Fatty acid metabolism in southern bluefin tuna (*Thunnus maccoyii*)

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

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Summary

Southern bluefin tuna (SBT, *Thunnus maccoyii*) is a highly valuable aquaculture species but little is known of its exact nutritional requirements due to the prohibitively high costs of classical feeding trials with this species. As an alternative approach, this thesis reports on the use of a recently established SBT cell line (designated SBT-E1) to investigate fatty acid metabolism and mitochondrial biogenesis in this species. Addition of the C_{18} polyunsaturated fatty acids (PUFA) 18:3n-3 or 18:2n-6 to the cell culture medium had little effect on cell proliferation, whereas addition of the long-chain PUFA 20:4n-6, 20:5n-3 or 22:6n-3 significantly reduced cell proliferation, especially at higher concentrations and especially for DHA. Addition of vitamin E to the culture medium overcame this effect, suggesting that it was due to oxidative stress. The fatty acid profiles of the total lipid from the cells reflected those of the respective culture media except that 22:6n-3 was substantially more abundant in the cells than in the media. Fatty acid esterification occurred predominantly into phosphatidylcholine and phosphatidylethanolamine, the two most abundant lipid classes. The SBT-E1 cells showed very limited Δ6 fatty acyl desaturase (Fads) activity towards either 18:3n-3 or 18:2n-6 but substantial elongation of very long chain fatty acids (Elovl) activity towards 20:5n-3. The latter activity is usually attributable to an Elovl5 enzyme. Surprisingly though, there were much higher levels of Δ6Fads compared with Elovl5 gene expression in the SBT-E1 cells, suggesting that a different Elovl enzyme may catalyse this reaction in SBT. The cells also showed substantial β-oxidation of 18:3n-3 and 20:5n-3 but much less activity towards 18:0, 18:1n-9 or 18:2n-6. These results may explain the high 22:6n-3 to 20:5n-3 ratios found in the SBT tissue lipids. Serum deprivation did not

significantly affect gene expression of the mitochondrial markers citrate synthase (CS) and cytochrome *c* oxidase subunit 1 (COX1) in the SBT-E1 cells but it did significantly upregulate the expression of peroxisome proliferator activator receptor γ (PPARγ) co-activator 1 α (PGC-1α). This may indicate a role for PGC-1α in the cellular response to reduced fatty acid availability. Interestingly, the cells supplied with the reduced concentration of serum together with 20:5n-3 did not exhibit significantly increased PGC-1α gene expression, indicating that 20:5n-3 was able to overcome some of the effects of the reduced serum concentration. To further investigate the role of PGC-1α in SBT, a PGC-1α cDNA was cloned from SBT liver. This is the first report of a PGC-1α sequence from any tuna species. The PGC-1α cDNA corresponded to what is recognised as the canonical PGC-1α. When comparing different SBT tissues, PGC-1α gene expression was found to be high in the red muscle and the expression levels in the different tissues correlated well with the expression of its co-activation target, PPARγ. In contrast, the expression of PGC-1α did not appear to influence mitochondrial abundance, as indicated by the gene expression of CS and COX1. The results are discussed in light of the unusual physiology of tunas.

Declaration

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

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Chapter 1 – General Introduction

1.1 Bluefin Tunas

The three bluefin tuna species, Atlantic bluefin tuna (ABT, *Thunnus thynnus*), Pacific bluefin tuna (PBT, *Thunnus orientalis*) and southern bluefin tuna (SBT, *Thunnus maccoyii*) are targeted by commercial fisheries around the world as a high quality premium product. The main market for bluefin tunas is Japan and the main use is for the production of the Japanese raw fish delicacies sushi and sashimi. Bluefin tunas are typically captured as juveniles and then fattened in sea cages in the Spencer Gulf of South Australia (SBT), the Mediterranean Sea (ABT) and along the Pacific coast of Mexico and the southern coast of Japan (PBT). Fattening of the tunas is achieved by feeding them small pelagic fish (e.g. sardines, mackerels, herrings) sourced from wild-catch fisheries (Miyake *et al.* 2010, Mylonas *et al.* 2010, Musgrove *et al.* 2011). This technique is used for three main reasons. Firstly, and most importantly, it allows producers to make the most of the limited number of fish available as their capture from the wild is regulated by strict quotas due to concerns regarding their declining wild stocks (CCSBT, ICCAT). Secondly, it allows the farmers to increase the fat content of the tuna muscle (desired by Japanese sashimi consumers). Finally, it allows the farmers to hold fish back from the market at times when over supply or currency fluctuations bring sub-optimal prices (Miyake *et al.* 2010, Mylonas *et al.* 2010, Woodhams *et al.* 2013, Stephan and Hobsbawn 2014). In an additional effort to minimise the effects of quotas on the bluefin tuna market, several producers have attempted to develop captive breeding and aquaculture of tuna but with limited success (Sawada *et al.* 2005, Masuma *et al.* 2008, De Metrio *et al.* 2010, Bubner *et al.* 2012, Tsuda *et al.* 2012, Okada *et al.* 2014, Yúfera *et al.* 2014). These efforts have been largely successful in producing larvae and juveniles but a high fish mortality rate during the first year post-hatch currently limits the commercial viability of these ventures (Tsuda *et al.* 2012, CST, 2011, CST, 2012).

1.2 Southern bluefin tuna (*Thunnus maccoyii*)

SBT is possibly the most valuable scombrid fish in the southern hemisphere and Australia holds approximately 42% of the global fishing quota for this species (Timmiss 2011). Wild SBT spawn in the warm waters off the coast of Java and during their first year of life, they follow the Leeuwin current to migrate down the western coast of Australia into the cold waters of the Great Australian Bight (Proctor *et al.* 1995, Farley and Davis 1998, Ridgway and Condie 2004, Gunn *et al.* 2008). The juvenile SBT are captured in the Great Australian Bight using purse seine nets and transported to sea cages off the coast of Port Lincoln, South Australia where they are fattened before sale, predominantly to Japanese markets for use in the raw fish delicacies sushi and sashimi (Ottolenghi 2008, Kirchhoff *et al.* 2011). One of the challenges faced by producers in this process is that like all tunas, little is known of the specific nutritional requirements for SBT other than from studies of their natural prey (Masuma *et al.* 2008, Miyake *et al.* 2010, Mylonas *et al.* 2010, Itoh *et al.* 2011, Logan *et al.* 2011, Shimose *et al.* 2013, Woodhams *et al.* 2013, Metian *et al.* 2014, Stephan and Hobsbawn 2014). Thus, most aquaculturalists utilise small pelagic fish to feed SBT, mimicking their natural diet and thus presumably satisfying their nutritional requirements (Itoh *et al.* 2011, Musgrove *et al.* 2011). Whilst the use of small pelagic fish in SBT farming is currently viable, it is heavily reliant on the wild sardine fishery. This fishery is heavily exploited globally and is prone to high variability in catches (Freon *et al.* 2005). A higher demand for sardines and other small pelagic fish to feed the growing aquaculture industry, will also drive feed costs higher, leading to profit losses in the SBT aquaculture industry as this growth cannot be sustained by the current supply of wild-caught fish products (Mourente and Tocher 2009, Turchini *et al.* 2009). Furthermore, as SBT aquaculture develops,

hatchery-reared tuna may become available for grow-out, which, in turn, will increase the demand for small pelagic fish to use as feed.

1.3 Fish oil replacement in feeds for farmed fish

In their natural habitat, the flesh of top predator fish such as SBT is rich in omega-3 (n-3) long chain polyunsaturated fatty acids (LC-PUFA, defined as PUFA with ≥ 20 carbons and ≥ 3 double bonds (Morais *et al.* 2009)), particularly eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3) (Tocher 2003, Mourente and Tocher 2009). These fatty acids are synthesised almost exclusively by marine primary producers such as phytoplankton and they accumulate with increasing trophic level (Moreno *et al.* 1979, Tocher 2003, Perhar *et al.* 2012). Thus, the diet of piscivorous fish such as SBT is abundant in both 22:6n-3 and 20:5n-3. In the past 15-20 years, the growth of wild-catch fisheries production has failed to keep pace with the growth of aquaculture and as a result the use of wild-caught fish to feed farmed fish has been called into question (Tacon and Metian 2013, Metian *et al.* 2014). In other farmed fish species, such as Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*), significant progress has been made in the replacement of wild-catch fisheries products (fish oil (FO) and fish meal) with alternatives such as vegetable oils (VOs) and plant meals but this has not been the case for tunas (Tacon and Metian 2013, Metian *et al.* 2014). There are several reasons for this. The first is that Japanese sushi and sashimi consumers prefer wild-caught tunas or, at the very least, farmed tunas that have been fed their natural diet (Ottolenghi 2008). The second is that classical feeding trials with proper replication have not been possible due to the high commercial value of wild-caught bluefin tunas, the high costs of maintaining them in sea cages or purpose built facilities on land and the limited success of captive breeding programs (Mourente and Tocher 2009, Mylonas *et al.*

2010). The third reason is that replacement of FO with VO in feeds for farmed fish reduces their flesh concentrations of 22:6n-3 and 20:5n-3 (Miller *et al.* 2008, Turchini *et al.* 2009). Both 22:6n-3 and 20:5n-3 have well documented benefits for human and fish health (Fernández-Palacios *et al.* 1995, Izquierdo 1996, Sargent *et al.* 1999, Connor 2000, Calder and Yaqoob 2009). For example, 22:6n-3 is important in human brain and eye development and is present at especially high concentrations in these organs (Innis 2008). Consumption of 22:6n-3 and 20:5n-3 is also associated with reduced risk of cardiovascular disease and amelioration of the symptoms of inflammatory disorders such as rheumatoid arthritis (Monteiro *et al.* 2014, Yates *et al.* 2014). Both 22:6n-3 and 20:5n-3 are important modulators of the inflammatory response in vertebrates (Chapkin *et al.* 2009). The n-6 counterpart of 20:5n-3, arachidonic acid (20:4 n-6), is a precursor for pro-inflammatory eicosanoids, whereas 20:5n-3 is a precursor of anti-inflammatory eicosanoids (Calder 2009, Chapkin *et al.* 2009). Therefore, the balance between 20:5n-3 and 20:4n-6 is critical in regulating the vertebrate inflammatory response. In fish, both 22:6n-3 and 20:5n-3 are considered dietary essential fatty acids (EFA) and are necessary for correct larval development, nutrition, and good reproductive and gut health in marine fish (Fernández-Palacios *et al.* 1995, Izquierdo 1996, Sargent *et al.* 1999). Interestingly, tunas not only contain high levels of 22:6n-3 in their lipids, but importantly they also contain high levels of 22:6n-3 relative to 20:5n-3 (Morais *et al.* 2011). This ratio is important as it suggests that tunas preferentially retain the 22:6n-3 they consume rather than oxidising it (Tocher 2003, Morais *et al.* 2011). If tunas do, in fact, have this capability, it is possible that dietary FO may be partially substituted with VO without excessively negative impacts on the fatty acid composition of the flesh. However, this has not yet been investigated.

1.4 Polyunsaturated fatty acid (PUFA) metabolism

1.4.1 Fatty acid synthesis

A substantial amount of the dietary LC-PUFA obtained by fish is esterified and incorporated into the phospholipids that make up cellular membranes and/or is esterified and stored, most commonly as triacylglycerols (Duplus *et al.* 2000). However an interesting alternative to obtaining LC-PUFA from dietary sources is the ability of some species to endogenously synthesise these important fatty acids from shorter-chain, more saturated fatty acids. Fish, like all vertebrates, do not have the Δ12 or Δ15 fatty acyl desaturase (Fads) enzymes which convert the monounsaturated fatty acid (MUFA) oleic acid (18:1n-9) to linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3), respectively, and as a result they require 18:2n-6 and 18:3n-3 as dietary EFA (Ghioni *et al.* 1999, Tocher 2003). These EFA can be used as precursors in the *de novo* synthesis of LC-PUFA through a series of desaturation and elongation steps that occur in the endoplasmic reticulum (Figure 1.1). This pathway is the same for n-3 and n-6 fatty acids with 18:3n-3 serving as the precursor for the synthesis of n-3 LC-PUFA and 18:2n-6 as the precursor for the synthesis of n-6 LC-PUFA (Tocher 2003, Bell and Tocher 2009). In this process, the Δ6Fads enzyme introduces a double bond at the sixth position from the carboxyl (COOH) end of 18:3n-3 or 18:2n-6, yielding 18:4n-3 and 18:3n-6, respectively (Sprecher 2000). Then elongase of very long chain fatty acids 5 (Elovl5) adds two carbon atoms from malonyl CoA, yielding 20:4n-3 and 20:3n-6, respectively (Monroig *et al.* 2009). These first desaturation and elongation steps can also occur in the opposite order with Elovl5 elongating 18:3n-3 and 18:2n-6 to 20:3n-3 and 20:2n-6, respectively, and the Δ6Fads, which commonly possesses Δ8Fads activity, introducing a double bond at the eighth position from the carboxyl end to yield 20:4n-3 and 20:3n-6, respectively (Monroig *et al.* 2011). A Δ5 Fads then introduces a double bond at the fifth position from the carboxyl end,

yielding 20:5n-3 and 20:4n-6, respectively. Continuation of this process involves further elongation of 20:5n-3 and 20:4n-6 by Elovl5 and Elovl2, before Δ6Fads is again used in a desaturation step to yield 24:6n-3 and 24:5n-6. Prior to the final step, 24:6n-3 and 24:5n-6 are moved from the endoplasmic reticulum to the peroxisomes where partial oxidation of the fatty acids occurs, removing two carbons from the carboxyl end to yield 22:5 n-6 and 22:6n-3 (Fig. 1.1, Sprecher 2000). The final oxidation step in this process occurs in the peroxisomes to prevent complete β-oxidation of the fatty acid, as would be the case if oxidation occurred in the mitochondria (Sprecher 2000). It was recently discovered that the final elongation, desaturation and β-oxidation steps were able to be replaced by a single reaction catalysed by a Δ4Fads enzyme (Li *et al.* 2010). However, the only fish species that have been found to contain this enzyme are herbivorous or scavenging species, which have a substantially different diet to predatory fish such as SBT (Li *et al.* 2010, Morais *et al.* 2012).

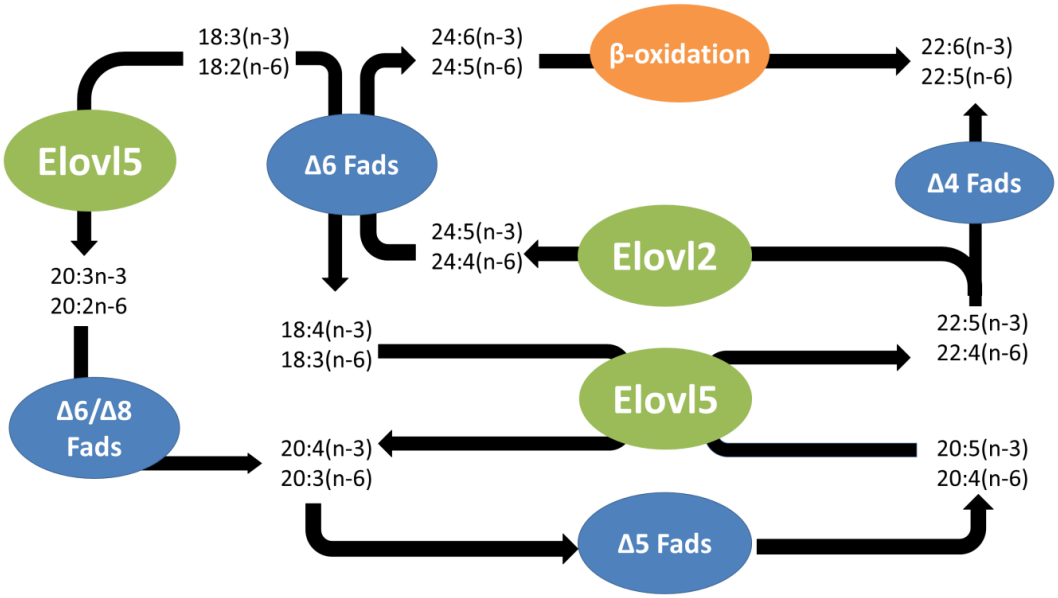


Figure 1.1: The long-chain polyunsaturated fatty acid biosynthesis pathway in vertebrates. (Li *et al.* 2010, Monroig *et al.* 2011, Morais *et al.* 2012). Figure adapted from Fig. 5 in Gregory et al. (2010).

The LC-PUFA biosynthesis pathway is important because it provides a means to produce 20:5n-3 and 22:6n-3, which are abundant in FO, from their precursor 18:3n-3, which is abundant in many VOs such as linseed or canola oil (Turchini *et al.* 2009). However many fish species, especially marine piscivores, have only limited capacity to convert 18:3n-3 to 20:5n-3 or 22:6n-3, presumably due to the abundance of 20:5n-3 and 22:6n-3 in their natural diet (Ghioni *et al.* 1999, Tocher and Ghioni 1999, Tocher 2003). The majority of work in the area of LC-PUFA biosynthesis in fish has been conducted with Atlantic salmon (*Salmo salar*) but also with species such as turbot (*Psetta maxima*) (Ghioni *et al.* 1999, Zheng *et al.* 2004), gilthead sea bream (*Sparus aurata*) (Tocher and Ghioni 1999, Zheng *et al.* 2004, Fountoulaki *et al.* 2009) and Atlantic cod (*Gadus morhua*) (Tocher *et al.* 2006, Monroig *et al.* 2011). Previously our group cloned a Δ6Fads cDNA (M. Gregory and K. Schuller, unpublished data) and an Elovl5 cDNA from SBT (Gregory *et al.* 2010). Functional characterization in yeast showed that the SBT Elovl5 gene product catalysed efficient C_{18} to C_{20} and C_{20} to C_{22} conversion of both n-3 and n-6 fatty acids but the SBT Δ6Fads cDNA did not code for a functional protein (Gregory *et al.* 2010). In contrast, Morais et al. (2011) successfully cloned and functionally characterised a Δ6Fads cDNA from Atlantic bluefin tuna (*Thunnus thynnus*) and showed that the gene product desaturated approximately 32% of the supplied 18:3 n-3 to 18:4 n-3 (Morais *et al.* 2011). Thus it appears that tunas do have a functional Δ6Fads. In mammals, the Δ6Fads is thought to catalyse the rate limiting step in LC-PUFA biosynthesis (de Antueno *et al.* 2001). However, this is apparently not always the case for fish. For example, Tocher et al. (1989) found that conversion of 18:3n-3 to 20:5n-3 was limited by Elovl5 rather than Δ5 or Δ6Fads in a turbot cell line. These results were confirmed by Ghioni et al. (1999) who found that a cell line from turbot showed limited Elovl5 activity, whereas a cell line from Atlantic salmon showed

limited Δ5 and possibly Δ6Fads activity. The inter-species variability in this pathway illustrates the requirement for species-specific investigations of *de novo* LC-PUFA biosynthesis. Furthermore, these cell lines were obtained from different fish tissues, with the turbot cells originating from fin tissue (Tocher *et al.* 1988) and the Atlantic salmon cell line originating from a mixture of heart, liver, kidney and spleen tissue (Nicholson and Byrne 1973). The expression of Fads and Elovl genes varies between different tissues and reflect the higher capacity for tissues such as liver and intestine for fatty acid biosynthesis (Tocher *et al.* 2006). Thus it is also likely that the different tissues of origin contributed to the differences in the capacity of these cell lines for *de novo* LC-PUFA biosynthesis. It is assumed that tunas would have limited capacity for endogenous production of LC-PUFA from C_{18} PUFA, due to the abundance of LC-PUFA in their natural diet (Mourente and Tocher 2009). Furthermore, since both Elovl5 and Δ6Fads have been isolated from bluefin tunas, and shown to efficiently elongate and desaturate fatty acids, respectively, it may be predicted that the rate-limiting step is catalysed by either Elovl2 or Δ5Fads, though this has not been tested.

1.4.2 Fatty acid β-oxidation

The second point to consider on the subject of fatty acid metabolism is the utilisation of the fatty acids for energy through β-oxidation. Fatty acids have been shown to increase mitochondrial proliferation and increase fatty acid β-oxidation by inducing expression of certain members of the peroxisome proliferator activator receptor (PPAR) family of transcription factors (Schoonjans *et al.* 1996, Torstensen and Stubhaug 2004). Interestingly, another study found that 22:6n-3 was β-oxidised at a greater rate than either 18:2n-6 or 18:3n-3 in the red (slow-twitch, aerobic) muscle of rainbow trout (*Oncorhynchus mykiss*) (Kiessling and Kiessling 1993). In contrast, hepatocytes from Atlantic salmon showed higher levels of 18:3n-3 β-oxidation

compared to 16:0 and 18:1n-9, but not 18:2n-6, 20:5n-3 or 22:6n-3 (Torstensen and Stubhaug 2004). These studies demonstrate that there are substantial differences between different species, and also that there can be differences in substrate preference for β-oxidation in different tissues.

1.5 Fatty acid β-oxidation and red muscle endothermy in tunas

Tunas are unusual amongst fishes in that certain of their tissues (i.e. their red muscle, eye, brain and viscera) are endothermic (Graham and Dickson 2004). This capacity to heat some, but not all tissues above ambient temperature is known as regional endothermy. In tunas, as in all vertebrates, red muscle metabolism is fuelled by the aerobic oxidation of fatty acids in the mitochondria, a process known as β-oxidation (Graham and Dickson 2004). Thus, the influence of fatty acids on mitochondrial abundance, function and specifically β-oxidation is an especially interesting subject to investigate in tunas. Interestingly, tuna red muscle is endothermic whereas their white (fast-twitch, glycolytic) muscle is not (Block *et al.* 1993, Altringham and Block 1997, Dickson and Graham 2004). It is proposed that continuous swimming, which is characteristic of tunas, necessitates constant contraction and relaxation of red muscle tissue which produces heat (Dickson and Graham 2004, Graham and Dickson 2004). The heat generated by the contraction of the red muscle is a by-product of two major inefficiencies. Firstly, there is inefficiency in the coupling of metabolic processes, such as the β-oxidation of fatty acids, to ATP synthesis in the muscle mitochondria and secondly in the coupling of ATP hydrolysis to muscle contraction (Brand 2000, Jubrias *et al.* 2008). Both of these processes contribute to the heat production that is necessary for endothermy in tuna red muscle. The constant contraction and relaxation of the red muscle in tunas requires a substantial amount of energy and, by extension, a high metabolic rate. Moyes *et al.* (1992) found that in

skipjack tuna (*Katsuwonas pelamis*), both the volume of mitochondria per volume of tissue and the surface area of the inner mitochondrial membrane per cubic centimetre of mitochondrial volume were significantly higher in red muscle compared to white muscle. The difference in these parameters translated to a >28 fold higher mitochondrial membrane surface area per cm^3 of muscle fiber in red muscle compared to white muscle. This physiology allows for an extremely high metabolic rate. In addition, high concentrations of myoglobin, a protein which increases the diffusion of oxygen into the cells, have been observed in tuna red muscle and this is also believed to be associated with the high respiratory capacity of the mitochondria in the red muscle tissue (Brill 1996, Kubo *et al.* 2008). The combination of these factors means that in red muscle the maximum metabolic rate is significantly higher in tunas than closely related ectothermic fishes (Dickson and Graham 2004). A further physiological adaptation that affords tuna red muscle the capacity for endothermy is that the heat release associated with the constant activity in tuna red muscle is conserved due to the medial location of their red muscle and the presence of vascular counter-current heat exchangers (*retia mirabilia*) between the gills and the red muscle (Dickson and Graham 2004, Graham and Dickson 2004, Sepulveda *et al.* 2007). The counter-current heat exchangers are a web of closely packed arteries and veins which enter and exit the red muscle, respectively (Cech *et al.* 1984). Heat is transferred from the warm, oxygen poor blood in the veins to the cold, oxygen rich blood entering the arteries from the gills, thus minimising heat loss when gas exchange occurs with the cold sea water in the gills (Dewar *et al.* 1994). This process is very efficient in tunas and conserves the majority of the heat produced in the red muscle tissue (Cech *et al.* 1984, Dewar *et al.* 1994). Both the muscle localisation and the adaptations to conserve heat in the red muscle differ from ectothermic scombrids, which exhibit a more lateral localisation of red muscle and lack the *retia* that reduce

heat loss (Block *et al.* 1993, Dickson and Graham 2004, Graham and Dickson 2004, Kubo *et al.* 2008).

