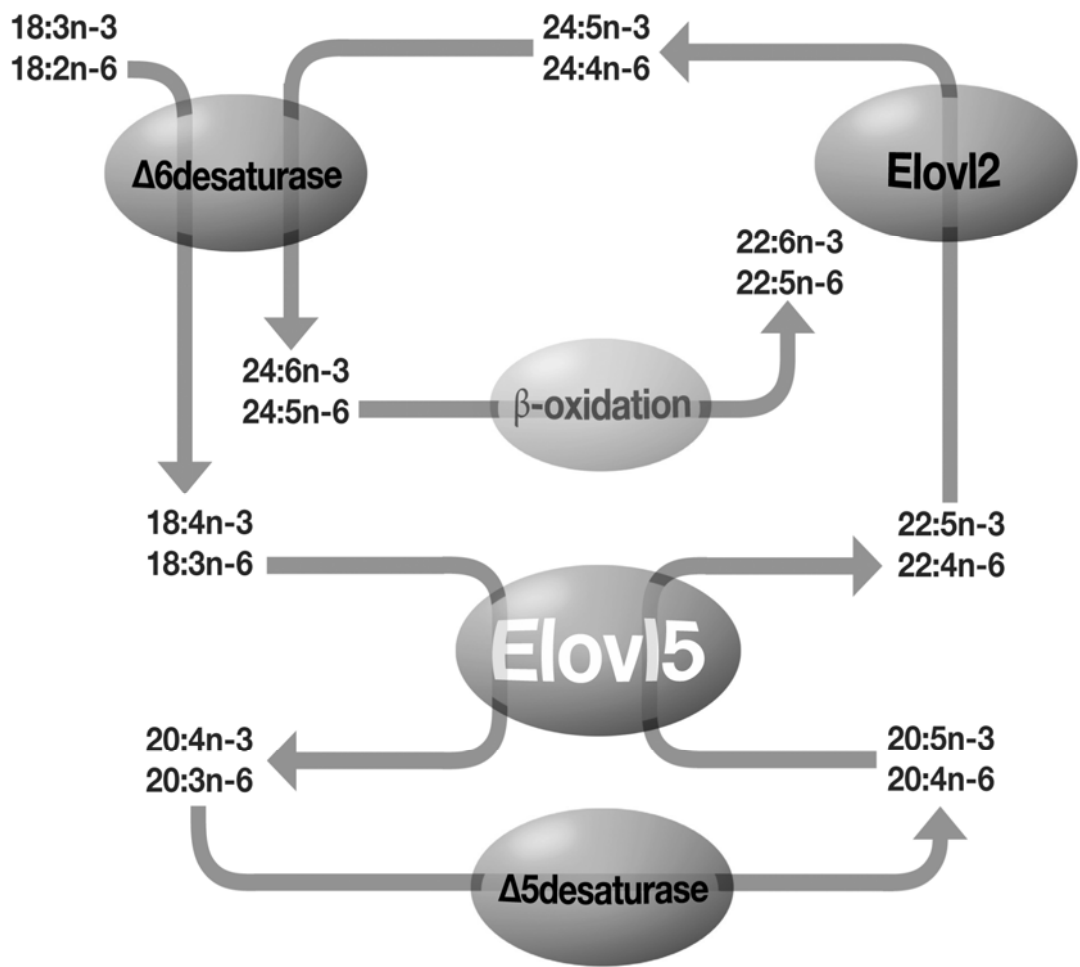
Figure 1.1 Long chain polyunsaturated fatty acid (LCPUFA) synthesis in vertebrates.

1.3 Aquaculture

Aquaculture is the farming of aquatic plants and animals (Bols 1991). The aim of aquaculture is to maximize the yield of useful organisms from the aquatic environment by controlling growth and reproduction whilst reducing mortality (Beveridge 2004). Aquaculture dates back 4000 years but it has not contributed large quantities of fish to the world food supply until recently (Beveridge 2004). The aquaculture industry is continuing to grow in response to increasing demand for a sustainable supply of seafood to support the world's growing population (Tidwell and Allan 2001). There has been an expansion of aquaculture all over the world, with species of interest including gilthead sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*) in the Mediterranean, Asian sea bass (*Lates calcarifer*) in the Indo-West Pacific region, olive flounder (*Paralichthys olivaceus*) in East Asia and turbot in Western Europe (Bengtson 2003). High-value species such as tuna, salmon and shrimp are in great demand in developed countries (Naylor *et al.* 2000; Tidwell and Allan 2001).

Little is known about the nutritional requirements of most fish species compared to the wealth of information available for terrestrial animals (Tidwell and Allan 2001). Herbivorous, omnivorous and carnivorous fish species have different dietary requirements and consequently different nutritional value for the human consumer. Herbivorous and omnivorous fish species use plant based proteins and oils more efficiently than carnivorous fish (Naylor *et al.* 2000). Herbivorous or omnivorous species, which commonly live in freshwater environments, are able to utilise C_{18} PUFA to synthesize LCPUFA (Sargent *et al.* 2002). In contrast, carnivorous fish frequently live in marine environments where fish meal and fish oil, in the form of small pelagic fish, supply the essential amino acids such as lysine and methionine and fatty acids such as EPA and DHA, which are not found in plant proteins or oils (Naylor *et al.* 2000). Carnivorous species have a dietary requirement for LCPUFA which is met by consuming lipid-rich prey (Regost *et al.* 2003; Tocher 2003).