Although adult tuna have the capacity for endothermy, larval tuna do not (Kubo *et al.* 2008). Although it has not been directly tested in SBT, a study in the closely related PBT showed that the red muscle of fish with a fork length of 55 cm was 2.6-4.4°C above the ambient water temperature, suggesting that at least some capacity for endothermy had developed by that size (Kubo *et al.* 2008). Fish of smaller sizes did not show muscle temperature elevation. Studies in SBT have shown that they reach a fork length of 55 cm during their first year of life, during their migration down the west coast of Australia from the warm waters off the coast of Java to the cold waters of the Great Australian Bight (Farley and Davis 1998, Farley *et al.* 2007, Gunn *et al.* 2008). Therefore, if SBT follow a similar developmental process to PBT, they would develop the capacity for endothermy during this migration. It is reasonable, therefore, to propose that the development of endothermy in juvenile SBT allows them to survive in the cold waters south of Australia. The effects of dietary fatty acids on mitochondrial abundance, β-oxidation and respiratory capacity could indicate a role for fatty acids in the development of endothermy (discussed in more detail in Section 1.6 below). Interestingly, it has been proposed that the most significant contributor to the large mortalities faced by juvenile tunas bred in captivity is the fact that the juvenile fish succumb to the cold water temperatures experienced in the sea cages after these fish have been transferred out of the warmer waters of the hatchery (Tsuda *et al.* 2012, CST, 2012). Therefore, an understanding of the development of endothermy in tunas and an assessment of whether this capability affects the survival of juveniles in colder waters, is clearly needed.

1.6 Is endothermy in tunas under transcriptional control?

1.6.1 Peroxisome proliferator activator receptors (PPARs)

The PPAR family comprises three members, termed PPARα, PPARγ and PPARβ/δ. PPARs modulate gene expression by forming a heterodimer with the retinoid X receptor (RXR) and binding to a peroxisome proliferator responsive element (PPRE) in the promotor regions of their target genes (Leaver *et al.* 2005, Barrera *et al.* 2008). The PPAR family of transcription factors regulates genes involved in lipid, fatty acid and carbohydrate metabolism as well as inflammation and regulation of the cell cycle (Barrera *et al.* 2008). For example, PPARα has been shown to stimulate gene expression of muscle-type carnitine palmitoyltransferase 1 (CPT-1β) (Baldán *et al.* 2004). This gene codes for a protein that allows fatty acid transport into the mitochondria in the initial and regulatory step of mitochondrial fatty acid β-oxidation (Baldán *et al.* 2004). PPARα is also strongly expressed in the red muscle of sea bream (*Sparus aurata*) (Leaver *et al.* 2005) and thus may support the aerobic metabolism in that tissue that is fuelled by fatty acid β-oxidation. PPARγ is strongly expressed in preadipocytes and has a widely recognised role in adipogenesis (Schoonjans *et al.* 1996, Guan *et al.* 2005). The PPARδ gene is expressed in early embryogenesis and, in mice, its expression precedes the expression of PPARα and PPARγ by a number of days (Kliewer *et al.* 1994). Mouse knockouts of PPARδ are nearly always embryo-lethal and it has been proposed that PPARδ may be a central regulator of fatty acid catabolism in muscle and adipose tissue (Luquet *et al.* 2005).

1.6.2 Peroxisome proliferator activator receptor γ co-activator 1α (PGC-1α)

PGC-1α was first discovered in mammals by Puigserver *et al.* (1998) as a result of research to determine the mechanism of regulation of adaptive thermogenesis. Thermogenesis is the ability to heat the body using metabolic means and adaptive

thermogenesis is the ability to regulate this process. Puigserver *et al.* (1998) investigated the regulation of uncoupling proteins (UCP), specifically UCP1. This protein uncouples electron transport from ATP synthesis in mammalian brown fat by increasing the permeability of the inner mitochondrial membrane to protons. Thus, the energy from the proton gradient is released as heat instead of being utilised for ATP synthesis. As a result, PGC-1α was discovered to be involved in the upregulation of UCP1 expression in response to cold temperatures (Puigserver *et al.* 1998). As its name suggests, PGC-1α does not bind DNA directly but rather co-activates other transcription factors that have DNA binding capability. The name PGC-1α was given as it was initially discovered through its co-activation of a PPARγ/RXR heterodimer in mammalian brown adipose tissue (Sears *et al.* 1996, Puigserver *et al.* 1998, Liu and Lin 2011). Interestingly, one of the defining characteristics of brown adipose tissue is the high number of mitochondria, which allow for substantial heat production through aerobic fatty acid oxidation, which is uncoupled from ATP synthesis (Sears *et al.* 1996). This is the basis of thermogenesis in the brown fat of human babies. Soon after its discovery, PGC-1α was found to also interact with nuclear respiratory factor 1 and 2 (NRF-1 and NRF-2, respectively) (Fig. 1.2, Wu *et al.* 1999). NRF-1 and NRF-2 are involved in mediating the expression of genes encoding proteins involved in the respiratory electron transport chain and regulating the balance between nuclear and mitochondrial gene expression required for mitochondrial biogenesis (Tiranti *et al.* 1995, Scarpulla 1997). Indeed, PGC-1α has been shown to play a central role in stimulating mitochondrial biogenesis in mammals (Moyes 2003, Liu and Lin 2011). Importantly, the interaction of PGC-1α with NRF-1 and NRF-2 has been shown to stimulate mitochondrial biogenesis in muscle tissue which, in turn, increases the ability for this tissue to β-oxidise fatty acids (Wu *et al.* 1999, Miura *et al.* 2008, Scarpulla *et al.* 2012). These

data are supported by transactivation studies which have shown that the activation of the CPT-1β promotor by PPARα was significantly increased in the presence of PGC-1α (Baldán *et al.* 2004) and thus PGC-1α has the potential to increase fatty acid β-oxidation. Further research revealed the PGC-1α also interacts with estrogen-related receptor α (ERRα), thyroid hormone receptor β (TRβ) and myocyte enhancer factor 2 (MEF2) (Fig. 1.2, Lin *et al.* 2005). Like NRF-1 and NRF-2, ERRα is associated with the transcriptional control of mitochondrial biogenesis (Schreiber *et al.* 2004, Mirebeau-Prunier *et al.* 2010). TRβ is one of a number of receptors that bind to sequences of DNA known as thyroid hormone (T_3) response elements (TREs) in the promoter region of various genes (Zhang and Lazar 2000). The binding of TRβ to these TREs either co-activates, or co-represses gene expression, with co-activation occurring when the T_3 ligand is bound to the receptor and co-repression occurring when the T_3 ligand is not bound to the receptor (Zhang and Lazar 2000). The presence of T_3 has been shown to promote mitochondrial biogenesis, increase both synthesis and degradation of fatty acids and lipids and control glucose homeostasis (Blennemann *et al.* 1992, Pucci *et al.* 2000, Chidakel *et al.* 2005). MEF2 is involved in muscle development and in coordinating the expression of muscle-specific transcripts (Yu *et al.* 1992, Black and Olson 1998). Furthermore, it has been shown to play a role in glucose utilisation through binding to the promoter of the glucose transporter 4 (GLUT4) gene whose protein product is required for glucose import into muscle cells (McGee and Hargreaves 2004). The co-activation of each of these transcription factors by PGC-1α demonstrates the critical role that PGC-1α plays in the regulation of mitochondrial abundance, metabolism and energy production.

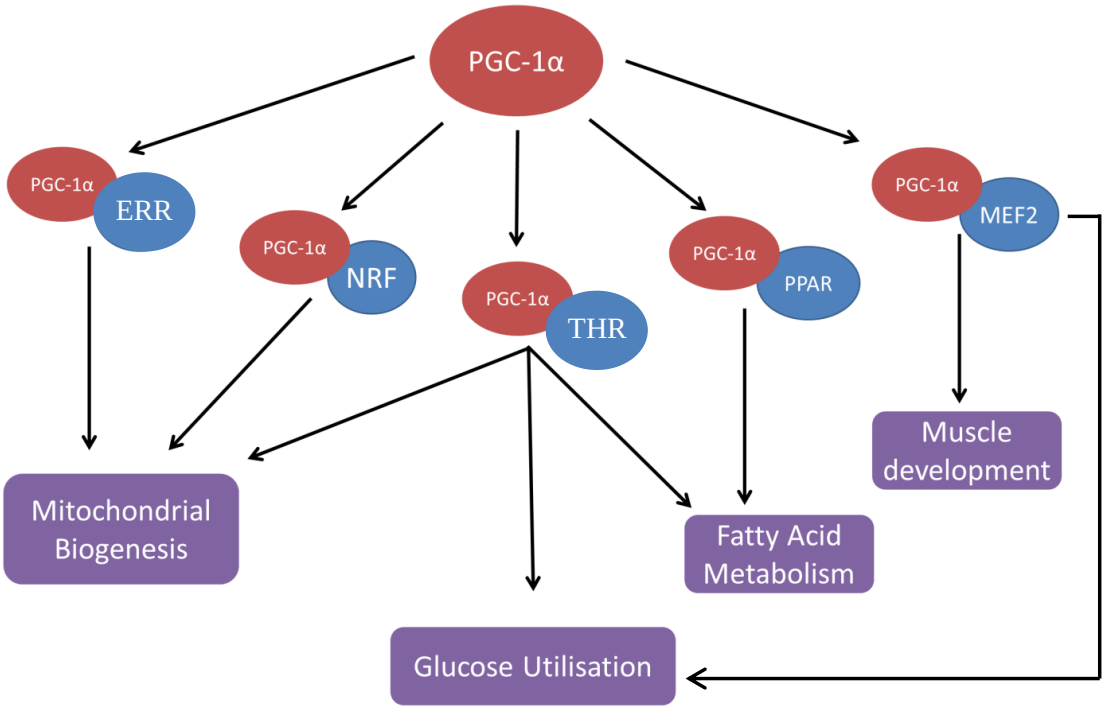


Figure 1.2: The transcriptional activation targets of PGC-1α and their downstream effects. ERR: Estrogen-related receptor, NRF: nuclear respiratory factor, THR: thyroid hormone receptor, PPAR: peroxisome proliferator activator receptor, MEF2: Myocyte enhancer factor 2. Figure kindly provided by Mr Arif Malik, Flinders University.

Since PGC-1α was discovered, two additional members of the PGC-1 family have been described, termed PGC-1β (Lin *et al.* 2002) and PGC related co-activator (PRC) (Andersson and Scarpulla 2001). Of these three proteins, PGC-1α has been the most extensively studied and is the member that is reported on in this thesis in light of its potential regulatory role in mitochondrial β-oxidation and its consequent potential involvement in red muscle endothermy in tunas. During embryogenesis, mammals, like juvenile SBT, are ectothermic It has been proposed that metabolic acclimation in ectotherms and adaptive thermogenesis in endotherms are regulated by a similar suite of regulatory genes (Seebacher 2009). PGC-1α gene expression has been shown to be sensitive to temperature changes in fish (LeMoine *et al.* 2008, Orczewska *et al.* 2010, Bremer *et al.* 2012) and is associated with changes in metabolic capacity leading to endothermy in birds (Walter and Seebacher 2007). Furthermore, there is substantial homology in several of the functional domains between mammalian and fish sequences of PGC-1α (LeMoine *et al.* 2010b). However, the role of PGC-1α in endothermy in fish has not yet been investigated. The majority of research on PGC-1α has been conducted with small model species such as zebrafish and goldfish which are ectothermic (LeMoine *et al.* 2008, LeMoine *et al.* 2010a, Bremer *et al.* 2012).

1.6.3 PGC-1α and mitochondrial biogenesis in fish

Whilst the central role of PGC-1α in controlling mitochondrial biogenesis and metabolism in mammals is well established (Moyes 2003, Miura *et al.* 2008, Liu and Lin 2011), work to define the role of PGC-1α in fish to date has focused on temperature acclimation and exercise in a few ectothermic species (LeMoine *et al.* 2008, LeMoine *et al.* 2010a, Orczewska *et al.* 2010, Bremer *et al.* 2012). Several studies have shown that cold acclimation increases the activity of mitochondrial

enzymes, the number of mitochondria per cell (as indicated by an increase in the activity of mitochondrial enzymes per gram muscle tissue), and β-oxidation capacity in several ectothermic fish species (Rodnick and Sidell 1994, Guderley and Johnston 1996, O'Brien 2011). However, the role of PGC-1α in regulating this process appears to be different in fish, than it is in mammals. For example, in goldfish (*Carassius auratus*), PGC-1α showed temperature-dependent gene expression in both red and white muscle, with significantly higher expression in fish reared at warmer temperatures (LeMoine *et al.* 2008). A similar study found a decrease in PGC-1α gene expression in white muscle, but not red muscle, during cold acclimation (4°C) in goldfish (Bremer *et al.* 2012). These findings were unexpected as PGC-1α was expected to stimulate mitochondrial proliferation in response to cold. Interestingly, PGC-1α gene expression has been shown to increase in the liver of goldfish reared at either warmer (35°C) or colder (4°C) temperatures, compared to the fish reared at a standard (20°C) temperature (LeMoine *et al.* 2008). This suggests that the changes in PGC-1α gene expression in these studies may have been part of a stress response to temperatures outside a normal range. In contrast, Orczewska *et al.* (2010) found no significant changes in PGC-1α gene expression in the red muscle or liver of three-spine stickleback (*Gasterosteus aculeatus*) during cold acclimation (8°C) compared to fish reared at a standard temperature (20°C). In addition, Bremer and Moyes (2011) found substantial inter-species variability in the enzyme activity of cytochrome *c* oxidase (COX), an indicator of mitochondrial abundance, and the gene expression of NRF-1, NRF-2 and PGC-1α in response to seasonal temperature variations. For example, the majority of species showed an increase in COX enzyme activity in winter but most species showed a decrease in PGC-1α gene expression in winter (Bremer and Moyes 2011). Furthermore, there was a lack of correlation between PGC-1α gene expression and NRF-1 and NRF-2 gene expression (Bremer

and Moyes 2011). The inter-species variability in the response of PGC-1α to cold acclimation in fish, combined with the fact that PGC-1α-induced mitochondrial proliferation has been clearly shown during cold acclimation in mammals, indicates that PGC-1α may not control mitochondrial proliferation in fish in the same manner as it does in mammals (Liang and Ward 2006, Ihsan *et al.* 2014). This is supported by LeMoine *et al.* (2010b) who found that fish species exhibited a higher rate of evolution in the PGC-1α gene when compared to mammalian species, suggesting that there may be substantial differences in the role of PGC-1α between fish and mammals. Although the role of PGC-1α in fish is less clear, there is a large amount of variability between species and there is still much that is unknown about its role in metabolic acclimation in response to thermal cues.

1.6.4 Is there a role for PGC-1α in red muscle endothermy in tunas?

The endothermic red muscle of tunas is characterised by having greater activities of mitochondrial enzymes per gram tissue compared with the ectothermic white muscle and this indicates that mitochondria are substantially more abundant in tuna red muscle than white (Moyes *et al.* 1992). Thus, it would be expected that PGC-1α gene expression would be substantially greater in tuna red muscle than white; however to date this has not been investigated.

1.7 Indicators of mitochondrial abundance and heat generation capacity

1.7.1 Citrate synthase (CS)

Citrate synthase (CS) is a nuclear encoded enzyme that is located in the mitochondrial matrix and is responsible for the condensation of acetyl CoA with oxaloacetate to form citrate in the initial and rate-limiting step of the citric acid cycle (Alp *et al.* 1976, Wiegand and Remington 1986). It is only found in mitochondria

and therefore it is an excellent candidate for the assessment of mitochondrial abundance and has been extensively used in this capacity (Moyes *et al.* 1992, Dickson and Graham 2004, Dalziel *et al.* 2005). For example, Moyes *et al.* (1992) found higher CS enzyme activity in the red muscle of both skipjack tuna (*Katsuwonus pelamis*) and common carp (*Cyprinus carpio*) compared to white muscle indicating greater abundance of mitochondria in the red muscle than the white. In addition, the activity of CPT, which is required for fatty acid transport into the mitochondria for β-oxidation, was also higher in red muscle of both species (Moyes *et al.* 1992). These results suggested that red muscle had a greater number of mitochondria and thus a greater capacity to utilise fatty acids to fuel oxidative metabolism, than white muscle. The endothermic capability of red muscle in tunas is made possible through the greater number of mitochondria in this tissue (Altringham and Block 1997, Dickson and Graham 2004). Thus, the transcription factors that stimulate mitochondrial biogenesis may play a role in endothermy in SBT. This suggests that PGC-1 gene expression should be greater in tuna red muscle than white.

1.7.2 Cytochrome c oxidase subunit 1 (COX1)

A second indicator of mitochondrial abundance and function is the cytochrome *c* oxidase subunit 1 (COX1) enzyme. The gene encoding this enzyme is located in the mitochondrial genome and encodes the largest core subunit of the cytochrome *c* oxidase (also known a complex IV), the final complex in the mitochondrial respiratory electron transport chain, which is located in the inner mitochondrial membrane (Khalimonchuk and Rödel 2005). Complex IV is responsible for the reduction of O_2 to H_2O and the pumping of protons across the inner mitochondrial membrane in the final step of the chain (Poyton *et al.* 1988, Wikström *et al.* 1997,

Khalimonchuk and Rödel 2005). The gene expression of COX1 may therefore be correlated to the surface area of the inner mitochondrial membrane and thus may be used as an indicator of mitochondrial respiratory capacity. A higher surface area of the inner mitochondrial membrane is associated with more copies of the proteins that make up the complexes within that membrane and, by extension, an increase in respiratory capacity. Thus, COX1 gene expression may be used as an indicator of mitochondrial respiratory capacity, due to either a) an increase in the number of mitochondria, or b) an increase in the surface area of the inner mitochondrial membrane.

1.7.3 Uncoupling proteins (UCPs)

Uncoupling proteins (UCPs) are involved in thermogenesis and, as their name suggests, they do this through the uncoupling of respiratory electron transport from oxidative phosphorylation. The energy obtained from the respiratory electron transport chain is thus released as heat rather than being stored in the terminal phosphate bond of ATP. Five UCPs, termed UCP1-5, have been identified in mammals and have been shown to play a role in thermogenesis (Ricquier and Bouillaud 2000, Kim-Han and Dugan 2005, Ramsden *et al.* 2012). Homologs of UCP1-5 have been identified in zebrafish, although some researchers have proposed that the zebrafish homolog of UCP3 is best characterised as a UCP2-like protein due to a difference in expression patterns between the mammalian and zebrafish UCP3 (Tseng *et al.* 2011). Since zebrafish are ectothermic, the discovery of these proteins has caused some debate about whether thermogenesis is actually the primary physiological role of these proteins (Stuart *et al.* 1999, Brand and Esteves 2005). Several studies have shown, however, that UCP2 is thermo-sensitive. The expression of UCP2 is upregulated during cold acclimation in zebrafish (Tseng *et al.* 2011),

downregulated during warm acclimation in Antarctic eelpout (*Pachycara brachycephalum*) and downregulated during cold acclimation in the temperate fish species common eelpout (*Zoarces viviparus*) (Mark *et al.* 2006). In Atlantic salmon, UCP2 is expressed to a low level in white muscle, red muscle, heart and liver, but is strongly expressed in white adipose tissue and myosepta, the latter of which is located along the spinal column (Torstensen *et al.* 2009). In tuna, this region corresponds to the location of red muscle tissue (Graham and Dickson 2004). Therefore, the expression of UCP2 in this region, if it is shown to occur, could play a role in red muscle endothermy in tunas. Furthermore, since tunas are regionally endothermic, they represent an intermediate state between mammals, which are true endotherms, and other fish, which are true ectotherms. Therefore the gene expression of UCPs, especially UCP2, provides an interesting subject for investigation in SBT.

1.8 Project aims

The overall aim of this project was to gain an understanding of the regulation of fatty acid metabolism in SBT.

Specifically, this project aimed to investigate:

1. The effects of various fatty acids on the proliferation of a newly established SBT cell line, designated SBT-E1
2. Fatty acid incorporation into cellular lipids in the SBT cell line
3. *De novo* synthesis of LC-PUFA from their C_{18} precursors in the SBT cell line
4. β-oxidation of various fatty acids in the SBT cell line
5. The effects of fatty acid deprivation or supplementation on the gene expression of Δ6Fads and Elovl5, which encode enzymes involved in LC-PUFA biosynthesis in the SBT cell line

6. The expression of genes encoding PGC-1α, PPARγ, CS, COX1 and UCP2 in the SBT cell line and in the red muscle, white muscle, liver and gill of SBT

The rationale for using the cell line is that the high commercial value of SBT makes it prohibitively expensive to conduct these investigations in whole fish. Thus, the cell line can be used as a model for understanding metabolism in SBT.

It was predicted that:

1. The proliferation of the SBT-E1 cells would increase in response to fatty acid supplementation
2. The supplied fatty acids would be incorporated in approximately equal amounts into the cellular lipids of the SBT-E1 cells
3. There would be low levels of *de novo* synthesis of LC-PUFA from their C_{18} precursors in the SBT cell line. Specifically, it was expected that there would be high levels of Δ6 desaturation and Elovl5-mediated elongation but low levels of Δ5 desaturation and Elovl2-mediated elongation
4. The SBT-E1 cells would show a preference for β-oxidation of n-3 fatty acids compared to other fatty acids as there is an abundance of n-3 fatty acids in the diet of wild tunas
5. The SBT-E1 cells supplied with the LC-PUFA synthesis precursors 18:3n-3 and 18:2n-6 would show higher expression of both Δ6 desaturase and Elovl5
6. The SBT-E1 cells supplied with different fatty acids would show an increase in the expression of PGC-1α, PPARγ CS, COX1, and UCP2

7. The PGC-1α, PPARγ CS, COX1, and UCP2 transcripts would be more abundant in red muscle than white muscle

Chapter 2 – General Methods

2.1 Standard cell culture

2.1.1 Standard cell culture materials and medium

The cells were from the recently established SBT-E1 cell line and stock cultures were maintained as previously described (Bain *et al.* 2013). Cells were routinely cultured in a 25°C incubator in 25 cm^2 or 75 cm^2 Corning® cell culture flasks. The standard cell culture medium was prepared by dissolving one sachet of Leibovitz's L-15 basal cell culture medium (Gibco™) in 1 L of ultrapure deionised water. A final concentration of 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.4) (Sigma-Aldrich) was added and the medium was vacuum-filtered through a 0.22 μM polyethersulfone sterilising filter (Corning) into a sterile 0.5 or 1 L plastic flask. A final concentration of 10% (v/v) fetal bovine serum (FBS, Gibco) was added to the flask to make the standard cell culture medium.

2.1.2 Revival of cells from cryostorage

To revive cells from cryostorage, a vial was taken from liquid nitrogen storage and the contents were rapidly thawed by vigorous rubbing with hands for 2-3 minutes. One mL of the thawed cell suspension was mixed with 4 mL of fresh medium and placed into a 25 cm^2 flask. The flask was capped and placed in a 25°C incubator to allow the cells to adhere to the flask. The medium in the flask was changed after 24 hours to remove any dead cells or cell debris. The cells were then returned to the incubator and allowed to proliferate before passaging.

2.1.3 Passaging of cells

Cells were cultured to approximately 80-90% confluence before passaging. The existing medium was decanted into a waste bottle and the cell monolayer was washed 3 times with phosphate buffered saline (PBS) to remove any residual

medium. A volume of 1 or 2 mL of trypsin/ethylenediaminetetraacetic acid (EDTA) solution (TE, 0.05% (w/v) trypsin, 0.004% (w/v) EDTA in PBS) was added to each of the 25 cm^2 or 75 cm^2 flasks, respectively, to cover the entire monolayer. The caps of the flasks were replaced and the cells were incubated for 3-5 minutes before dislodging the cells by tapping the flask against a hard surface. Cell detachment was verified under a microscope before a 5-10 mL volume of standard cell culture medium was added to inhibit the trypsin reaction and to resuspend the cells. Fresh 25 cm^2 or 75 cm^2 flasks were seeded at a 1:3 split ratio in 5 mL or 15 mL of the standard cell culture medium, respectively. The fresh flasks were capped and returned to the 25°C incubator for the cells to adhere and proliferate. The cells routinely reached 80-90% confluence and were ready for passaging after approximately 4-5 days.

2.1.4 Cryopreservation of cells

For cryopreservation for long-term storage, cells were detached from a confluent 75 cm^2 flask as described in Section 2.1.2.2 and the cell suspension was transferred to a 15 mL tube. The tube was then sealed and centrifuged at 1,000 *g* for 5 min. The supernatant was decanted into a waste bottle and the cell pellet was resuspended in 3 mL of the standard cell culture medium but with 10% (v/v) dimethylsulfoxide (DMSO, Sigma-Aldrich) added. One mL of the cell suspension was transferred to each of three cryopreservation tubes. The tubes were then sealed and placed in a freezer box insulated with cotton wool. The freezer box was placed in a -80°C freezer for a minimum of 24 hours before the tubes were transferred to storage containers in liquid nitrogen.

2.2 Experimental cell culture

2.2.1 Cell culture materials and medium

The cells were cultured as described in Section 2.1 except that the FBS concentration was set at 2, 5 or 10% (v/v), depending on the experiment.

2.2.2 Counting of cells

The cells were washed and detached from the flasks using TE solution as described in Section 2.1.3. Once detached, the standard cell culture medium or the standard cell culture medium with the FBS concentration reduced to 2% or 5% (v/v) was added to inhibit the trypsin reaction. Twenty µl of the cell suspension was mixed with 20 µl of 0.4% (w/v) Trypan blue dye (Sigma-Adrich) in PBS and a small volume of the mixture was added to a haemocytometer slide that had been sterilised with 70% (v/v) ethanol. Viable cells exclude the Trypan blue dye and are not stained whereas non-viable cells stain blue. Of the nine squares on the haemocytometer slide, the number of viable cells in each of the four corner squares was counted under a phase contrast light microscope at 20X magnification and the average of the four squares was calculated. Each of the four squares measures 1 mm^2 and the solution depth is 0.1 mm so the average cell count from each square equals the number of cells per 0.1 mm^3. Since 1 mL is equal to 1 cm^3, the cell concentration in cells per mL (cm^3) was calculated by multiplying the average cell count by 10^4 (0.1 $mm^3 \times 10^4 = 1\ cm^3$). This number was then multiplied by 2 to account for the Trypan blue dye dilution factor to obtain the concentration of the cell suspension.

2.2.3 Passaging of cells

The cells were harvested from a confluent 75 cm^2 cell culture flask and resuspended in the standard cell culture medium or the standard cell culture medium but with the FBS concentration reduced to 2% (v/v) or 5% (v/v). A subsample of the cell suspension was counted as described above (Section 2.2.2) and the cells were seeded into 96, 24 or 6-well plates or 25 or 75 cm^2 flasks (depending on the experiment) at a density of 25,000 cells/cm^2. After 4 hours to allow the cells to attach, the medium was decanted and replaced with the experimental medium. The cells were then cultured and analysed using specific methods that are described in detail in the appropriate chapter.

2.2.4 Harvest of cells

At the conclusion of each experiment, the cells were washed and detached from the flasks using TE solution as described in Section 2.1.3. Once the cells had detached, the trypsin reaction was inhibited by addition of standard cell culture medium but with the FBS concentration reduced to 2% (v/v). The cell suspension was centrifuged at 1,000 *g* for 5 min and the supernatant was discarded. The cell pellet was washed with PBS and centrifuged at 1,000 *g* for 5 min and the supernatant was discarded. The washed cell pellet was then stored at -80°C or used immediately for lipid, RNA or DNA extraction.

2.3 Confirming the identity of the SBT-E1 cell line

2.3.1 The need to confirm cell line identity

In laboratories that handle multiple cell lines that proliferate under similar conditions, cross-contamination is possible (Romano *et al.* 2009). For example, in fish cell culture, Winton *et al.* (2010) found that many of the cells currently believed

to be the widely used Epithelioma papulosum cyprini (EPC) cell line, that was originally isolated from common carp (*Cyprinus carpio*) (Fijan *et al.* 1983), were from fathead minnow (FHM, *Pimephales promelas*). This highlights the importance of conducting regular tests to verify the identity of cells in culture. A common method used to genetically identify fish species was first described by Ward *et al.* (2005). This method uses the variation in sequence of the mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) gene to differentiate between different species on a genetic level. This method was used to verify the identity of the SBT-E1 cell line.

2.3.2 Determining the species of origin of the SBT-E1 cell line using polymerase chain reaction

2.3.2.1 DNA extraction and polymerase chain reaction

Genomic DNA was extracted using a DNeasy® mini kit (Qiagen) according to the manufacturer's instructions. The genomic DNA was used as the template for polymerase chain reaction (PCR) using a GoTaq® PCR master mix (Promega), according to the manufacturer's protocol together with the F1 (5´-TCAACCAACCACAAAGACATTGGCAC-3´) and R1 (5´-TAGACTTCTGGGTGGCCAAAGAATCA-3´) primers described by Ward *et al.* (2005).

2.3.2.2 Agarose gel electrophoresis

A 5 µl subsample of the PCR reaction was loaded into a gel containing 1.5% (w/v) agarose (Amresco). The gel was immersed in TAE buffer (40 mM Tris (Sigma-Aldrich), 20 mM acetic acid (BDH chemicals), 1 mM EDTA) and was stained with

SYBR® Safe DNA stain (Invitrogen). A DNA size marker (2-log DNA ladder, New England Biolabs (NEB)) was added to one lane as a reference and the PCR products were separated by electrophoresis at 80 volts for 30-40 min. The gel was photographed using a Gel Doc™ EZ imaging system (Bio-Rad) and gel photographs were annotated using Photoshop and/or Illustrator (Adobe).

2.3.2.3 PCR product purification

The remainder of the PCR product was purified using a Wizard® SV gel and PCR clean-up system (Promega) according to the manufacturer's instructions. After elution, a 2 µl subsample of the purified PCR product was used to estimate the DNA concentration using a NanoDrop 1000 (Thermo Scientific).

2.3.2.4 Sequencing of the purified PCR product

Approximately 50-100 ng of DNA was combined with 9.6 pmol of the corresponding forward or reverse primer for each PCR product and sent to the Australian Genome Research Facility (AGRF) to be sequenced using a BigDye® Terminator (BDT) v3.1 Cycle Sequencing Kit on an Applied Biosystems 3730 capillary sequencer.

2.3.2.5 Sequence analysis

The returned sequence was subjected to analysis using the basic local alignment search tool (BLAST) to confirm a match to the fragment of the reported mitochondrial genome sequence from SBT tissue corresponding to the *cox1* gene in GenBank® (Accession number NC_014101.1). The sequence returned from the PCR product of the *cox1* gene from the SBT-E1 cell genomic DNA showed a high degree

of identity with the reported sequence for *cox1* from the GenBank® database (Fig. 2.1). There were only 2 divergent bases in the SBT-E1 sequence when compared to the reported SBT *cox1* sequence, an 'A' deletion at base number 17 and a G/C substitution at base number 200. The high degree of similarity (>99%) indicates that the SBT-E1 cells originated from SBT and are not contaminated with cell lines that had previously been used in our laboratory, including primary hepatocytes from yellowtail kingfish (YTK, *Seriola lalandi*), and the EPC/FHM cell line described above.

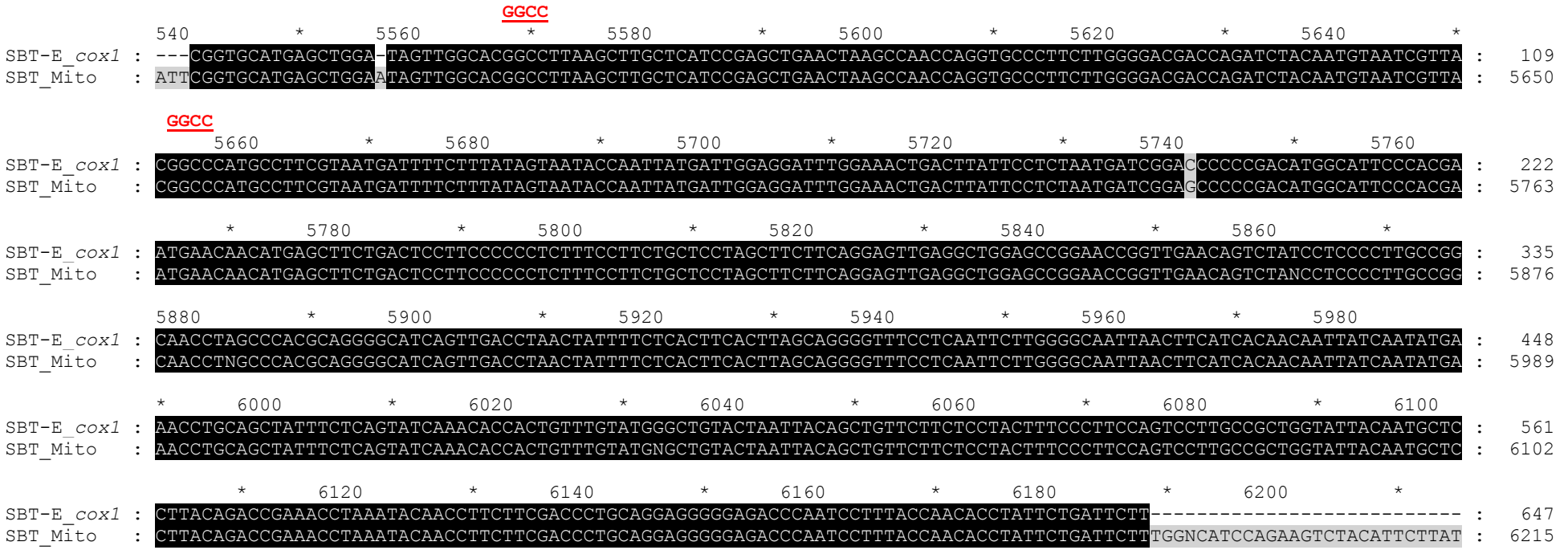


Figure 2.1: Sequence alignment of the SBT-E1 *cox1* amplicon (SBT-E_*cox1*) and a portion of the reported SBT mitochondrial genome sequence corresponding to the *cox1* gene (SBT_Mito) (NC_014101.1). The *cox1* gene sequence from the SBT-E1 cells contained a single base deletion at base 5557 and a G/C substitution at base 5741.

2.3.3 Determining the species of origin of the SBT-E1 cells using restriction enzyme digestion of the cox1 PCR product

A quick, alternative method of species identification was developed for the SBT-E1 cells (Dr P.A. Bain, unpublished data). This method used the *cox1* PCR product, generated as described in Section 2.3.2.1. The PCR product was subjected to digestion using a *Hae*III restriction enzyme (NEB), according to the manufacturer's protocol. The digestion of the SBT *cox1* amplicon with *Hae*III would result in three fragments with sizes of 28, 85 and 534 base pairs, respectively (Fig. 2.1). The genomic DNA, the PCR products and the PCR products that had been digested with *Hae*III were separated by electrophoresis as described in Section 2.3.2.2. Sequence data for SBT, YTK, common carp and fathead minnow were obtained from the National Centre for Biotechnology Information (NCBI) Nucleotide database to compare the expected fragment size for each species to the fragments obtained from the *Hae*III digestion. A summary of these sequences, including indications of *Hae*III recognition sites (GGCC), is shown in Fig. 2.2. Each of these species would yield a PCR product of a similar size when amplified using the method described in Section 2.3.1. However, digestion of the PCR products with *Hae*III would yield unique fragment lengths (Fig. 2.2). The results of PCR amplification of the *cox1* gene from the SBT-E1 cells and the subsequent digestion of this product with *Hae*III are shown in Fig. 2.3. The *cox1* gene showed good amplification (Fig. 2.3, Lane 6) and digestion with *Hae*III showed two bands of approximately 550 base pairs (bp) and 100 bp, respectively (Fig. 2.3, lane 7) which indicates that the SBT-E1 cells are indeed likely to be of SBT origin and are not contaminated with cells from another cell line. The other samples shown in Fig. 2.3 were from the same experiment conducted on an alternate morphology of the SBT cell line, designated SBT-F, which is not reported on in this thesis.

Figure 2.2: Sequence alignment of *cox1* genomic DNA sequences from yellowtail kingfish (YTK, *Seriola lalandi*, DQ521001.1), common carp (CC, *Cyprinus carpio*, EU417775.1) and fathead minnow (FHM, *Pimephales promelas*, JX516786.1). Each sequence contained one GGCC recognition site for the *Hae*III restriction enzyme, which is indicated by the bars in red (CC), green (FHM) or blue (YTK). Based on these sequence data, digestion of the *cox1* amplicon with *Hae*II would result in two fragments for YTK (304 bp and 336 bp) and CC (65 bp and 590 bp) and three fragments for FHM (6 bp, 308 bp and 338 bp).

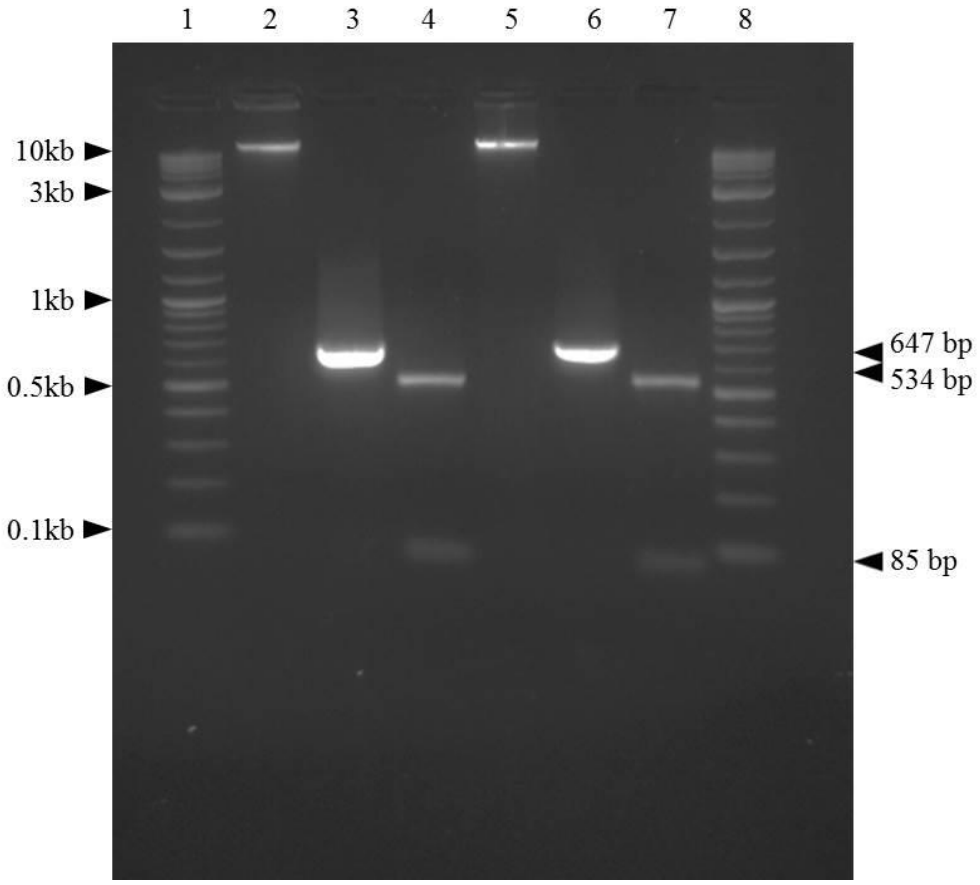


Figure 2.3: Verification of the species of origin of the SBT-E1 cell line using a *Hae*III restriction enzyme digest of a 647 bp amplicon of the cytochrome *c* oxidase subunit 1 (*cox1*) gene. Lane 1: DNA size marker (NEB); Lane 2: genomic DNA from the SBT-F cell line; Lane 3: *cox1* PCR product from SBT-F genomic DNA; Lane 4: *Hae*III digestion of the *cox1* PCR product from SBT-F genomic DNA; Lane 5: genomic DNA from the SBT-E1 cell line; Lane 6: *cox1* PCR product from SBT-E1 genomic DNA; Lane 7: *Hae*III digestion of the *cox1* PCR product from the SBT-E1 genomic DNA; Lane 8: DNA size marker (NEB).

2.4 Fatty acid incorporation and metabolism by the SBT-E1 cells

2.4.1 PUFA supplementation of the SBT-E1 cells

Purified 18:3n-3, 18:2n-6, 20:5n-3, 20:4n-6 and 22:6n-3 were obtained from Cayman Chemical Company in solution in ethanol. Cell culture tested Vitamin E ((+)-α-Tocopherol) was obtained from Sigma-Aldrich in an oil solution. The solutions were diluted in 200-proof molecular biology grade ethanol (Sigma-Adrich) to a concentration of 10 mg/mL for the PUFA or 0.2 M for the vitamin E. To prepare the PUFA-supplemented media, the molar concentrations of the 10 mg/mL PUFA stock solutions were calculated using the molecular weights of the fatty acids and then the appropriate volume of each solution was mixed with 10 mM bovine serum albumin (BSA, Sigma-Aldrich) at a molar ratio of 2:1 (PUFA:BSA) before mixing with the culture medium at a final concentration of 0-20 µM. Vitamin E supplemented media were prepared by adding the diluted vitamin E solution to the PUFA supplemented media to a final concentration of 0 to 2 mM. Control medium was prepared using pure ethanol as a vehicle control in place of the PUFA and/or vitamin E solutions. The cells were passaged into 96, 24 or 6-well plates or 25 or 75 cm^2 flasks (depending on the experiment) in standard cell culture medium (Section 2.1.1) but with the FBS concentration set at either 2, 5 or 10% (v/v) FBS according to the protocols described in Section 2.2. After the cells had adhered to the surface of the plate or flask, the medium was decanted and replaced with the PUFA and/or vitamin E supplemented medium.

2.4.2 Fatty acid profiles of the total lipid from the SBT-E1 cells

The cells were cultured under the experimental cell culture conditions (Section 2.2) in standard cell culture medium with the following modifications. The FBS concentration was set at either 10% (v/v) or 2% (v/v), with fatty acids and/or vitamin

E added as described in Section 2.4.1. The cells were harvested as described in Section 2.2.4 and total lipid was extracted from the cells and fatty acid methyl esters (FAME) were produced using a chloroform/isopropanol lipid extraction method and a sulphuric acid-mediated fatty acid methylation method as previously described (Gregory *et al.* 2010). The FAME were analysed as previously described (Gregory *et al.* 2010). Briefly, the FAME were analysed using a Hewlett-Packard 6890 gas chromatograph (GC) with a 50 m capillary column. The injector and detector temperatures were set to 250^{o}C and 300^{o}C, respectively. The initial oven temperature was 140^{o}C and rose to 220^{o}C at a rate of 5^{o}C per minute before a 3 minute hold at 220^{o}C. Individual FAME were identified by comparison to a reference mixture of known standards (GLC-463, NuCheck Prep Inc.).

2.4.3 Lipid class composition of the total lipid from the SBT-E1 cells

The SBT-E1 cells were cultured under standard cell culture conditions (Section 2.1) and harvested as described in Section 2.2.4. Total lipid was extracted in chloroform/methanol (2:1, v/v) (Merck) containing 0.05% (w/v) butylated hydroxytoluene (BHT) (Tocher *et al.* 1988). Lipid classes were separated by high performance-thin layer chromatography (HPTLC) as previously described (Bell *et al.* 1993) and individual classes were visualised by charring at 160^{o}C for 15 min after the HPTLC plate had been sprayed with 3% (w/v) copper acetate (Sigma-Aldrich) in 8% (v/v) phosphoric acid (Merck). The relative abundance of each lipid class was determined by scanning densitometry using a ChemiDoc^{TM} MP imaging system (Bio-Rad). The identity of each lipid class was determined by running authentic standards in lanes next to those containing the SBT-E1 lipid extracts.

2.4.4 Fatty acid profiles of individual phospholipid classes from the SBT-E1 cells

The SBT-E1 cells were cultured under standard cell culture conditions (Section 2.1) and harvested as described in Section 2.2.4. Total lipid was extracted from the SBT-E1 cells and the different classes of lipids were separated as described in Section 2.4.3. The separated lipids were visualised by spraying the plates with fluorescein 5-isothiocyanate in methanol and the areas of silica gel corresponding to the different lipid classes were scraped from the plates into separate vials. FAME were produced from the different lipid classes and analysed as described in Section 2.4.2.

2.4.5 Fatty acid composition of the total lipid and the phospholipids from SBT tissues

Tissue samples were obtained from SBT specimens with fork lengths of 114–130 cm during a normal commercial harvest from a tuna farm located near Port Lincoln, South Australia. The fish were gilled and gutted and the tail was removed. Samples of the tail muscle, liver, intestine and kidney were frozen on dry ice. Upon return to the laboratory, total lipid was extracted from the tissues using chloroform/methanol (2:1 v/v) containing 0.05% (w/v) butylated hydroxyanisole using the method described by Bligh and Dyer (1959) with some modifications as described by Makrides et al. (1996). Subsamples of the total lipid were separated into polar lipids (mostly phospholipids) and neutral lipids using thin layer chromatography (TLC) (Pahl et al., 2010). Briefly, the subsamples were dried under a stream of nitrogen, reconstituted in 150 μL of chloroform/methanol (9:1 v/v) and then applied in streaks to TLC plates. The plates were developed in petroleum spirit/diethyl ether/acetic acid (180/30/2 by volume) and the separated lipid classes were visualised by drying the plates, spraying them with fluorescein 5-isothiocyanate in methanol and then exposing them to UV light. The bands of silica gel corresponding to the

phospholipids were scraped into glass vials and FAME were produced and analysed as described in Section 2.4.2.

2.4.6 Esterification of [1-^{14}C]-labelled fatty acids into individual lipid classes in the SBT-E1 cells

The ^{14}C-labelled fatty acids were purchased from Perkin Elmer NEN® (Perkin Elmer, Cambridge, UK) at a specific activity of 50-55 mCi mmol^{-1}. Complexes of the labelled fatty acids with fatty acid free-bovine serum albumin (FAF-BSA) in Hanks' Balanced Salt Solution (HBSS) were prepared as described previously (Ghioni *et al.* 1999). To label the cells, they were cultured in the standard culture medium, harvested and resuspended in the same medium but with the FBS concentration reduced to 5% (v/v). The resuspended cells were seeded at a density of 25,000 cells cm^{-2} into the required number of 75 cm^2 cell culture flasks in a final volume of 15 mL of the culture medium with 5% (v/v) FBS. Once the cells had adhered to the flasks (after approximately 4 h), 0.25 μCi (0.33 μM) of each [1-^{14}C]- labelled fatty acid complexed with FAF-BSA was added to the appropriate flasks and the cells were incubated at 25°C for 72 h.

The cells that had been incubated with [1-^{14}C]-labelled fatty acids were detached from the flasks as described in Section 2.2.4. The detached cells were pelleted by centrifugation and then washed with 1% (w/v) FAF-BSA in HBSS to remove any residual [1-^{14}C]-labelled fatty acids. Total lipid was extracted as described above and the different classes of lipids were separated on HPTLC plates developed in methyl acetate/isopropanol/chloroform/ methanol/0.25% (w/v) aqueous KCl (25/25/25/10/9, by volume) as described previously (Vitello and Zanetta 1978). The separated lipids were visualised by placing the plate in a tank saturated with iodine vapour. The areas

of silica gel corresponding to the different classes of lipids were scraped from the plate into separate vials and radioactivity was assayed using a TRI-CARB 2000CA scintillation counter (United Technologies Packard, Pangbourne, UK). The transformed spectral index of external standard (tSIE) value was used as a measure of quenching. This value was used to calculate the counting efficiency according to the equation, efficiency = (tSIE × 0.033) + 56 based on a quenching calibration curve. This efficiency value was then used to convert counts per minute (cpm) to disintegrations per minute (dpm) using the equation dpm = (cpm / efficiency) × 100.

2.4.7 Metabolism of incorporated [1-^{14}C]-labelled fatty acids by desaturation and/or elongation in the SBT-E1 cells

The cells were incubated with [1-^{14}C]-labelled fatty acids as described in Section 2.4.6 and total lipid was extracted as described in Section 2.4.3. FAME were then produced by incubating the total lipid with 1% (v/v) H_2SO_4 in methanol at 55 °C for 16 h (Christie 1993). The FAME were extracted as described previously (Tocher and Harvie, 1988) and dissolved in 100 μL of hexane containing 0.01% (w/v) BHT. The dissolved FAME were separated on a TLC plate (20 × 20 × 0.25 cm) impregnated with 10% (w/v) $AgNO_3$ in acetonitrile as described previously (Ghioni *et al.* 1999). Areas of silica gel corresponding to individual FAME were detected by autoradiography (Kodak MR2 X-ray film, 16 days exposure at room temperature), scraped from the plate and assayed for radioactivity as described in Section 2.4.5.