Aquaculture of carnivorous fish continues to rise despite the increasing feed supply problems the industry is encountering (Sargent *et al.* 2002). The farming of

carnivorous fish is not a sustainable practice due to the large requirement for fish meal and fish oil, which can only be obtained from other fish (Naylor *et al.* 2000; Pauly *et al.* 2005). Wild fish stocks, particularly small pelagic fish such as anchovy, mackerel, herring and sardine, are currently being depleted in an attempt to supply the n-3 fatty acids found in the farmed species (Naylor *et al.* 2000; Jenkins *et al.* 2009). The use of pelagic fish for feeds increases the financial cost of farming fish, with fish oil costing US\$1800 per tonne in July 2008 (Francis *et al.* 2009). When pelagic fish, which are perfectly suitable for human consumption, are used for animal feeds, a large proportion of the nutritional value to humans is lost during the absorption through the gut of the carnivore (Pauly *et al.* 2005; Naylor *et al.* 2000). For carnivorous fish species, the biomass of fish consumed is 2.5-5.0 times the biomass of the farmed fish that is produced (Naylor *el al.* 2000). The projected growth of aquaculture will not be sustainable if it follows the current farming practices of high fish meal and fish oil diets (Naylor *et al.* 2000; Bengtson 2003).

1.4   Oils in aquaculture

There is a great need to find alternative sources of oils for fish feeds. The relative abundance of plants compared to fish make vegetable oils a good alternative as a more sustainable source of oil. Vegetable oils are rich in C_{18} PUFA, such as ALA and LA, but low in n-3 LCPUFA, such as EPA and DHA (Regost *et al.* 2003; Whelan and Rust 2006). Conversely, fish oils are rich in n-3 LCPUFA but low in C_{18} PUFA. The partial substitution of valuable fish oils with more readily available vegetable oils such as rapeseed, linseed, soya, palm and olive oils in aquaculture feeds is accepted within the aquaculture industry (Naylor *et al.* 2000; Bell and Sargent 2003). However, it is inevitable that the flesh fatty acid profile will be affected because it is a reflection of the dietary fatty acids (Francis *et al.* 2009). Previous work has shown that Atlantic salmon can survive and grow on a vegetable oil-based diet but the concentration of EPA and DHA in their flesh was lowered (Tocher *et al.* 2003). The reduction in flesh n-3 LCPUFA after feeding vegetable oil-based diets has been reported many times in Atlantic salmon, as well as in brown trout and Arctic charr (Tocher *et al.* 2001; Bell *et al.* 2002). Consequently, an increase in the n-6:n-3 ratio will occur (Naylor *el al.* 2000). Thus, the marketability

and flavour of the fish will change and the human health value will be lowered, causing further imbalances in the human diet (Sargent *et al.* 2002).

To enable fish to synthesize DHA from ALA, all of the enzymes in the LCPUFA synthesis pathway must be active (Tocher 2003). The salmonid, Atlantic salmon, has the full complement of LCPUFA synthesis enzymes, including an Elovl2, which is the only functionally characterised Elovl2 in a non-mammalian vertebrate (Morais *et al.* 2009). Increasing the vegetable oil concentration in the diets of Atlantic salmon, brown trout and Arctic charr resulted in an increase in the hepatic fatty acyl desaturation and elongation activities (Tocher *et al.* 2001; Bell *et al.* 2002). However, the reason for the increase in enzymatic activity in fish fed vegetable oil is unclear. The normally high dietary n-3 LCPUFA level may suppress the LCPUFA synthesis enzymes due to the competitive nature of the pathway. Replacing the dietary fish oil with vegetable oil reduces the preformed LCPUFA and increases the C_{18} precursors, which may increase enzyme activity. On the other hand, marine fish are widely considered to be unable to synthesize LCPUFA from C_{18} PUFA precursors, possibly due to an enzyme deficiency or structural modification of one or more of the key enzymes in the synthesis pathway (Regost *et al.* 2003; Tocher 2003; Agaba *et al.* 2004). A study with the marine species turbot showed that a vegetable oil-based diet reduced growth and lowered EPA and DHA concentrations in the flesh, even after an 8 week fish oil finishing diet (Regost *et al*. 2003). Turbot have a better ability to elongate fatty acids rather than desaturate them possibly due to $\Delta5$desaturase and $\Delta6$desaturase deficiencies (Regost *et al*. 2003). To ensure aquaculture continues as a sustainable industry a deeper understanding of fish LCPUFA synthesis must be gained.