2.4.8 β-oxidation of [1-^{14}C]-labelled fatty acids in the SBT-E1 cells

Peroxisomal and mitochondrial β-oxidation of [1-^{14}C]-labelled fatty acids was determined essentially as described previously but with some modifications (Tocher *et al.* 2004). The cells were incubated with [1-^{14}C]-labelled fatty acids as described in

Section 2.4.5 and at the end of the incubation, a 0.5 mL subsample of the medium was taken and set aside. The remainder of the medium was discarded, the cell monolayer was washed with PBS and the cells were detached with TE solution (Section 2.2.4). The isolated cells were washed in HBSS containing 1% (w/v) FAF-BSA before being resuspended in HBSS alone and homogenised using an ULTRA-TURRAX® (IKA®) mechanical homogeniser. A subsample (0.5 mL) of the homogenate was taken and set aside. One-hundred microlitres of 6% (w/v) FAF-BSA in water was added to the subsamples of both the culture medium and the homogenised cells before acid-insoluble products were precipitated by the addition of 1.0 mL of ice-cold 4 M $HClO_4$. The samples were centrifuged at 3,500 *g* for 10 min and 0.5 mL of the supernatant was mixed with 4 mL of scintillation fluid and radioactivity was assayed as described in Section 2.4.6. The data for the culture medium and the cells were combined to give the rate of β-oxidation per 10^6 cells.

2.5 Validation of primers for use in quantitative real-time PCR (qRT-PCR)

2.5.1 Harvest of SBT-E1 cells

The SBT-E1 cells were cultured under standard culture conditions (Section 2.1) and harvested as described in Section 2.2.4.

2.5.2 Harvest of liver tissue from a farmed SBT

Liver tissue was collected from a farmed SBT during a commercial harvest from a sea cage off the coast of Port Lincoln, South Australia in June 2014. The tissue was placed in RNA*later*® at ambient temperature before the tissue was transported back to Flinders University and stored at -20°C.

2.5.3 Primer design for qRT-PCR

A search of the National Centre for Biotechnology Information (NCBI) Nucleotide database was conducted for β-actin, elongation factor 1α (ELF-1α), Δ6Fads, Elovl5, PGC-1α, PPARγ, CS, COX1 and UCP2 cDNA sequences. Full or partial cDNA sequences for fish species for each target gene were then downloaded. The sequences for the different species for each of the target genes were aligned using ClustalX2 (Larkin *et al.* 2007) and annotated using GeneDoc (Nicholas *et al.* 1997). The accession numbers for each of the individual sequences that were used are included in Table 2.1. Primers for each of the genes described above were designed using the Primer3 software (Koressaar and Remm 2007, Untergasser *et al.* 2012). The primers were designed within regions that were highly conserved between the different fish species. The primers were designed to amplify a small (<200bp) product as small products are amplified most efficiently and therefore yield the most accurate qRT-PCR results. Each primer set was further analysed using the OligoAnalyser® 3.1 software (Integrated DNA Technologies) to ensure the selected primer pairs bound DNA with a similar melting temperature with minimal potential to form primer dimers within the reaction mixtures. Following analysis, primer sets for each gene were chosen and then they were synthesised by GeneWorks (Thebarton, South Australia). The sequences for each of the primer sets are shown in Table 2.2.

Table 2.1: Species names and accession numbers of cDNA sequences that were aligned to create primers for each target gene

Target gene	Common name	Scientific name	Accession number
β-Actin	Southern bluefin tuna	*Thunnus maccoyii*	JX157141.1
	Atlantic bluefin tuna	*Thunnus thynnus*	GU046791.1
	Gilthead seabream	*Sparus aurata*	AF384096.1
	Atlantic salmon	*Salmo salar*	NM_001123525.1
ELF-1α	Atlantic bluefin tuna	*Thunnus thynnus*	FM995222.1
	Nile tilapia	*Oreochromis niloticus*	NM_001279647.1
	Atlantic salmon	*Salmo salar*	AF321836.1
	Zebrafish	*Danio rerio*	DQ083545.1
Δ6Fads	Southern bluefin tuna	*Thunnus maccoyii*	HM032095.1
	European seabass	*Dicentrarchus labrax*	EU647692.1
	Atlantic salmon	*Salmo salar*	NM001123575.2
	Turbot	*Scophthalmus maximus*	AY546094.1
	Zebrafish	*Danio rerio*	AF309556.1
Elovl5	Southern bluefin tuna	*Thunnus maccoyii*	GQ204105.1
	Atlantic salmon	*Salmo salar*	NM_001136552.1
	Senegalese sole	*Sola senegalensis*	JN793448.1
	Common carp	*Cyprinus carpio*	KF924199.1
	Zebrafish	*Danio rerio*	NM_200453.1
PGC-1α	Swordfish	*Xiphias gladius*	FJ710607.1
	White sturgeon	*Acipenser transmontanus*	FJ710613.1
	Rainbow trout	*Onchorhynchus mykiss*	FJ10605.1
	Grass carp	*Ctenopharyngodon idella*	JN195739.1
	Zebrafish	*Danio rerio*	AY998087.1
PPARγ	Pacific bluefin tuna	*Thunnus orientalis*	AB574331.1
	Japanese seabass	*Lateolabrax japonicas*	DQ345545.1
	Gilthead seabream	*Sparus aurata*	AY590304.1
	Zebrafish	*Danio rerio*	DQ017619.1
	Atlantic salmon	*Salmo salar*	AJ292962.1
CS	Atlantic cod	*Gadus morhua*	DQ059757.1
	Yellowfin tuna	*Thunnus albacares*	AY461848.1
	Bigeye tuna	*Thunnus obesus*	AY461849.1
	Zebrafish	*Danio rerio*	BC045362.1

COX1	Southern bluefin tuna	*Thunnus maccoyii*	NC_014101.1
	Bigeye tuna	*Thunnus obesus*	HM071005.1
	Yellowfin tuna	*Thunnus albacares*	HM071006.1
UCP2	Bicolor damselfish	*Stegastes partitus*	XM_008280962.1
	Atlantic salmon	*Salmo salar*	BT047080.1
	Gilthead seabream	*Sparus aurata*	JQ859959.1
	Common carp	*Cyprinus carpio*	AJ243486.1
	Zebrafish	*Danio rerio*	NM_131176.1

Table 2.2: A summary of the primer sets used to assess gene expression using qRT-PCR in the SBT-E1 cells. The estimated amplicon sizes were based on known sequence data for SBT or other fish species. The β-actin primer set was designed by Agawa *et al* (2012). All other primer sets were designed by the author of this thesis.

Target gene	**Forward primer sequence (5'→3')**	**Reverse primer sequence (5'→3')**	**Estimated size**
β-Actin	ACCCACACAGTGCCCATCTA	TCACGCACGATTTCCCTCT	155 bp
ELF-1α	CCCCTGGACACAGAGACTTC	GCCGTTCTTGGAGATACCAG	119 bp
Δ6Fads	CCGTGCACTGTGTGAGAAAC	CAGTGTAAGCGATAAAATCAGCTG	152 bp
Elovl5	CCCACCAACATTTGCACTCA	GTCCTGGCAGTAGAAGTTGT	189 bp
PGC-1α1	ATGGCGTGGGACAGGTGTA	AGTCGCTGACATCCAGCTCT	131 bp
PGC-1α2	AACAACACCCTTCTGCCATC	GTTTGCTGCCTGGTTCTCTC	135 bp
PPARγ	TCTTCAGACGCACAATCAGG	GTGACATGCCAACATTGAGG	129 bp
CS	CTGGACTGGTCCCACAACTT	GGACAGGTAGGGGTCAGACA	165 bp
COX1	GGAGCTGTATTCGCCATTGT	AGGAAGTGCTGTGGGAAGAA	143 bp
UCP2	AGACCATTGCCAGGGATGAA	CACGGCATGTTGTCTGTCAT	157 bp

2.5.4 RNA extraction from the SBT-E1 cells

Total RNA was extracted from cell pellets (Section 2.5.1) using a RNeasy® Mini kit (Qiagen) according to the manufacturer's protocol. Optional steps in the protocol such as on-column DNase digestion (to eliminate genomic DNA) and re-centrifugation of the column assembly before elution of the RNA (to reduce carry-over of ethanol and guanidine salts) were carried out according to the manufacturer's protocol to avoid any negative impacts on downstream processes such as inhibition of cDNA synthesis or PCR amplification. The concentration of the eluted RNA was estimated using a NanoDrop 1000 spectrophotometer and the RNA was frozen at -80°C or used immediately for cDNA synthesis.

2.5.5 RNA extraction from SBT tissues

The tissue from Section 2.5.2 was removed from -20°C storage, taken out of RNA*later*® and dabbed dry on a paper towel. The tissue was ground to a fine powder in liquid nitrogen in a mortar and pestle. Approximately 20 mg of the tissue powder was placed in a liquid nitrogen-cooled 1.5 mL tube after the liquid nitrogen had evaporated but before the tissue had thawed. A total of 350 µl of Buffer RLT (Qiagen) was added and RNA was extracted using an RNeasy® fibrous tissue mini kit (Qiagen) according to the manufacturer's protocols except that EconoSpin® mini spin columns (Epoch Life Sciences) were used in place of the RNeasy® mini spin columns. Optional steps in the protocol were completed as described in Section 2.5.4. The concentration of the eluted RNA was estimated using a NanoDrop 1000 spectrophotometer and the RNA was frozen at -80°C or used immediately for cDNA synthesis.

2.5.6 cDNA synthesis

A total of 1 µg of RNA was reverse transcribed using a SuperScript® III First-Strand Synthesis System (Invitrogen) according to the manufacturer's instructions. Briefly, oligo $(dT)_{20}$ primers supplied with the kit were used to synthesise cDNAs from messenger RNAs (mRNAs) present in the RNA extract. The resulting cDNA was diluted 5-fold in nuclease free water and frozen at -20°C in preparation for downstream applications.

2.5.7 Initial testing of the qRT-PCR primers using standard PCR

PCR reactions were set up for each primer set using the reagents shown in Table 2.3. Amplification was conducted in a Perkin Elmer GeneAmp® PCR System 2400 thermal cycler. The PCR cycling conditions were denaturation at 95°C for 5 min followed by 45 cycles of denaturation at 95°C for 30 s, primer annealing at 55°C for 30 s and extension at 72°C for 30 s, followed by a final extension step at 72°C for 5 min. A 5 µl subsample of the PCR reaction was subjected to electrophoresis as described in Section 2.3.2.2. The results of the electrophoresis showed that each of the primer sets facilitated the amplification of a single product (data not shown).

Table 2.3: The reagents used for standard PCR reactions.

Reagent	Stock concentration	Volume added to PCR reaction	Final concentration
GoTaq® Flexi buffer (Promega)	5 x	10 µl	1 x
Deoxynucleotides (dNTPs)	10 mM	1 µl	200 µM
$MgCl_2$	25 mM	1 µl	500 µM
Forward primer	10 µM	1 µl	200 µM
Reverse primer	10 µM	1 µl	200 µM
ThermoPol® Taq polymerase (NEB)	5 units/µl	0.5 µl	2.5 units
cDNA	From 50 ng RNA	5 µl	n.a.
Nuclease free water	n.a.	30.5 µl	n.a.

2.5.8 ***Confirmation of PCR specificity by sequencing of PCR amplicons***

PCR was conducted as described in Section 2.5.7 and the PCR products were purified as described in Section 2.3.2.3. A 2 µl subsample of the purified PCR product was used to estimate the DNA concentration using a NanoDrop 1000 spectrophotometer. The PCR product was sequenced according to the protocol described in Section 2.3.2.4 using the forward and reverse primers that were used to amplify the PCR product. The results from the sequencing using the reverse primer were converted to the reverse complement and aligned to the sequence obtained using the forward primer using ClustalX2.1 (Larkin *et al.* 2007). The consensus sequence was analysed using BLAST to confirm its identity to the target gene. The consensus sequence was then aligned to the other fish sequences used to design the primers (Figs. 2.4-2.13) to show similarity to other fish species. The annealing locations of the respective primers are indicated on each of the figures.

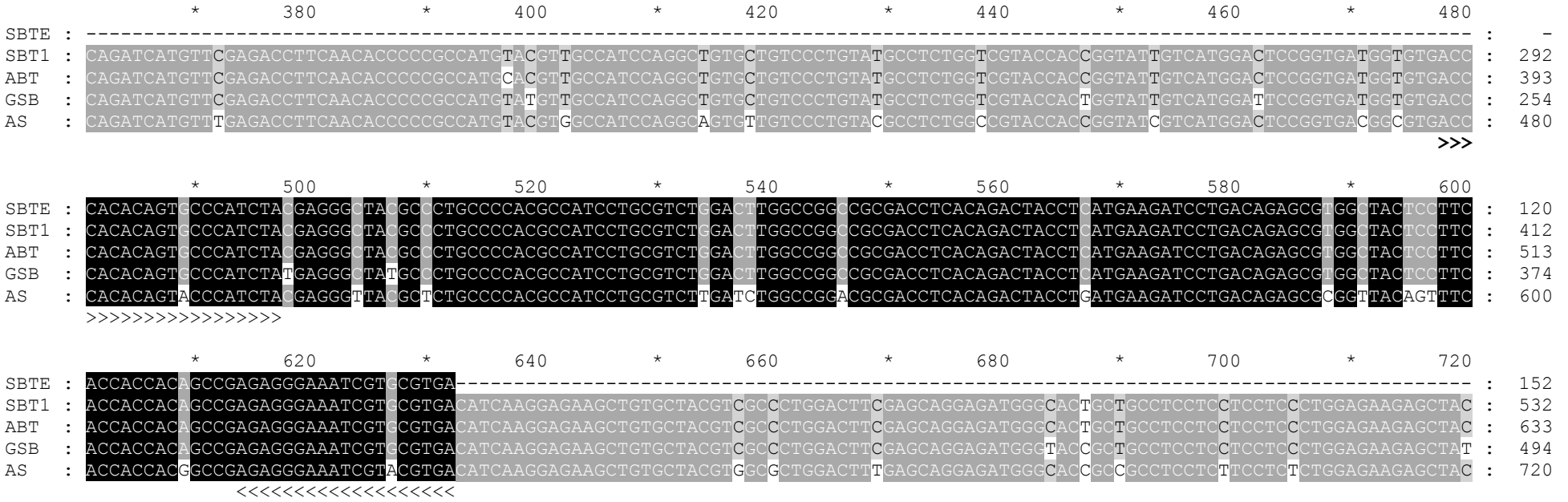


Figure 2.4: An alignment of β-actin nucleotide sequences from various fish species. The SBTE sequence was obtained from cDNA from the SBT-E1 cells using the primers shown in Table 2.2. The regions where the forward and reverse primers anneal are indicated by >>> and <<<, respectively. The other sequences were downloaded from the NCBI Nucleotide database for southern bluefin tuna (SBT1, *Thunnus maccoyii* JX157141.1), Atlantic bluefin tuna (ABT, *Thunnus thynnus*, GU046791.1), gilthead seabream (GSB, *Sparus aurata*, AF384096.1) and Atlantic salmon (AS, *Salmo salar*, NM_001123525.1). Shading represents the degree of conservation across all aligned sequences where black = 100%, dark grey = 70%, light grey = 50% and no shading = <50%.

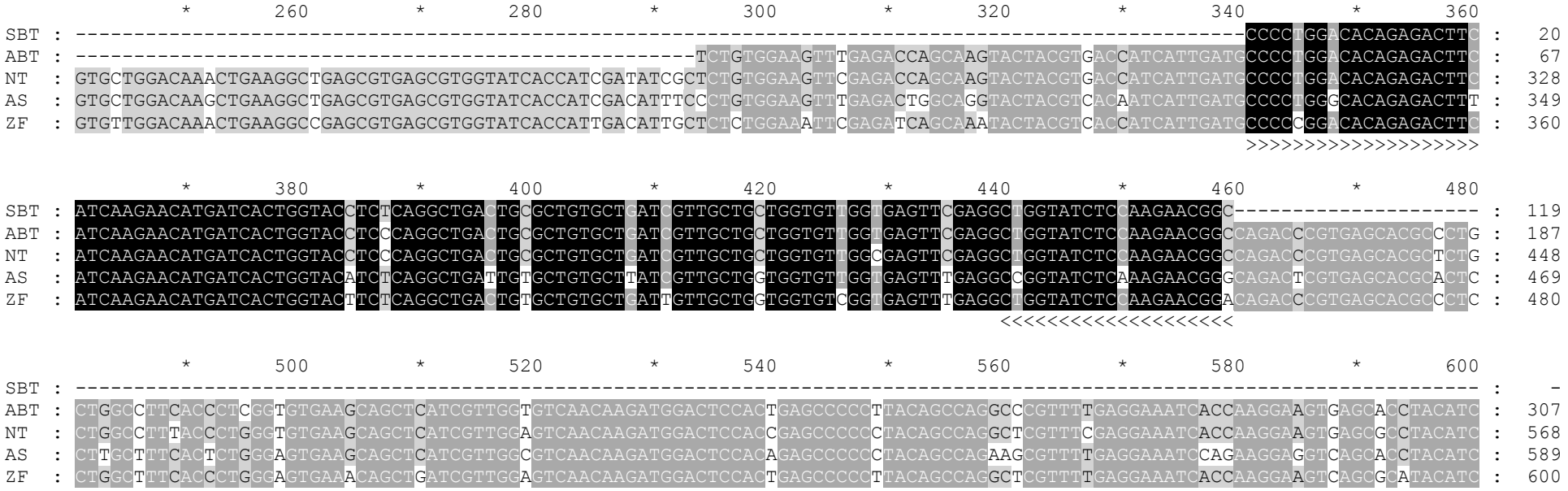


Figure 2.5: Alignment of ELF-1α nucleotide sequences from various fish species. The SBT sequence was obtained from cDNA from the SBT-E1 cells using the primers shown in Table 2.2. The regions where the forward and reverse primers anneal are indicated by >>> and <<<, respectively. The sequences were downloaded from the NCBI Nucleotide database for Atlantic bluefin tuna tuna (ABT, *Thunnus thynnus*, FM995222.1), nile tilapia (NT, *Oreochromis niloticus*, NM_001279647.1), Atlantic salmon (AS, *Salmo salar*, AF321836.1) and zebrafish (ZF, *Danio rerio*, DQ083545.1). Shading represents the degree of conservation across all aligned sequences where black = 100%, dark grey = 70%, light grey = 50% and no shading = <50%.

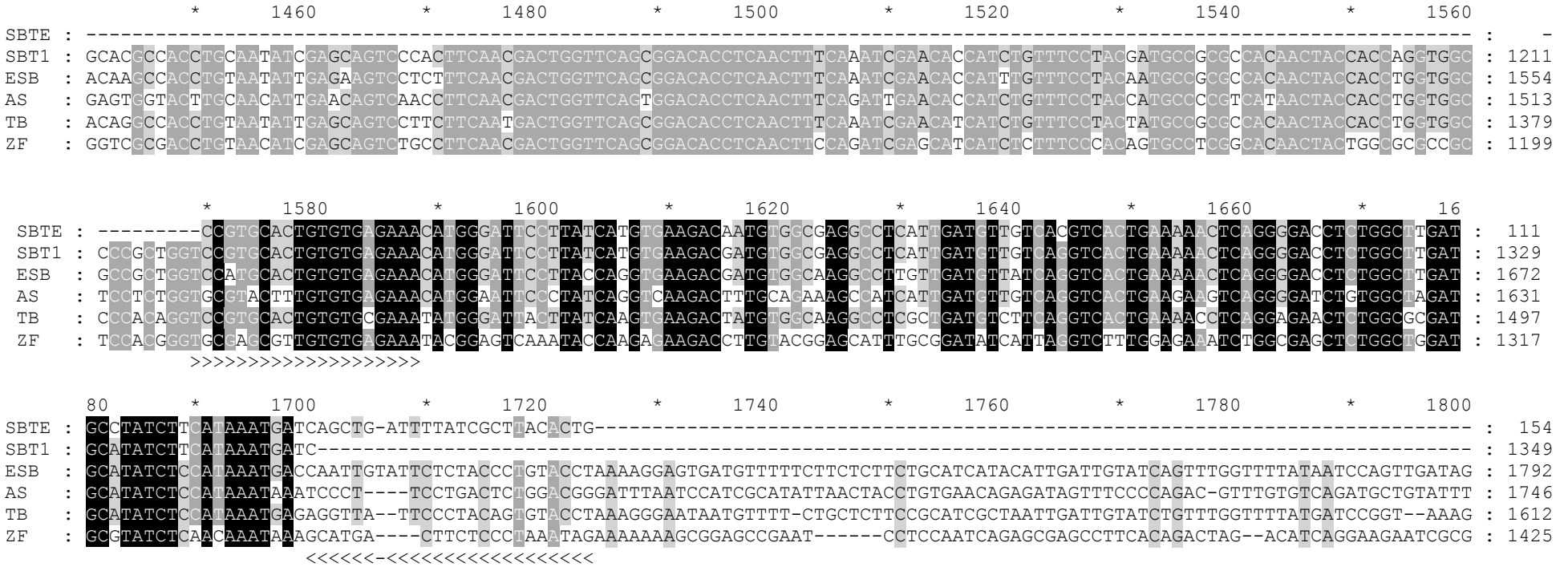


Figure 2.6: Alignment of Δ6Fads nucleotide sequences from various fish species. The SBTE sequence was obtained from cDNA from the SBT-E1 cells using the primers shown in Table 2.2. The regions where the forward and reverse primers anneal are indicated by >>> and <<<, respectively. The sequences were downloaded from the NCBI Nucleotide database for southern bluefin tuna (SBT1, HM032095.1), European seabass (ESB, *Dicentrarchus labrax*, EU647692.1), Atlantic salmon (AS, *Salmo salar*, NM_001123575.2) turbot (TB, *Scophthalmus maximus*, AY546094.1) and zebrafish (ZF, *Danio rerio*, AF309556.1). The shading represents the degree of conservation across all aligned sequences where black = 100%, dark grey = 70%, light grey = 50% and no shading = <50%.

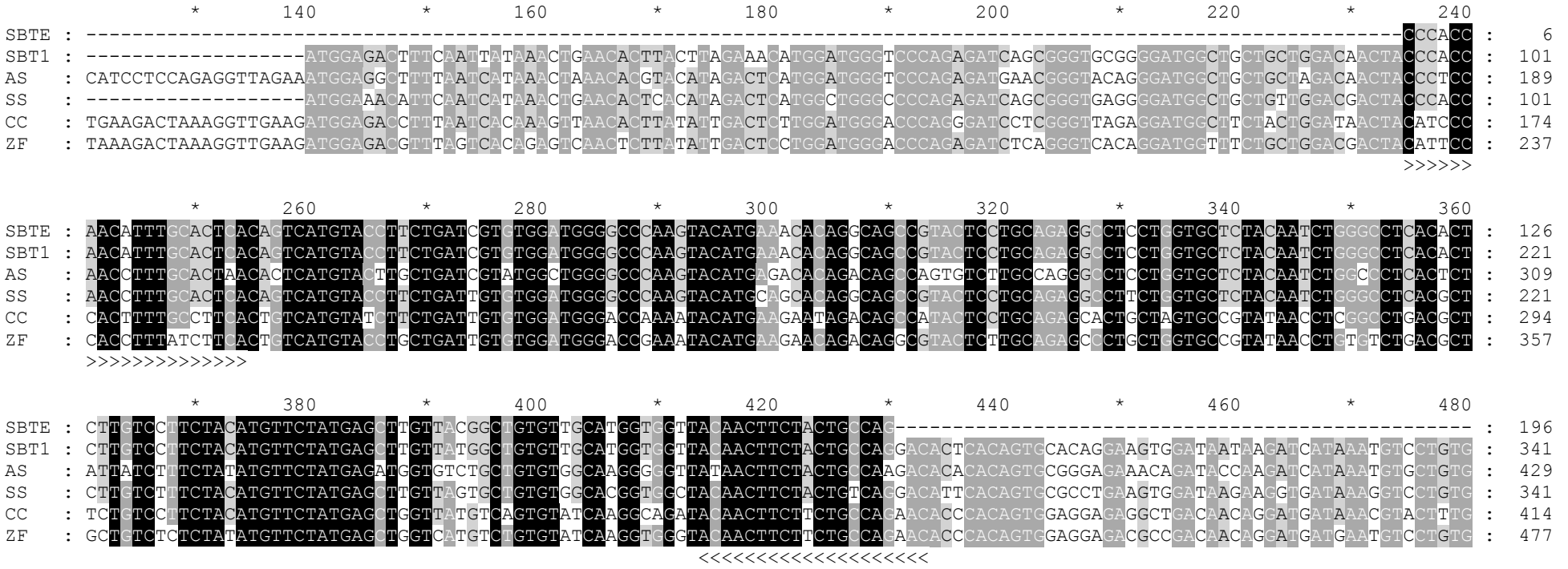


Figure 2.7: Alignment of Elovl5 nucleotide sequences from various fish species. The SBTE sequence was obtained from cDNA from the SBT-E1 cells using the primers shown in Table 2.2. The regions where the forward and reverse primers anneal are indicated by >>> and <<<, respectively. The sequences were downloaded from the NCBI Nucleotide database for southern bluefin tuna (SBT1, GQ204105.1), Atlantic salmon (AS, *Salmo salar*, NM_001136552.1) Senegalese sole (SS, *Solea senegalensis*, JN793448.1), common carp (CC, *Cyprinus carpio*, KF924199.1) and zebrafish (ZF, *Danio rerio*, NM_200453.1). The shading represents the degree of conservation across all aligned sequences where black = 100%, dark grey = 70%, light grey = 50% and no shading = <50%.

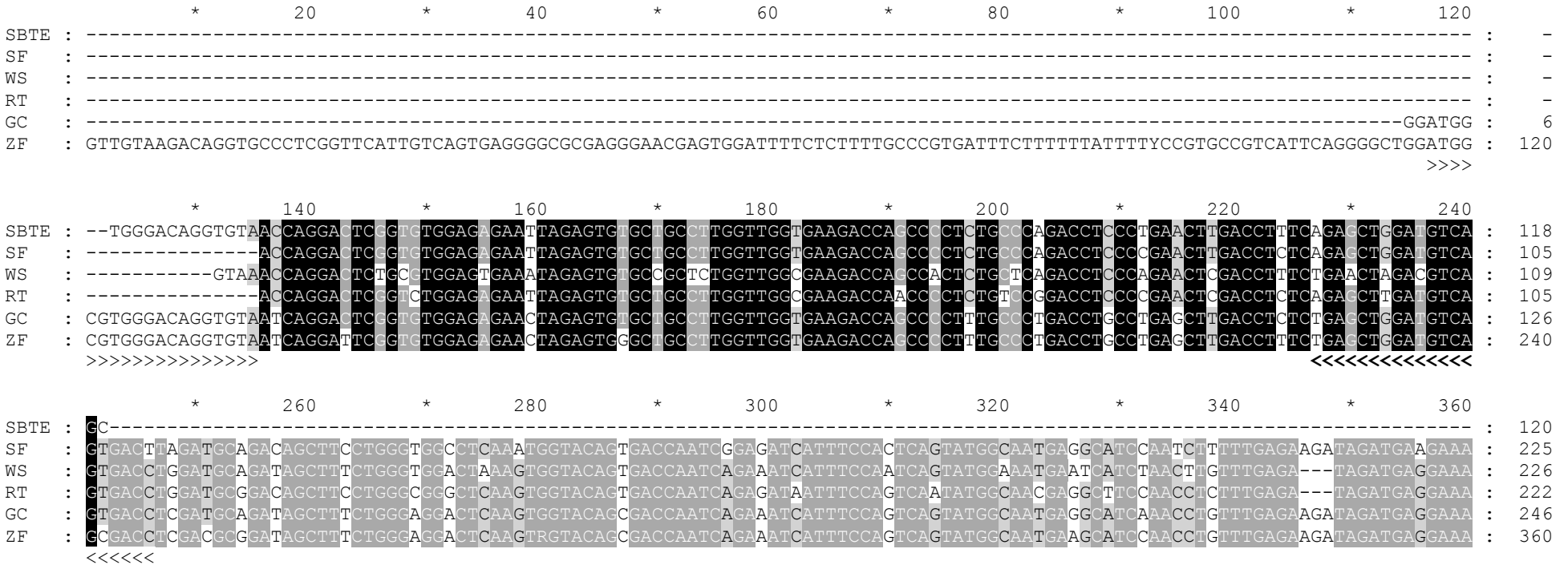


Figure 2.8: Alignment of PGC-1α nucleotide sequences from various fish species. The SBTE sequence was obtained from cDNA from the SBT-E1 cells using the PGC-1α1 primers shown in Table 2.2. The regions where the forward and reverse primers anneal are indicated by >>> and <<<, respectively. The sequences were downloaded from the NCBI Nucleotide database for swordfish (SF, *Xiphias gladius*, FJ710607.1), white sturgeon (WS, *Acipenser transmontanus*, FJ710613.1), rainbow trout (RT, *Oncorhynchus mykiss* FJ710605.1) grass carp (GC, *Ctenopharyngodon idella*, JN195739.1) and Zebrafish (ZF, *Danio rerio* AY998087.1). The shading represents the degree of conservation across all aligned sequences where black = 100%, dark grey = 70%, light grey = 50% and no shading = <50%.

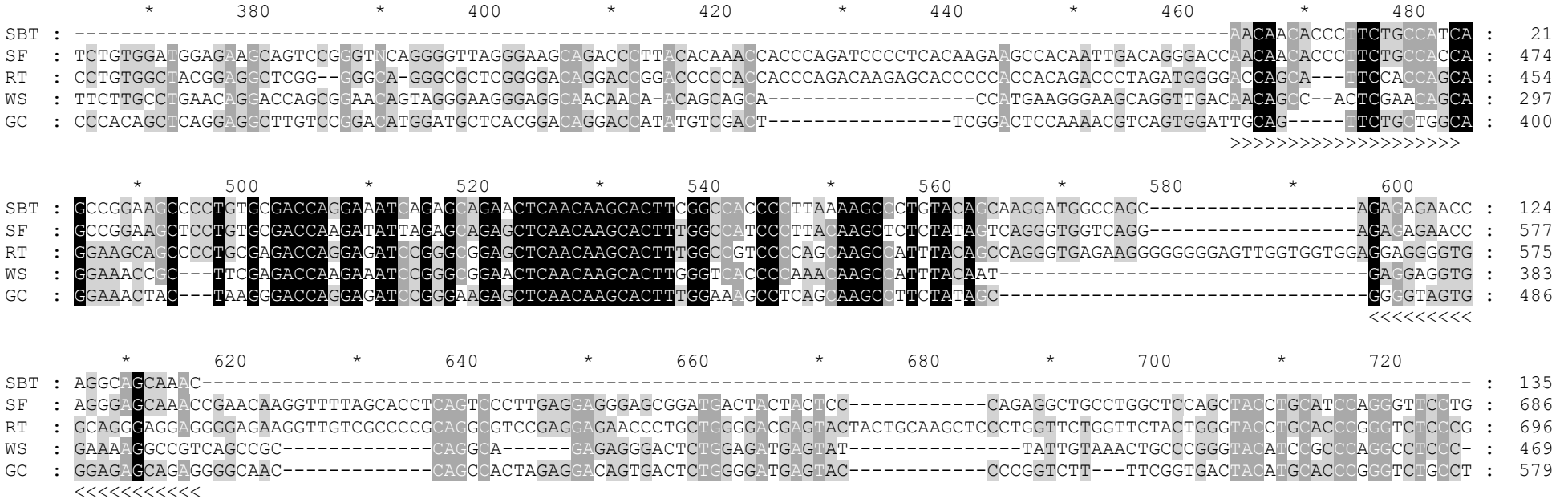


Figure 2.9: Alignment of PGC-1α nucleotide sequences from various fish species. The SBT sequence was obtained from SBT liver tissue using the PGC-1α2 primers shown in Table 2.2. The regions where the forward and reverse primers anneal are indicated by >>> and <<<, respectively. The sequences were downloaded from the NCBI Nucleotide database for swordfish (SF, *Xiphias gladius*, FJ710607.1), white sturgeon (WS, *Acipenser transmontanus*, FJ710613.1), rainbow trout (RT, *Oncorhynchus mykiss* FJ710605.1), grass carp (GC, *Ctenopharyngodon idella*, JN195739.1) and zebrafish (ZF, *Danio rerio* AY998087.1). The shading represents the degree of conservation across all aligned sequences where black = 100%, dark grey = 70%, light grey = 50% and no shading = <50%.

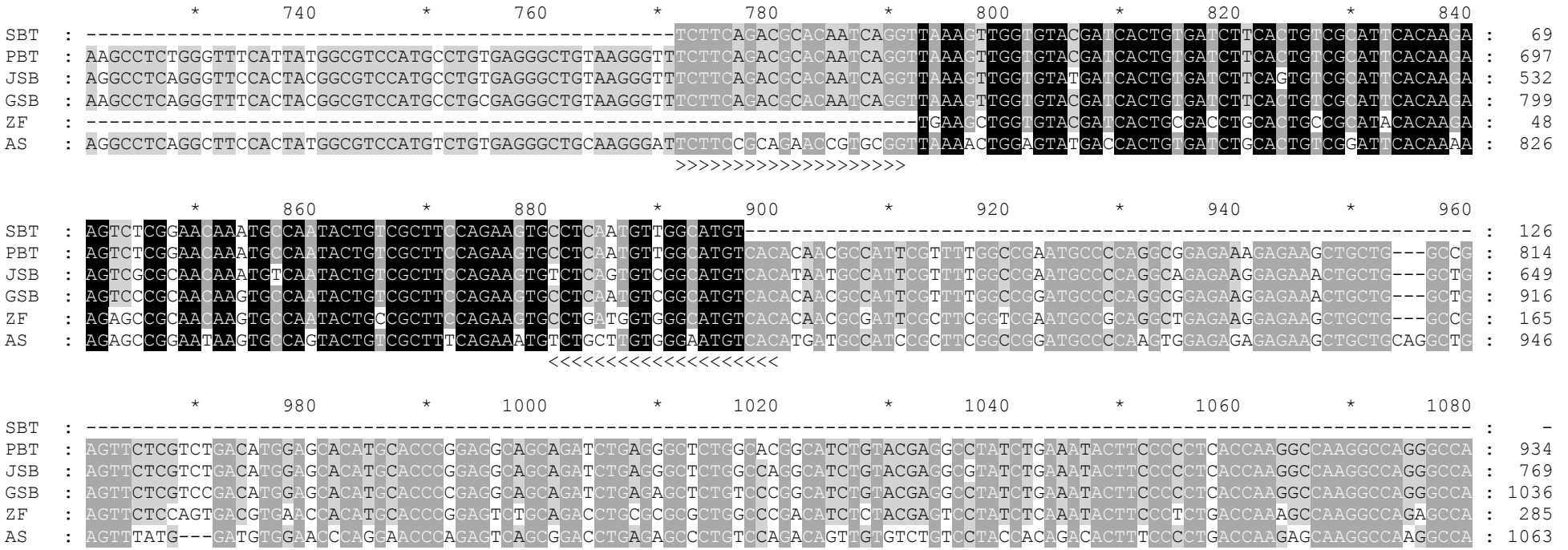


Figure 2.10: Alignment of PPARγ nucleotide sequences from various fish species. The SBT sequence was obtained from cDNA from the SBT-E1 cells using the primers shown in Table 2.2. The regions where the forward and reverse primers anneal are indicated by >>> and <<<, respectively. The sequences were downloaded from the NCBI Nucleotide database for Pacific bluefin tuna (PBT, *Thunnus orientalis*, AB574331.1), Japanese seabass (JSB, *Lateolabrax japonicas*, DQ345545.1), gilthead seabream (GSB, *Sparus aurata*, AY590304.1), Zebrafish (ZF, *Danio rerio* DQ017619.1) and Atlantic salmon (AS, *Salmo salar*, AJ292962.1). The accession numbers for the sequences are described in Table 2.1. The shading represents the degree of conservation across all aligned sequences where black = 100%, dark grey = 70%, light grey = 50% and no shading = <50%.

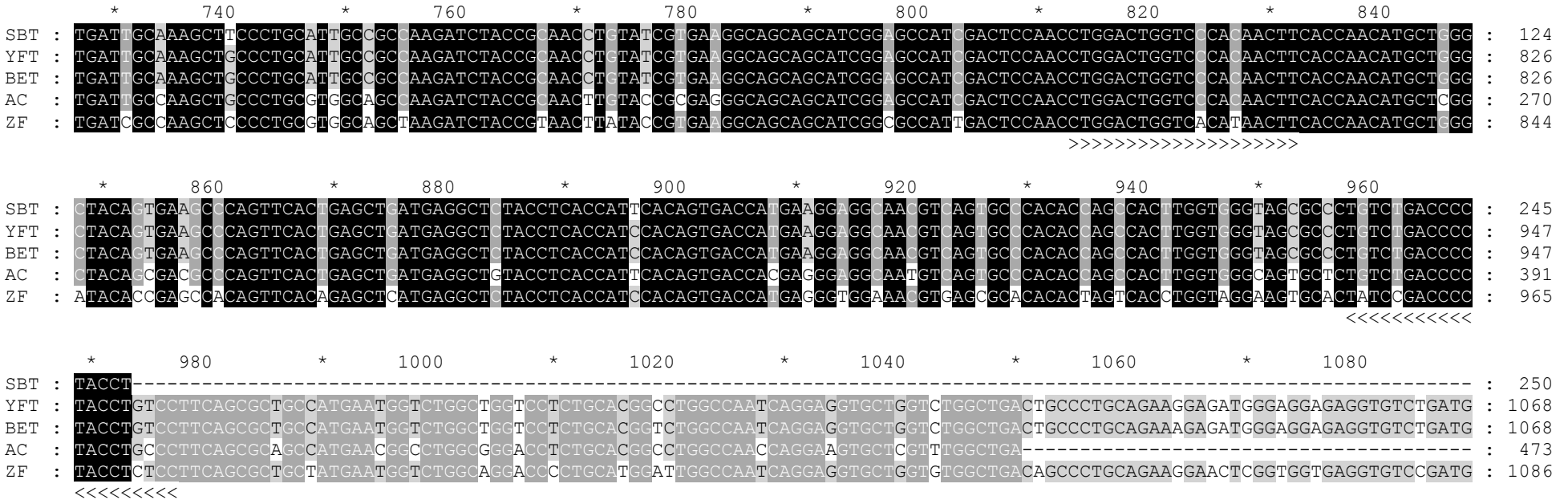


Figure 2.11: Alignment of CS nucleotide sequences from various fish species. The SBT sequence was obtained from cDNA from the SBT-E1 cells using the primers shown in Table 2.2. The regions where the forward and reverse primers anneal are indicated by >>> and <<<, respectively. The sequences were downloaded from the NCBI Nucleotide database for Atlantic cod ((AC, *Gadus morhua*, DQ059757.1), yellowfin tuna (YFT, *Thunnus albacares*, AY461848.1), bigeye tuna (BET, *Thunnus obesus*, AY461849.1) and zebrafish (ZF, *Danio rerio*, BC045362.1). The shading represents the degree of conservation across all aligned sequences where black = 100%, dark grey = 80%, light grey = 60% and no shading = <60%.

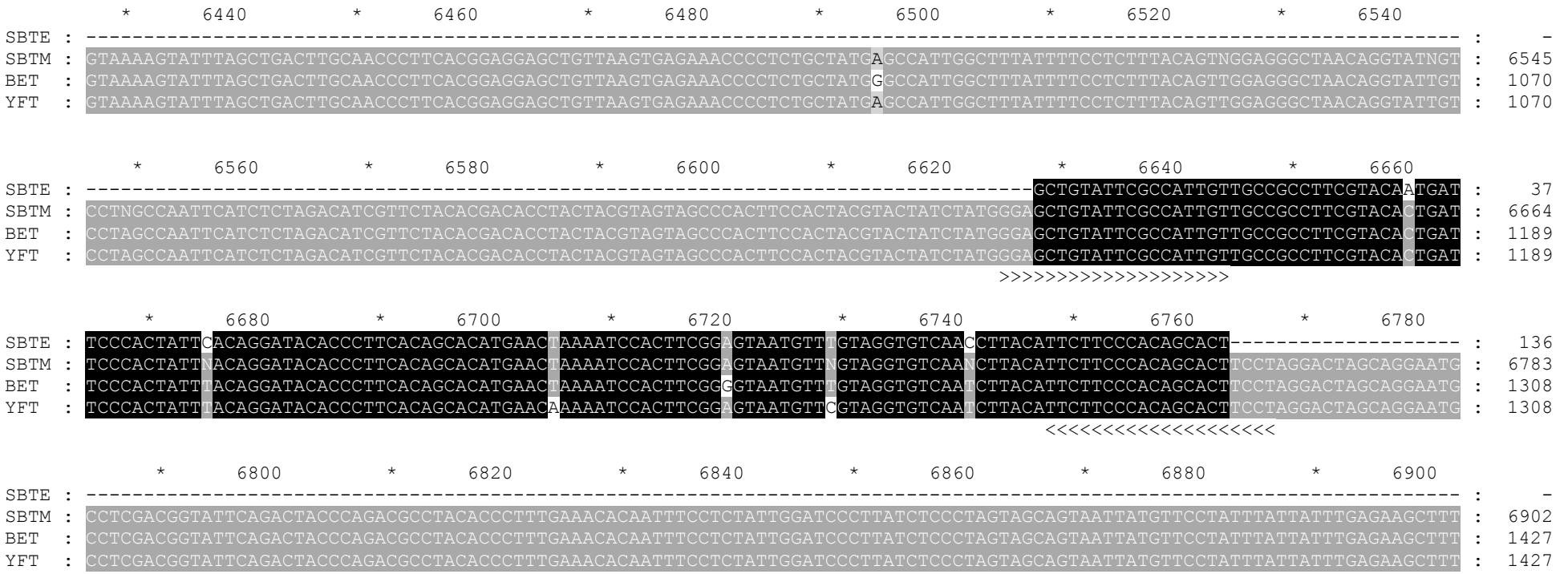


Figure 2.12: Alignment of COX1 nucleotide sequences from various fish species. The SBTE sequence was obtained from cDNA from the SBT-E1 cells using the primers shown in Table 2.2. The regions where the forward and reverse primers anneal are indicated by >>> and <<<, respectively. The sequences were downloaded from the NCBI Nucleotide database for southern bluefin tuna mitochondria mitochondria (SBTM, NC_014101.1), bigeye tuna (BET, *Thunnus obesus*, HM071005.1) and yellowfin tuna (YFT, *Thunnus albacares*, HM071006.1). The shading represents the degree of conservation across all aligned sequences where black = 100%, dark grey = 70%, light grey = 50% and no shading = <50%.

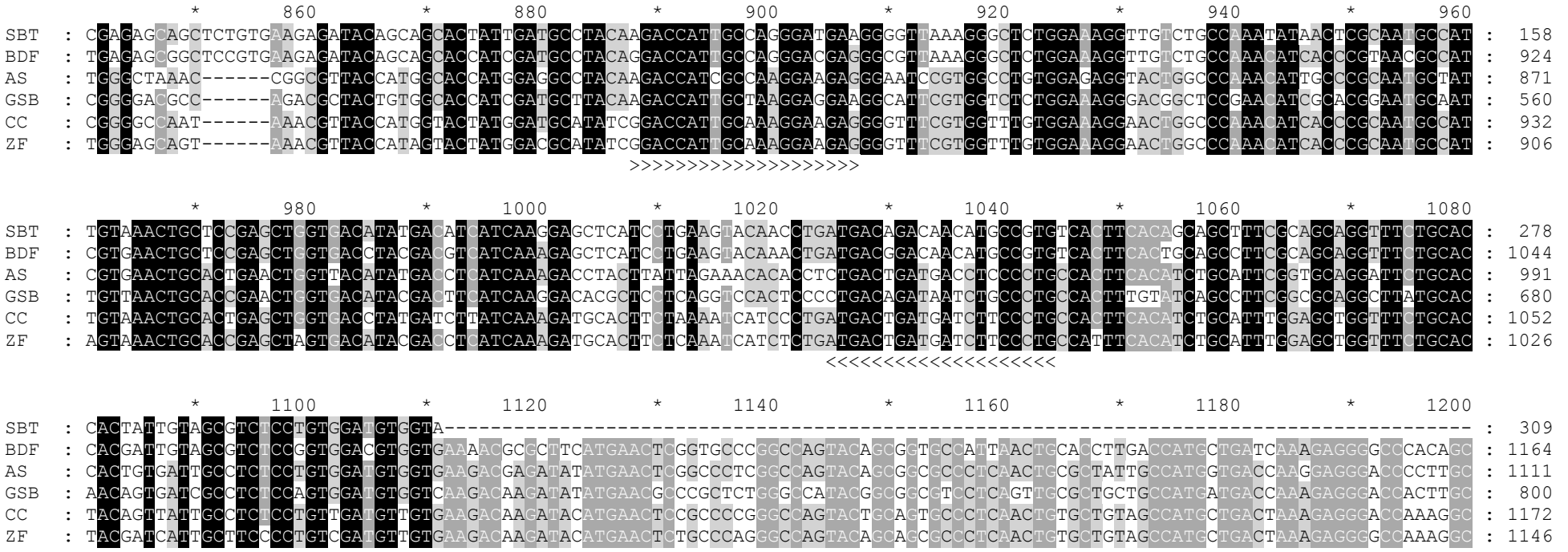


Figure 2.13: Alignment of UCP2 nucleotide sequences from various fish species. The SBTE sequence was obtained from cDNA from the SBT-E1 cells using the primers shown in Table 2.2. The regions where the forward and reverse primers anneal are indicated by >>> and <<<, respectively. The sequences were downloaded from the NCBI Nucleotide database for bicolor damselfish (BDF, *Stegastes partitus*, XM_008280962.1), Atlantic salmon (AS, *Salmo salar*, BT047080.1), gilthead seabream (GSB, *Sparus aurata*, JQ859959.1), common carp (CC, *Cyprinus carpio*, AJ243486.1) and zebrafish (ZF, *Danio rerio*, NM_131176.1). The accession numbers for the sequences are described in Table 2.1. The shading represents the degree of conservation across all aligned sequences where black = 100%, dark grey = 70%, light grey = 50% and no shading = <50%.

2.5.9 Determination of the amplification efficiency for each primer set

The accuracy of qRT-PCR, in terms of quantifying the abundance of a particular transcript, is dependent on the efficiency of the DNA amplification reaction. A 100% efficient amplification reaction represents exact doubling of the number of copies of the target DNA with each PCR cycle (Kubista *et al.* 2006). To ensure the accuracy of the qRT-PCR reactions, the amplification efficiency of each primer set was determined. PCR was conducted as described in Section 2.5.7 and the PCR product was purified as described in Section 2.3.2.3. The purified PCR product was then used as the template to determine primer efficiency through qRT-PCR analysis. This involved several steps. Firstly, an estimate of the number of transcript copies/µl in the purified PCR product was calculated using the copy number calculator from the URI genomics and sequencing centre (http://cels.uri.edu/gsc/cndna.html). The calculator used the following equation: $copies = ((A \times N/(L \times 10^9 \times 650)$, Where A = the concentration of the cDNA template (i.e. the PCR product) (ng/µl), N = Avogadro's number (6.022 x 10^{23} molecules per mole) and L = the length of the cDNA template (i.e. PCR product) (bp). A 10-fold dilution series of the purified PCR product was conducted in nuclease free water (Ambion™) to give concentrations relative to the original product in the range of 10^{-3} to 10^{-9}. Five µl of each of these dilutions was used as template for the subsequent qRT-PCR reactions. Each of the reactions was conducted in triplicate. The qRT-PCR reactions were conducted using a Platinum® SYBR® Green qPCR SuperMix-UDG kit (Invitrogen), according to the manufacturer's protocol except that a half volume (25 µl) reaction size was used. The qRT-PCR reactions were performed and the resulting data were analysed using a Rotorgene 3000 thermal cycler (Corbett Research). The fluorescence curves produced for each sample were used to calculate the threshold cycle (Ct) values. These Ct values were the cycle at which the fluorescence signal rose above

background fluorescence, and they were calculated by the thermal cycler using log-standardised fluorescence curves. These Ct values were used to construct a standard curve and the slope of the linear regression from this standard curve was used to estimate the amplification efficiency for each of the primer pairs. An amplification efficiency value of 1.0 represents perfect doubling of the desired amplicon with each PCR cycle and is derived from a slope value of -3.33. This value represents perfect doubling with each PCR cycle on a log-linear scale (i.e. a single transcript copy will amplify to 10 copies in 3.33 PCR cycles).