1.5 The southern bluefin tuna (SBT) aquaculture industry in South Australia

Perciformes are the largest order of vertebrates and the most diversified of all fish orders (Nelson 1994). Most Perciformes are marine fish, with 21% living in freshwater (Nelson 1994). Within the Perciformes, the family Scombridae contains 49 species of mackerels and tunas (Nelson 1994). There are five genera of *Thunnini*

(tunas), *Allothunnus*, *Auxis*, *Euthynnus*, *Katsuwonus* and *Thunnus* which contain 14 species (Nelson 1994). Southern bluefin tuna (SBT, *Thunnus maccoyii)* is the second most valuable aquaculture species in Australia (Mazur *et al.* 2010). Over ninety eight percent of the Australian SBT quota is farmed, with all farming occurring offshore of Port Lincoln, South Australia (SA) (Love and Langenkamp 2003; Buckee 2004). The juvenile SBT are caught during their migration from December to April in the Great Australian Bight, in the Southern Ocean. These SBT, which weigh approximately 15 kg, are towed in nets back to the calmer waters of Spencer Gulf. The SBT are transferred into sea cages and mainly fed a diet of pilchards and mackerel which provides them with their LCPUFA requirements. During the grow-out phase the SBT reach 30-40 kg in size. At this point in time, SBT aquaculture is a means to raise wild fish to marketable size in captivity (Naylor *el al.* 2000). Pelagic fish stocks are sufficient for the current fattening process but as the industry grows and captive breeding techniques are improved, wild fish stocks will not be able to sustain this industry.

The SBT are exported mostly to Japan's Tsukiji fish market as fresh, chilled or frozen whole tuna. Since 1990, the SBT aquaculture industry in SA has continued to expand to produce approximately 9,000 tonnes of gilled and gutted SBT annually (David Ellis, Tuna Boat Owners Association, pers. comm.). The estimated commercial value per annum is AU$150 - 300 million (David Ellis, pers. comm.). The scale of tuna farming in the Mediterranean, North America and Mexico has increased and this has elevated global production of farmed tuna (Love and Langenkamp 2003). Australian tuna farmers are experiencing an increase in market competition because all countries are targeting the one market, Japan. There is pressure on Australian tuna farmers to maintain quality while reducing production costs.

Tuna species have unique fatty acid profiles which contain high levels of DHA and a very high DHA/EPA ratio compared to other marine fish species (Mourente and Tocher 2003). The level of DHA found in tuna is higher than the level found in the diet (Sargent *et al.* 2002). The mechanism determining the possible selective accumulation and retention of the large quantity of DHA originating from their carnivorous diet is unknown (Saito *et al.* 1997; Sargent *et al.* 2002; Mourente and

Tocher 2003). The tuna's brain and eye heating systems and swimming muscles are likely to be fuelled by fatty acid oxidation, in particular the selective catabolism of saturated and monounsaturated fatty acids, rather than PUFA (Sargent *et al.* 2002). This may boost the DHA content in tuna, as the SBT flesh contains over 44% DHA and has a high DHA/EPA ratio of 9 (Nichols *et al.* 1998). These values are elevated compared with other tuna species found in Australian waters. The yellowfin tuna (*Thunnus albacores*) and albacore (*Thunnus alalunga*) contain 38.9% and 34.3% DHA, respectively and have DHA/EPA ratios of 7 and 6, respectively (Nichols *et al.* 1998).

Research into the nutrition of SBT is expensive because they are such large and valuable fish. Whole SBT are unobtainable due to their very high economic value and the inability to routinely breed this species, which limits their supply. SBT tissue can be obtained and used to elucidate the enzymatic regulation in the LCPUFA synthesis pathway.

1.6 The yellowtail kingfish (YTK) aquaculture industry in South Australia

The yellowtail kingfish (YTK, *Seriola lalandi*) is a member of the family Carangidae found in cool, temperate waters of the Pacific and Indian Oceans (Fowler *et al.* 2003). The YTK aquaculture industry in Spencer Gulf, SA is quickly growing and continuing to increase production. Hatcheries at Port Augusta and Arno Bay have a spawning season from August to December where larvae are initially fed Artemia and then manufactured feeds (Fowler *et al.* 2003). After 60-80 days the fingerlings are approximately 5 g in weight and are transferred to sea cages. The YTK are maintained for 1-2 years in sea cages between Port Lincoln and Fitzgerald Bay in the Spencer Gulf until they have reached 1-5 kg and are ready for harvest (Fowler *et al.* 2003).

The YTK are exported around the world, particularly to Europe and Japan (Clean Seas Tuna, www.cleanseas.com.au). The production of YTK from SA in 2007/2008 was 3,370 tonnes (Clean Seas Tuna, www.cleanseas.com.au). The YTK fillets have a

short shelf life which decreases marketability and market time, thus resulting in a decrease in the distribution distance, product options and convenience. A short shelf life increases the reliance on correct handling by fishmongers, as well as increasing discounting and wastage. Lipid oxidation causes the flesh to brown, particularly on the surface during storage.

The life cycle is closed in YTK which provides the opportunity to obtain juveniles for experimental needs such as dietary trials or primary cell line development. The accessibility of juvenile YTK is much better than SBT. YTK can be used as a model marine species for SBT, which are both farmed in the same geographic region.