The fluorescence curves, along with the standard curves are shown in Figs 2.14-2.23. A linear regression was fitted to the data in each figure and the slope of the line was used to determine the amplification efficiency as described above. A summary of the equations of the linear regressions obtained from the standard curves and the corresponding amplification efficiencies for each of the primer sets is shown in Table 2.4. Each of the primer sets showed acceptable amplification efficiency in the range of 0.91-1.0, with the lowest efficiency of 0.91 seen for β-actin and the highest efficiency of 1.0 seen for Elovl5.

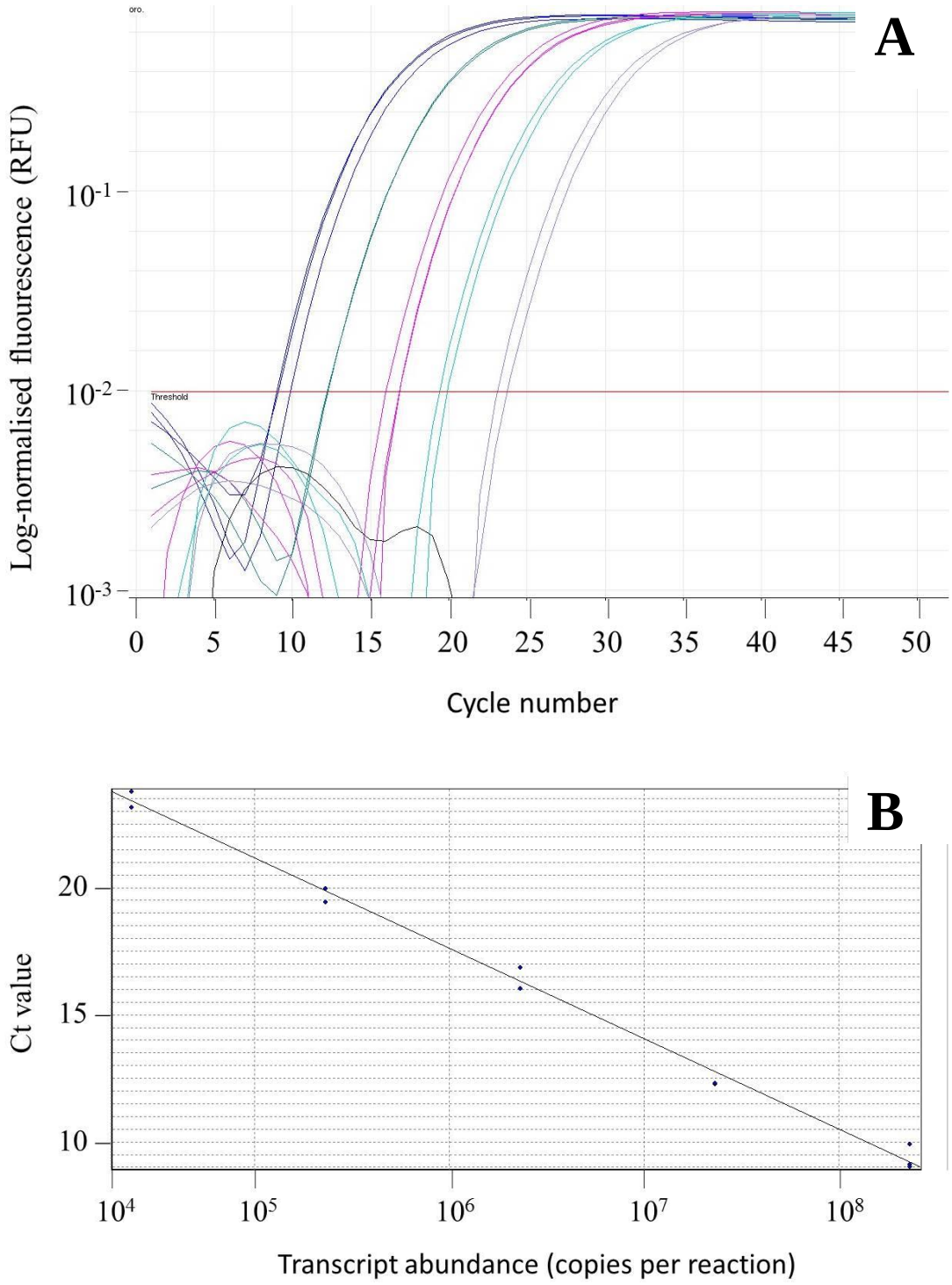


Figure 2.14: Fluorescence curve (A) and standard curve (B) obtained using serial dilutions of a purified β-actin PCR product as the template for qRT-PCR. The cycle at which the fluorescence intensity crossed a defined threshold (Ct value) was calculated using the fluorescence curve. The standard curve was constructed by plotting the Ct values against the copy number. The equation for the standard curve was $y = -3.552x + 38.922$. The slope of the line corresponded to an amplification efficiency of 0.91.

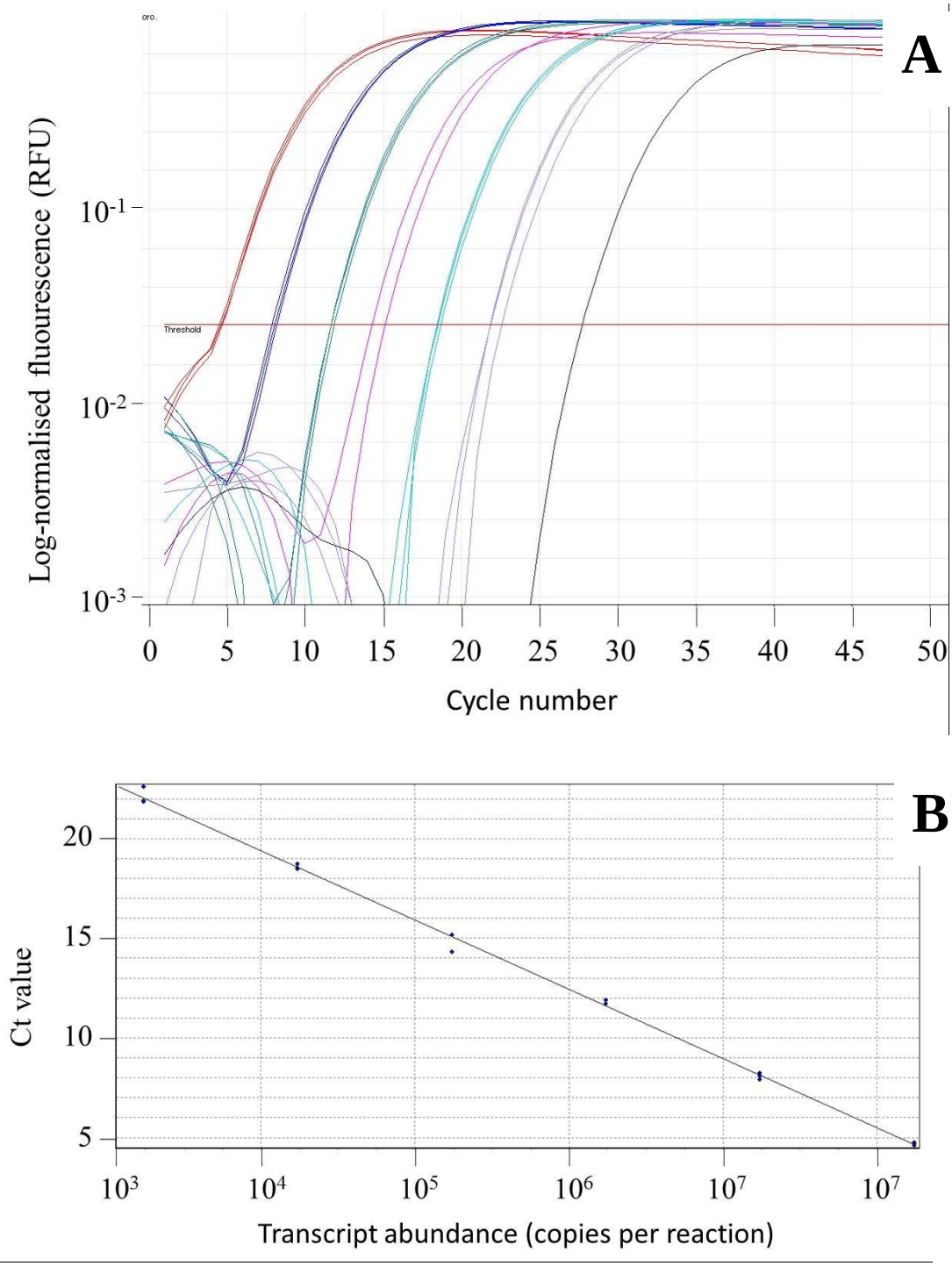


Figure 2.15: Fluorescence curve (A) and standard curve (B) obtained using serial dilutions of a purified ELF-1α PCR product as the template for qRT-PCR. The cycle at which the fluorescence intensity crossed a defined threshold (Ct value) was calculated using the fluorescence curve. The standard curve was constructed by plotting the Ct values against the copy number. The equation for the standard curve was $y = -3.476x + 33.293$. The slope of the line corresponded to an amplification efficiency of 0.94.

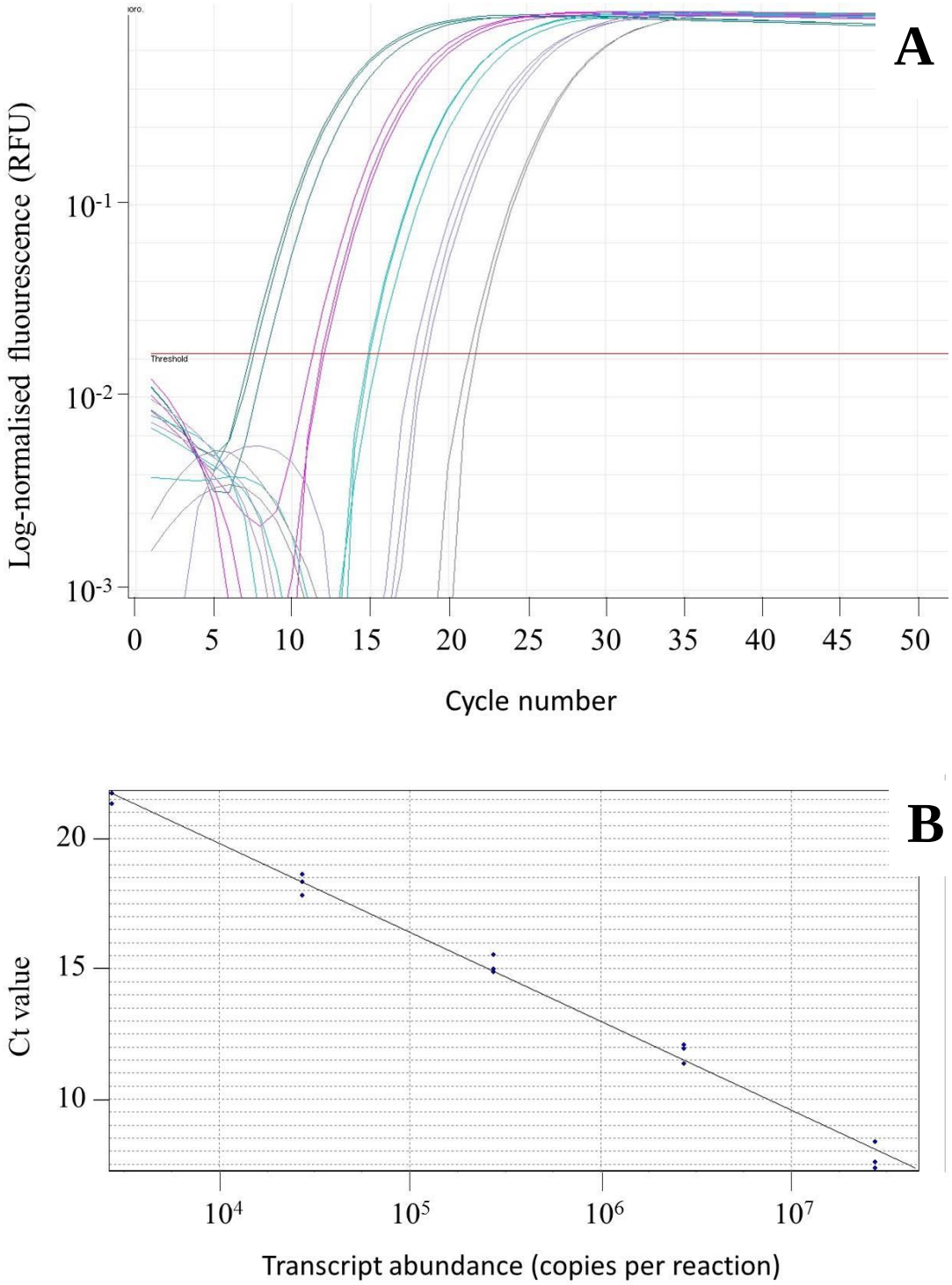


Figure 2.16: Fluorescence curve (A) and standard curve (B) obtained using serial dilutions of a purified Δ6Fads PCR product as the template for qRT-PCR. The cycle at which the fluorescence intensity crossed a defined threshold (Ct value) was calculated using the fluorescence curve. The standard curve was constructed by plotting the Ct values against the copy number. The equation for the standard curve was $y = -3.411x + 33.452$. The slope of the line corresponded to an amplification efficiency of 0.96.

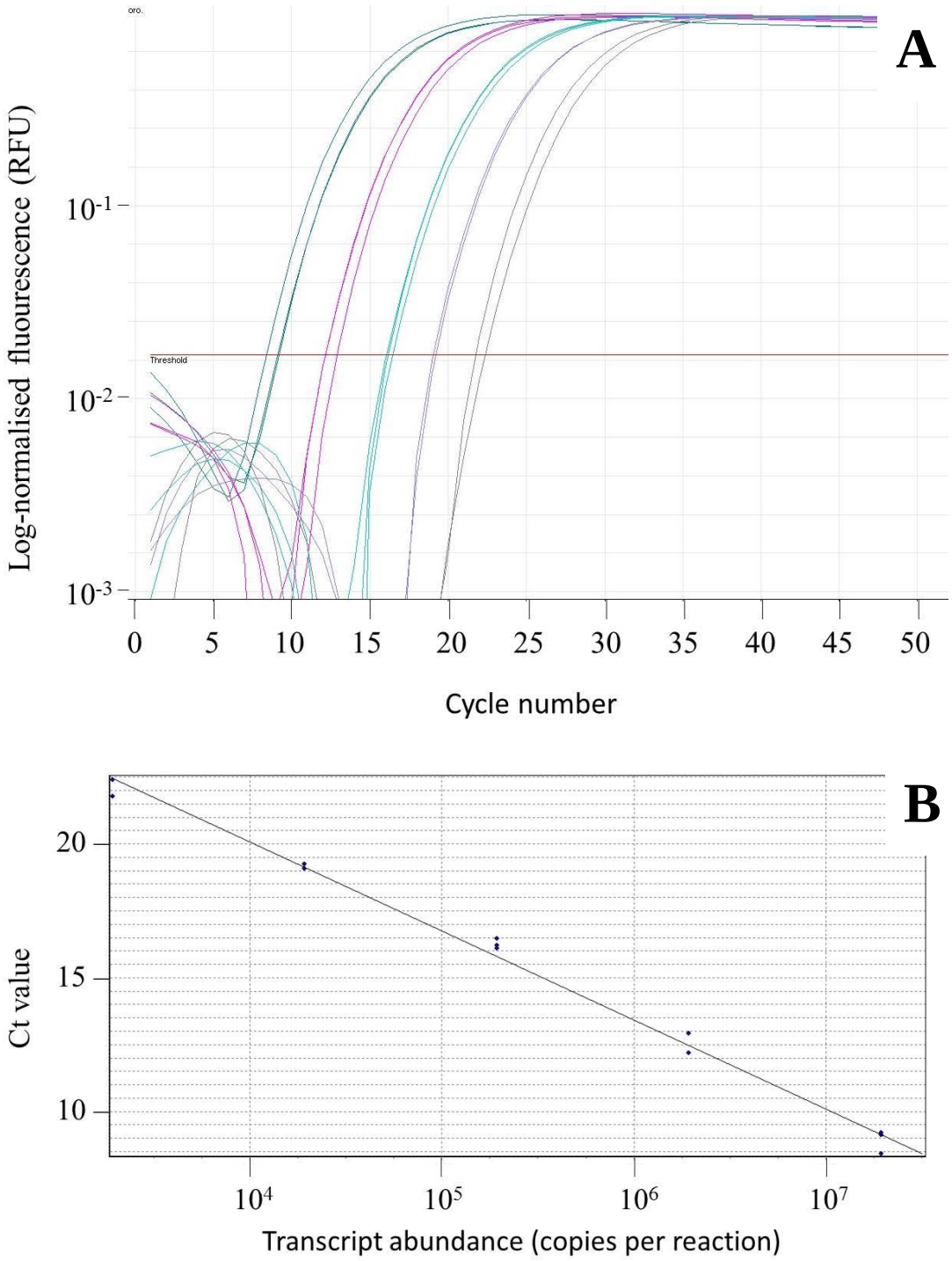


Figure 2.17: Fluorescence curve (A) and standard curve (B) obtained using serial dilutions of a purified Elovl5 PCR product as the template for qRT-PCR. The cycle at which the fluorescence intensity crossed a defined threshold (Ct value) was calculated using the fluorescence curve. The standard curve was constructed by plotting the Ct values against the copy number. The equation for the standard curve was. $y = -3.33x + 33.397$. The slope of the line corresponded to an amplification efficiency of 1.0.

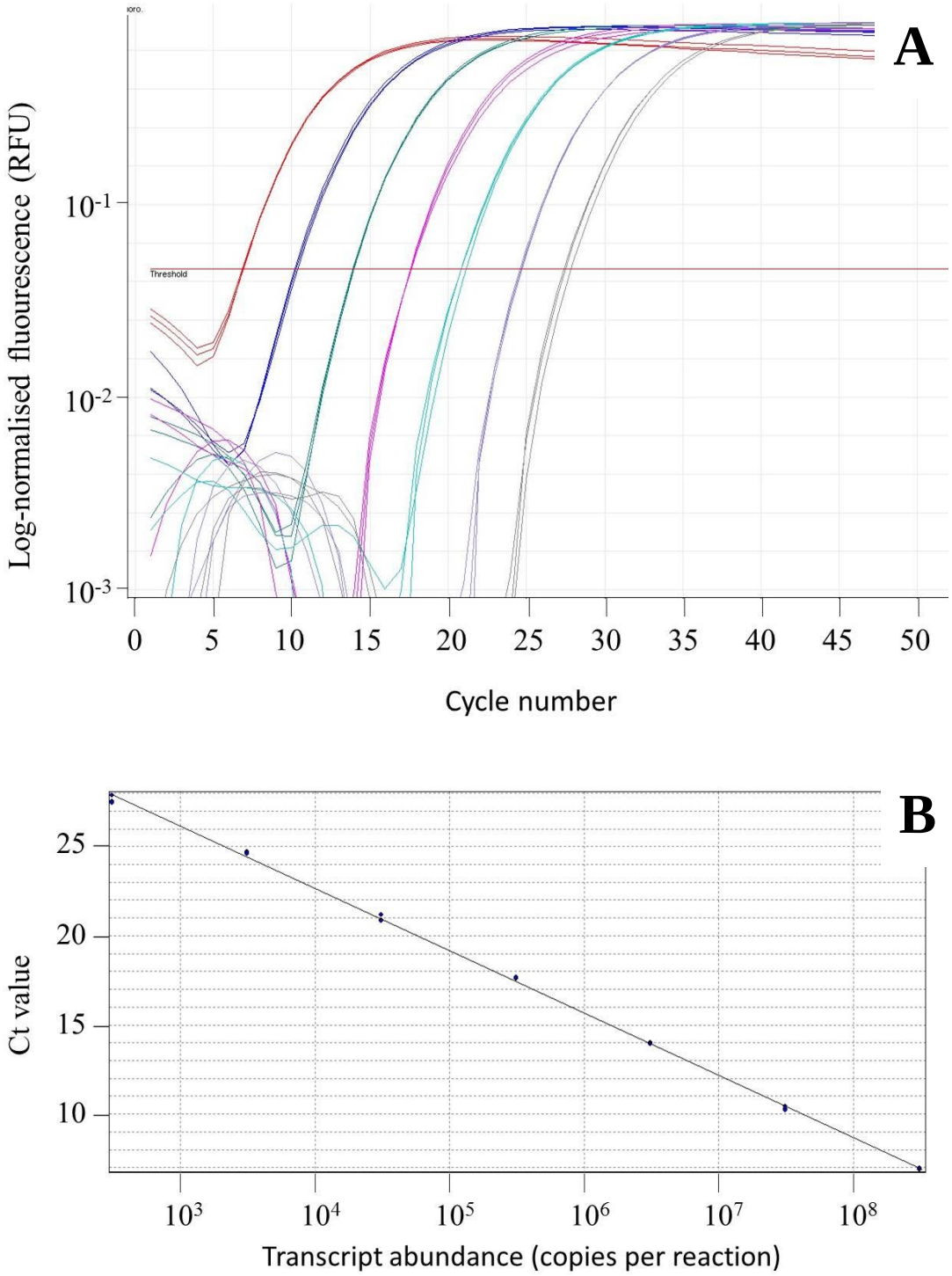


Figure 2.18: Fluorescence curve (A) and standard curve (B) obtained using serial dilutions of a purified PGC-1α PCR product from the SBT-E1 cell line (obtained using the PGC-1α1 primers) as the template for qRT-PCR. The cycle at which the fluorescence intensity crossed a defined threshold (Ct value) was calculated using the fluorescence curve. The standard curve was constructed by plotting the Ct values against the copy number. The equation for the standard curve was $y = -3.493x + 36.627$. The slope of the line corresponded to an amplification efficiency of 0.93.

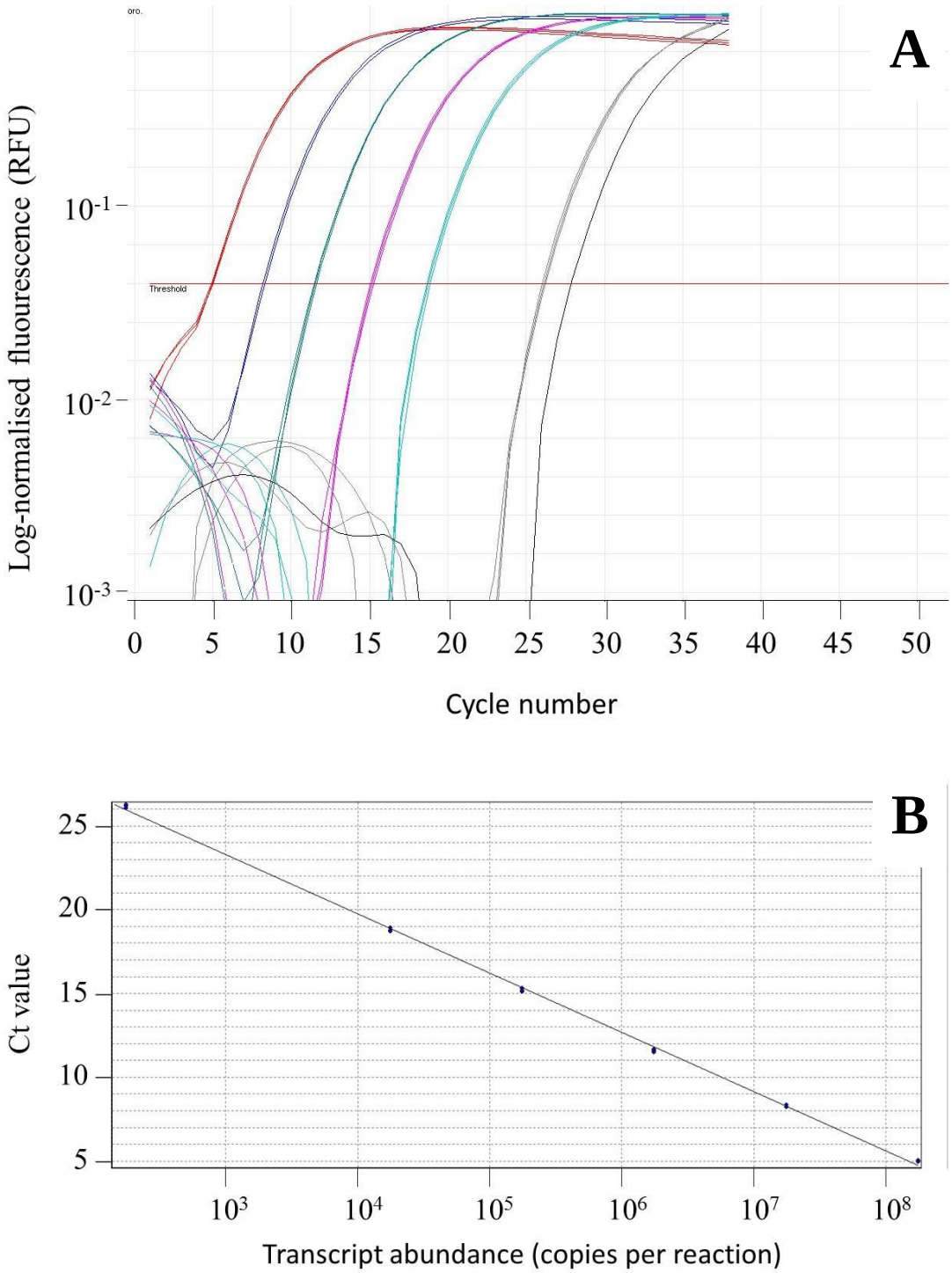


Figure 2.19: Fluorescence curve (A) and standard curve (B) obtained using serial dilutions of a purified PGC-1α PCR product from SBT liver tissue (obtained using the PGC-1α2 primers) as the template for qRT-PCR. The cycle at which the fluorescence intensity crossed a defined threshold (Ct value) was calculated using the fluorescence curve. The standard curve was constructed by plotting the Ct values against the copy number. The equation for the standard curve was $y = -3.536x + 33.893$. The slope of the line corresponded to an amplification efficiency of 0.92.

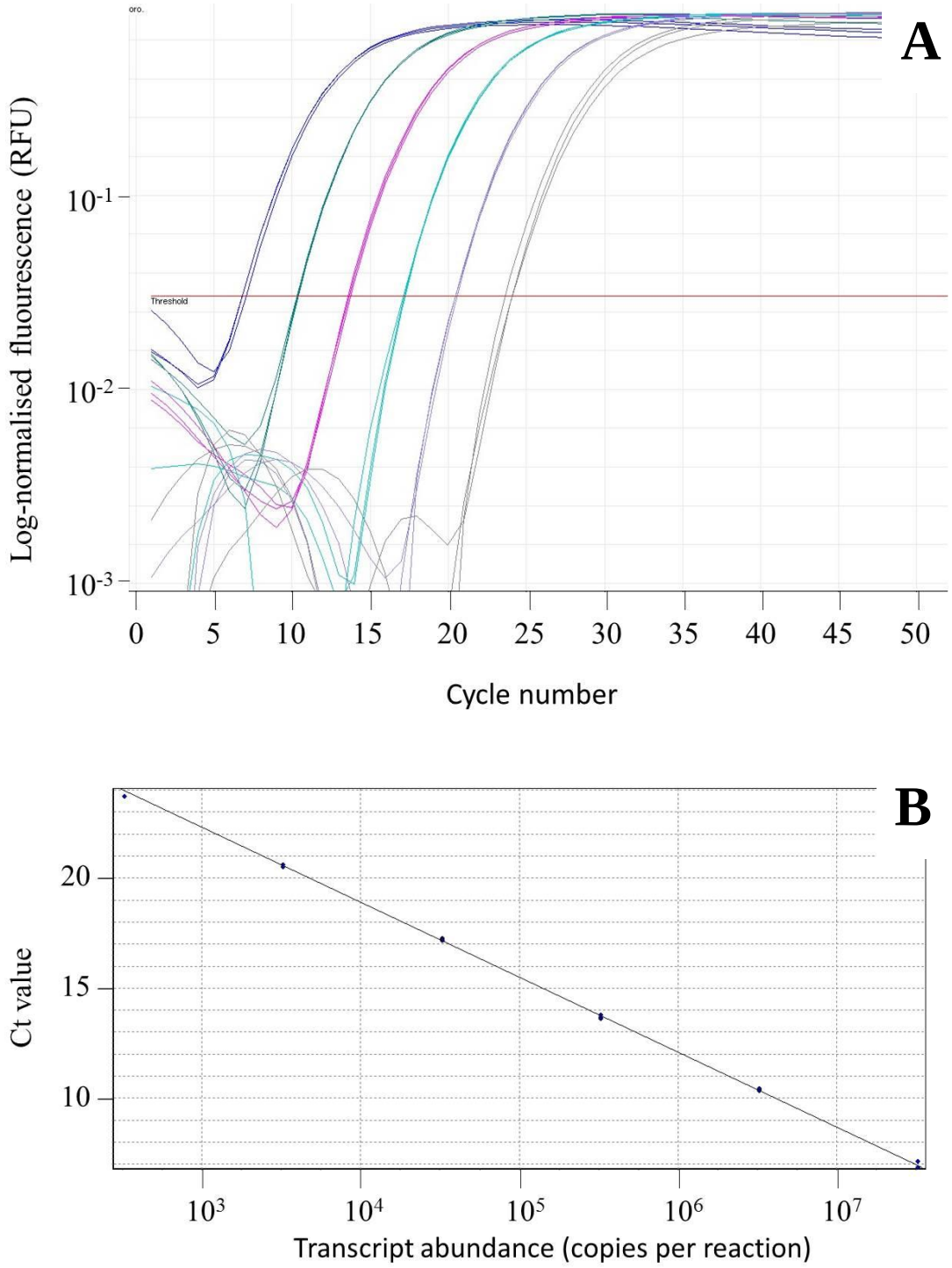


Figure 2.20: Fluorescence curve (A) and standard curve (B) obtained using serial dilutions of a purified PPARγ PCR product as the template for qRT-PCR. The cycle at which the fluorescence intensity crossed a defined threshold (Ct value) was calculated using the fluorescence curve. The standard curve was constructed by plotting the Ct values against the copy number. The equation for the standard curve was $y = -3.405x + 32.521$. The slope of the line corresponded to an amplification efficiency of 0.97.

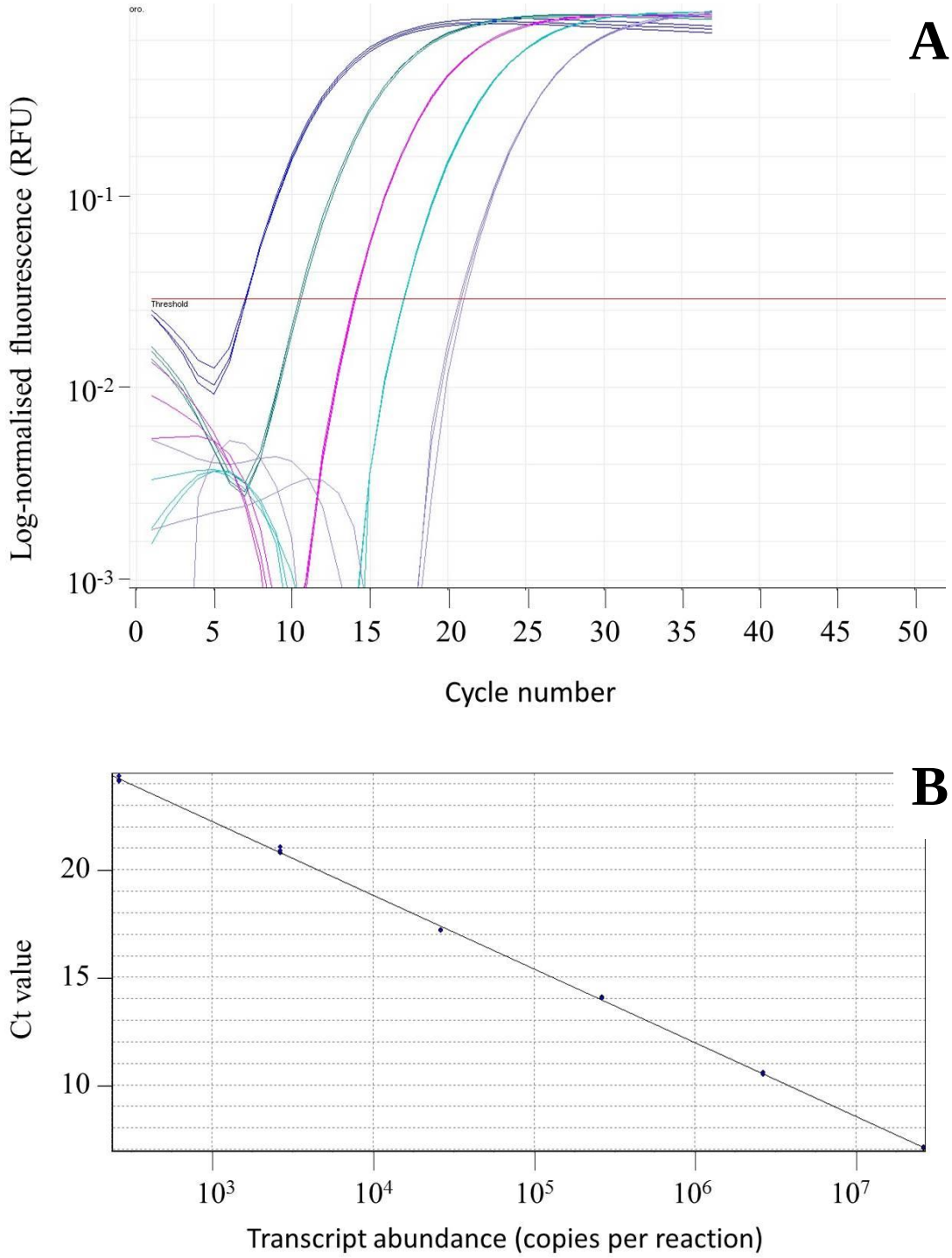


Figure 2.21: Fluorescence curve (A) and standard curve (B) obtained using serial dilutions of a purified CS PCR product as the template for qRT-PCR. The cycle at which the fluorescence intensity crossed a defined threshold (Ct value) was calculated using the fluorescence curve. The standard curve was constructed by plotting the Ct values against the copy number. The equation for the standard curve was $y = -3.476x + 32.457$. The slope of the line corresponded to an amplification efficiency of 0.94.

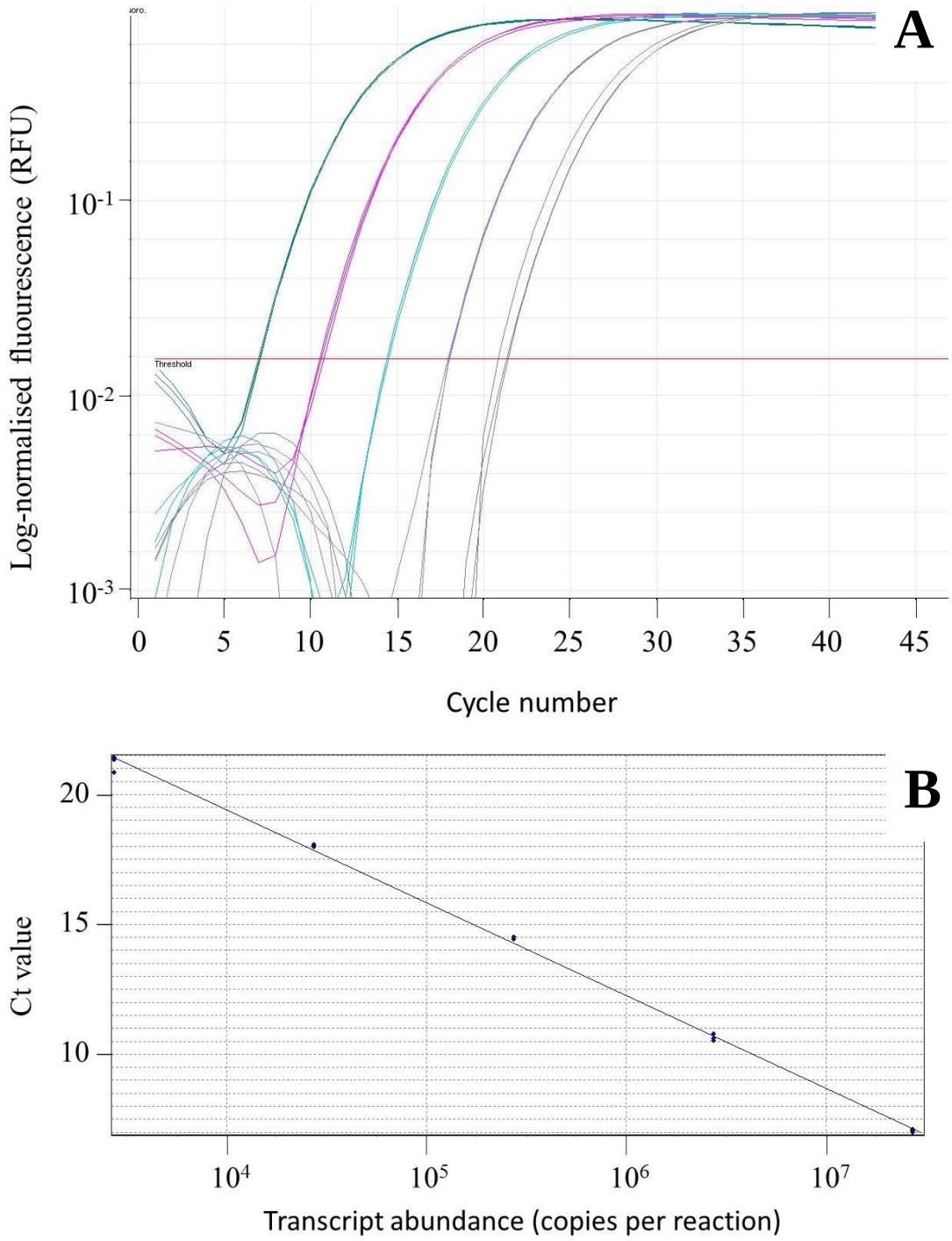


Figure 2.22: Fluorescence curve (A) and standard curve (B) obtained using serial dilutions of a purified COX1 PCR product as the template for qRT-PCR. The cycle at which the fluorescence intensity crossed a defined threshold (Ct value) was calculated using the fluorescence curve. The standard curve was constructed by plotting the Ct values against the copy number. The equation for the standard curve was $y = -3.569x + 33.676$. The slope of the line corresponded to an amplification efficiency of 0.91.

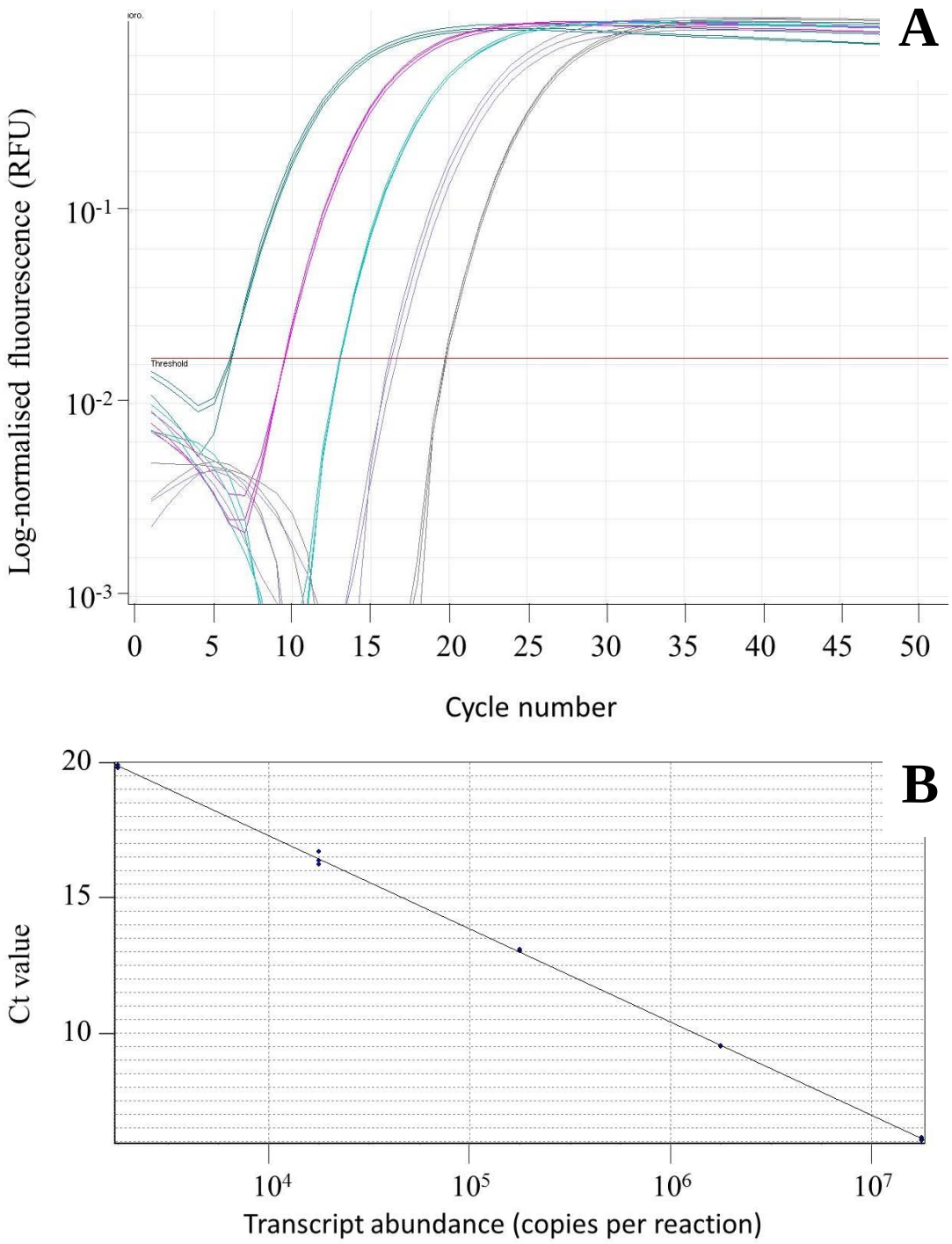


Figure 2.23: Fluorescence curve (A) and standard curve (B) obtained using serial dilutions of a purified UCP2 PCR product as the template for qRT-PCR. The cycle at which the fluorescence intensity crossed a defined threshold (Ct value) was calculated using the fluorescence curve. The standard curve was constructed by plotting the Ct values against the copy number. The equation for the standard curve was $y = -3.434x + 31.008$. The slope of the line corresponded to an amplification efficiency of 0.96.

Table 2.1: The amplification efficiencies of the primer sets selected for analysis of gene expression by qRT-PCR. Product size was determined by sequencing. The amplification efficiencies were estimated from the slopes of the linear regressions from the standard curves (Figs. 2.14-2.23). Efficiencies in the range of 0.9 – 1.0 were considered acceptable for use in qRT-PCR.

Target gene	Calculated Product size (bp)	Amplification Efficiency
β-Actin	155	0.91
ELF-1α	119	0.94
Δ6Fads	154	0.96
Elovl5	189	1.0
PGC-1α1	131	0.93
PGC-1α 2	135	0.92
PPARγ	129	0.95
CS	165	0.96
COX1	143	0.91
UCP2	157	0.96

2.5.10 Melt curve analysis

At the conclusion of each qRT-PCR reaction, a melt curve was produced by heating the reaction mixtures to 72°C for 45 seconds and then raising the temperature by 1°C every 5 seconds until the temperature reached 95°C and measuring the change in fluorescence during this time. PCR products with different nucleotide sequences dissociate at different temperatures and thus this method provided a simple way to verify that a single product was produced by the qRT-PCR reaction. The melt curves for each of the primer sets are shown in Figs. 2.24-2.34 and each shows a single dominant peak with one exception. The first set of primers for PGC-1α (PGC-1α1) produced a single product when used with the SBT-E1 cell line cDNA as template (Fig. 2.28). However, when they were used with the cDNA template from SBT tissue they produced multiple products (Fig. 2.29). Therefore, a second set of primers for PGC-1α was designed (PGC-1α2) and this second set of primers amplified a single product using the cDNA template from the SBT tissue (Fig. 2.30).

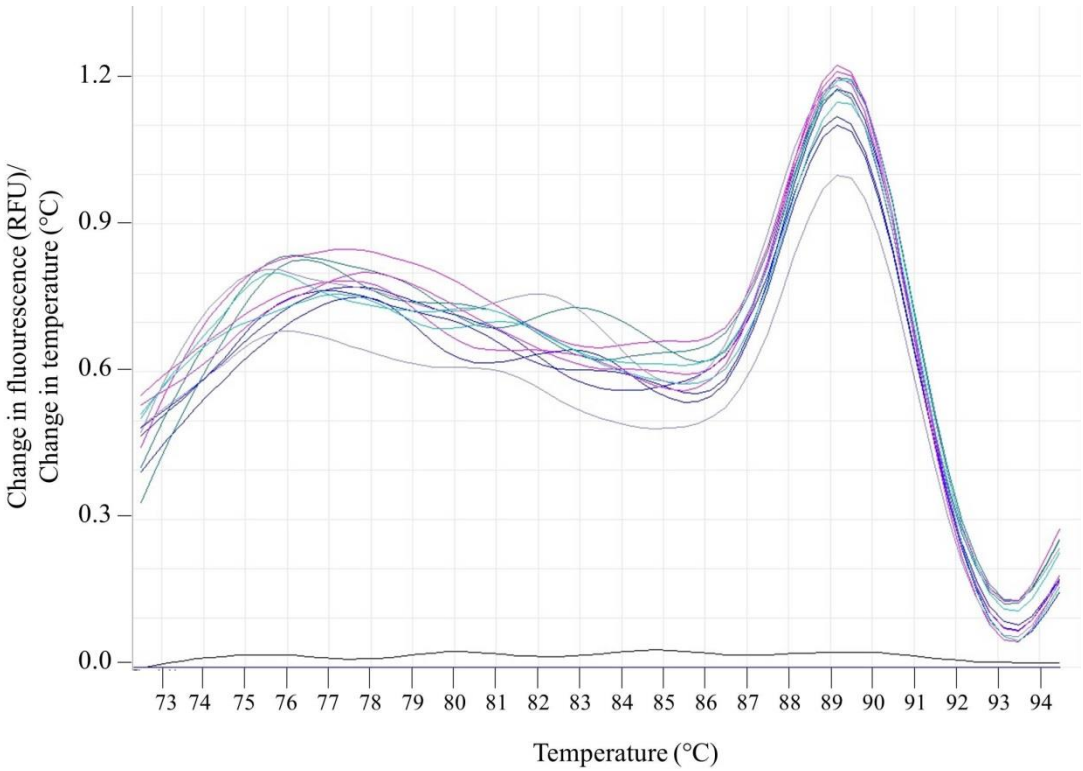


Figure 2.24: Melt curve for β-actin amplicons produced using qRT-PCR. Peaks on the graph correspond to the dissociation of different double stranded DNA products. The graph shows a single dominant peak at approximately 89.2°C, which shows that a single dominant product was produced by the qRT-PCR reaction.

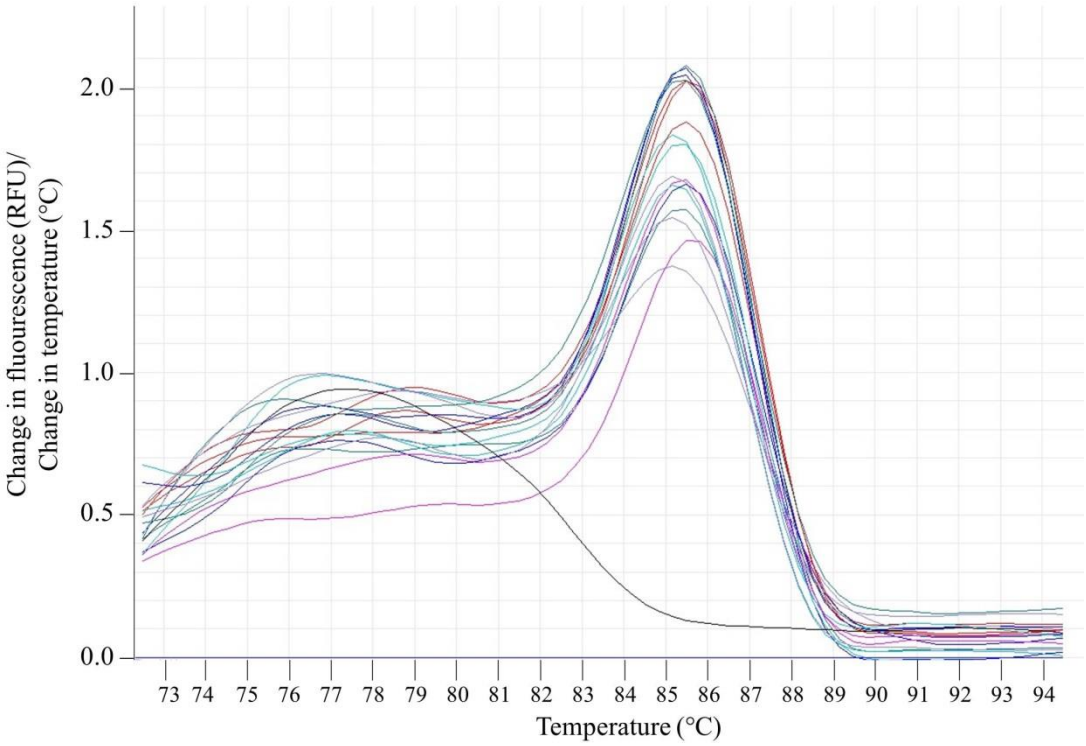


Figure 2.25: Melt curve for ELF-1α amplicons produced using qRT-PCR. Peaks on the graph correspond to the dissociation of different double stranded DNA products. The graph shows a single dominant peak at approximately 85.5°C, which shows that a single dominant product was produced by the qRT-PCR reaction.

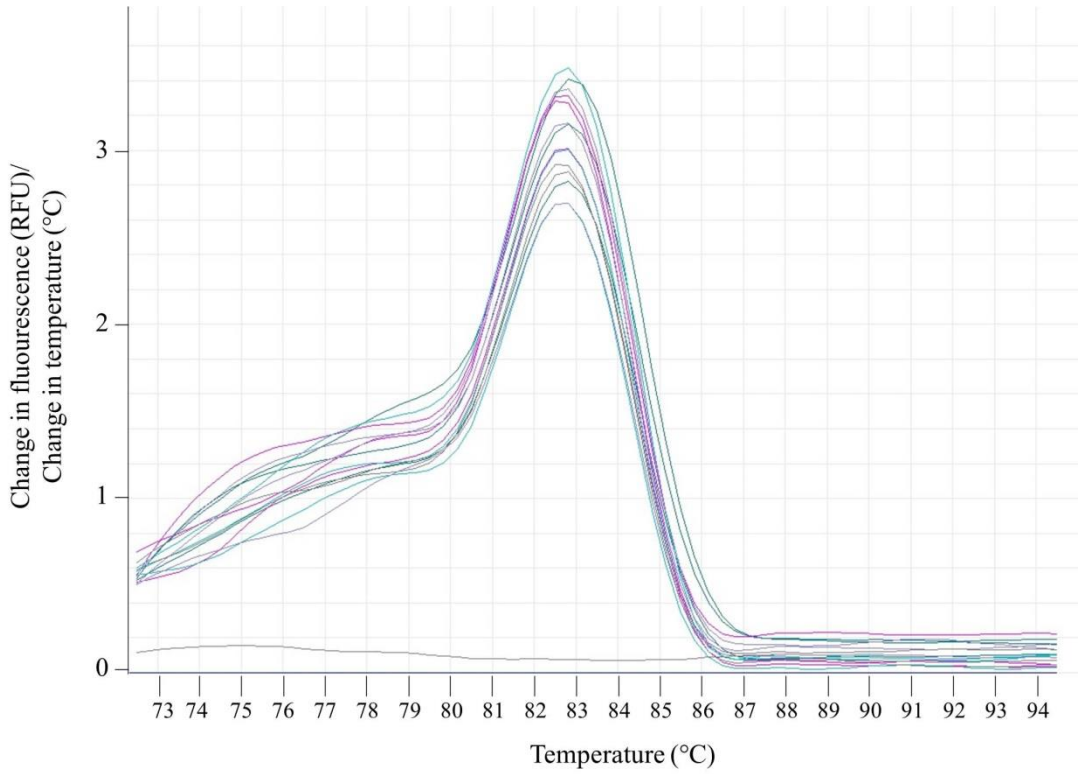


Figure 2.26: Melt curve for Δ6Fads amplicons produced using qRT-PCR. Peaks on the graph correspond to the dissociation of different double stranded DNA products. The graph shows a single dominant peak at approximately 82.7°C, which shows that a single dominant product was produced by the qRT-PCR reaction.

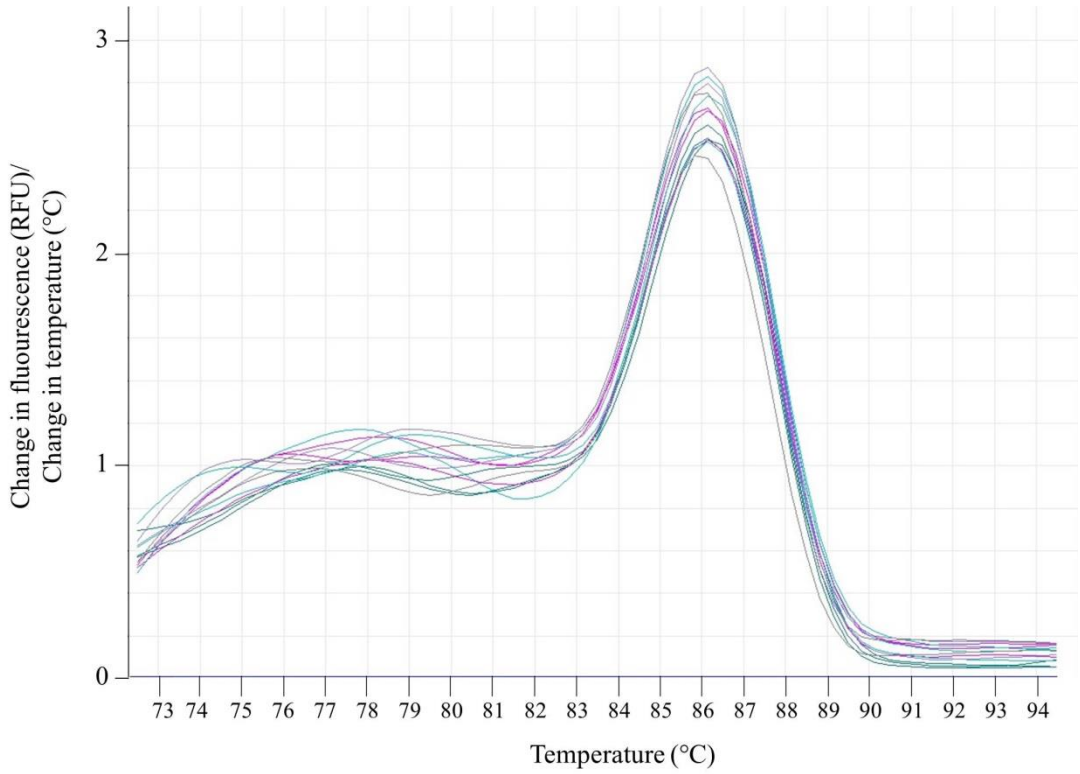


Figure 2.27: Melt curve for Elovl5 amplicons produced using qRT-PCR. Peaks on the graph correspond to the dissociation of different double stranded DNA products. The graph shows a single dominant peak at approximately 86.1°C, which shows that a single dominant product was produced by the qRT-PCR reaction.

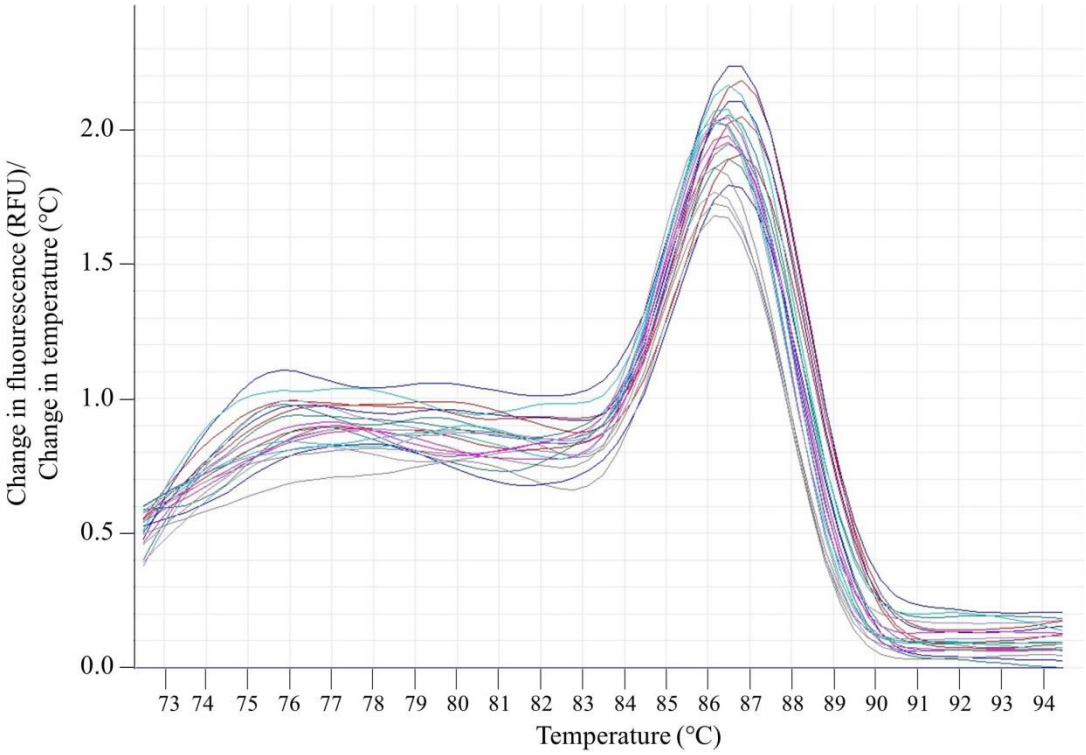


Figure 2.28: Melt curve for amplicons that were obtained using the PGC-1α1 set of qRT-PCR primers using cDNA from the SBT-E1 cell line. Peaks on the graph correspond to the dissociation of different double stranded DNA products. The graph shows a single dominant peak at approximately 86.5°C, which shows that a single dominant product was produced by the qRT-PCR reaction.

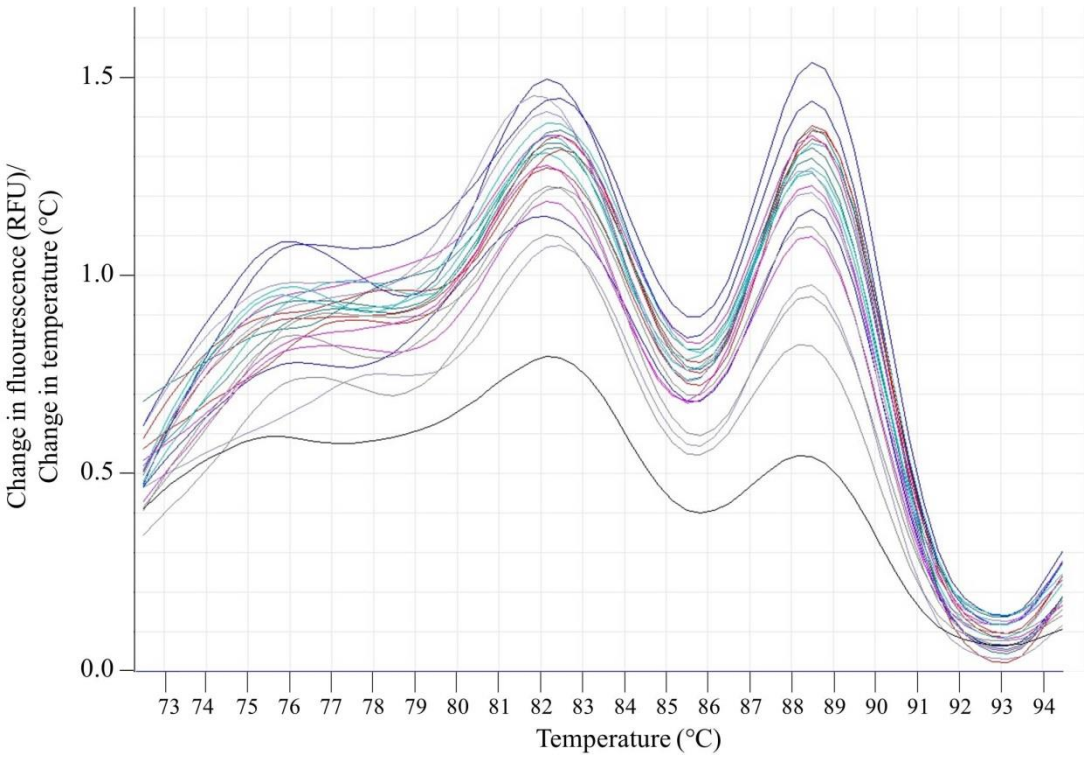


Figure 2.29: Melt curve for amplicons that were obtained using the PGC-1α1 set of qRT-PCR primers using cDNA from SBT liver tissue. Peaks on the graph correspond to the dissociation of different double stranded DNA products. The graph shows two dominant peaks at approximately 82.2°C and 88.5°C, which shows that multiple products were produced by the qRT-PCR reaction.

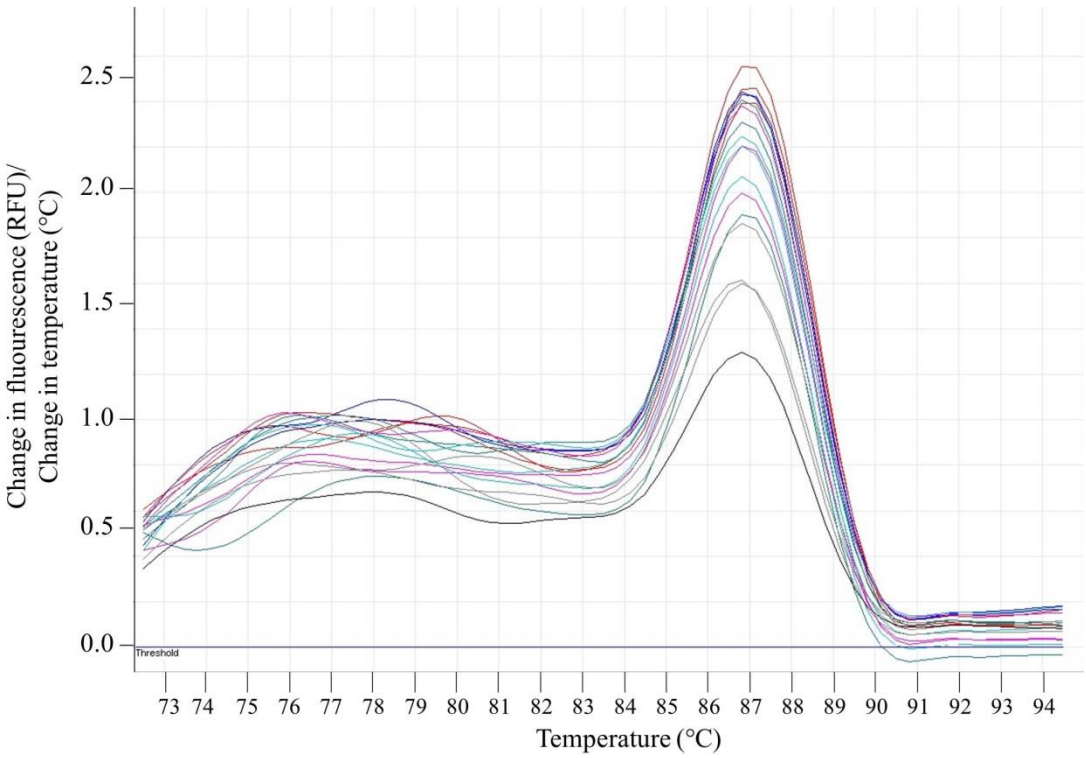


Figure 2.30: Melt curve for amplicons that were obtained using the PGC-1α2 set of qRT-PCR primers using cDNA from SBT liver tissue. Peaks on the graph correspond to the dissociation of different double stranded DNA products. The graph shows a single dominant peak at approximately 86.9°C, which shows that a single dominant product was produced by the qRT-PCR reaction.

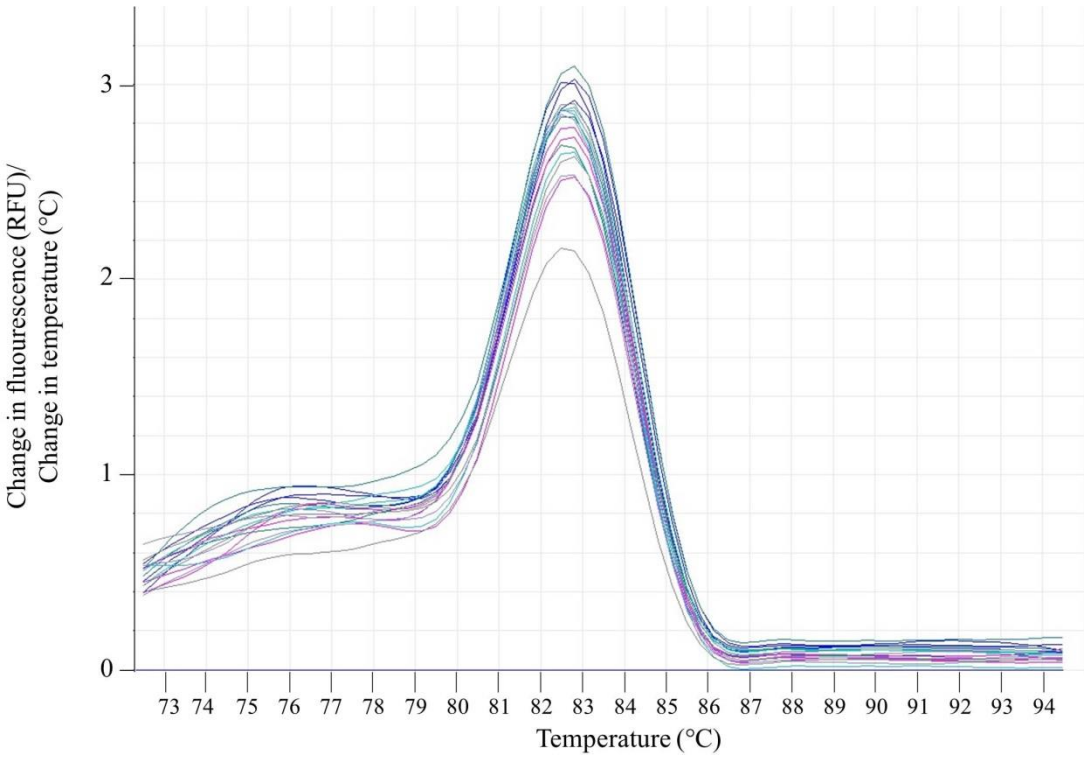


Figure 2.31: Melt curve for PPARγ amplicons produced using qRT-PCR. Peaks on the graph correspond to the dissociation of different double stranded DNA products. The graph shows a single dominant peak at approximately 82.7°C, which shows that a single dominant product was produced by the qRT-PCR reaction.

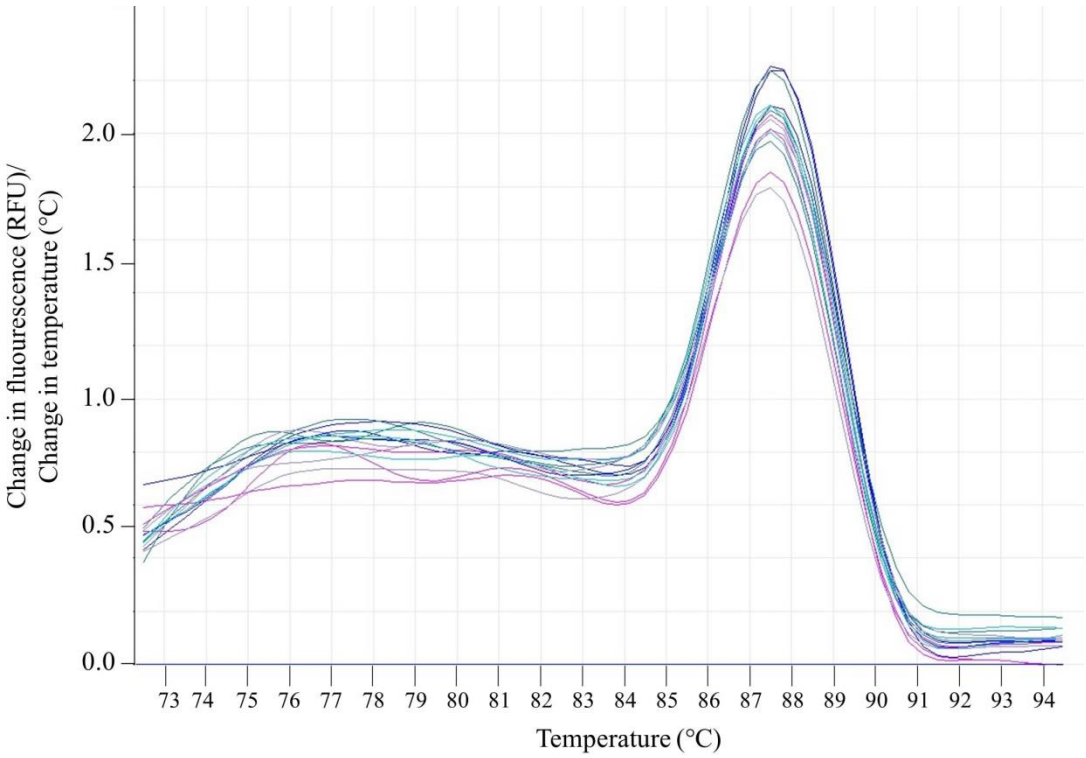


Figure 2.32: Melt curve for CS amplicons produced using qRT-PCR. Peaks on the graph correspond to the dissociation of different double stranded DNA products. The graph shows a single dominant peak at approximately 87.5°C, which shows that a single dominant product was produced by the qRT-PCR reaction.

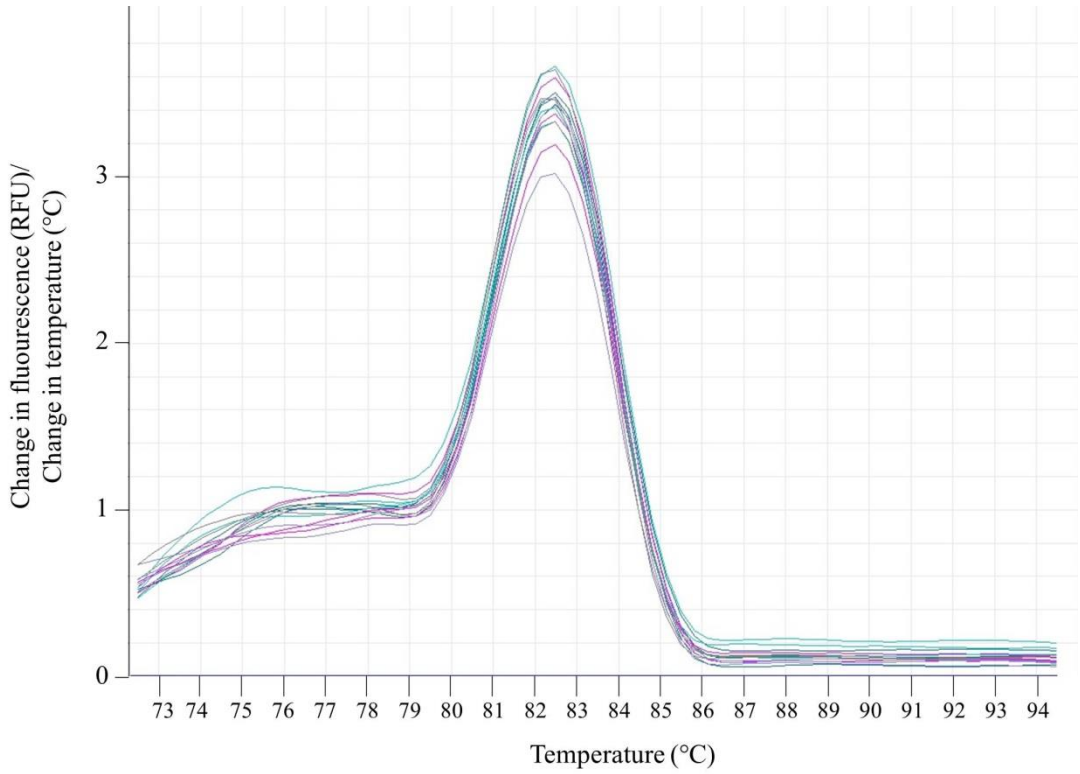


Figure 2.33: Melt curve for COX1 amplicons produced using qRT-PCR. Peaks on the graph correspond to the dissociation of different double stranded DNA products. The graph shows a single dominant peak at approximately 82.4°C, which shows that a single dominant product was produced by the qRT-PCR reaction.

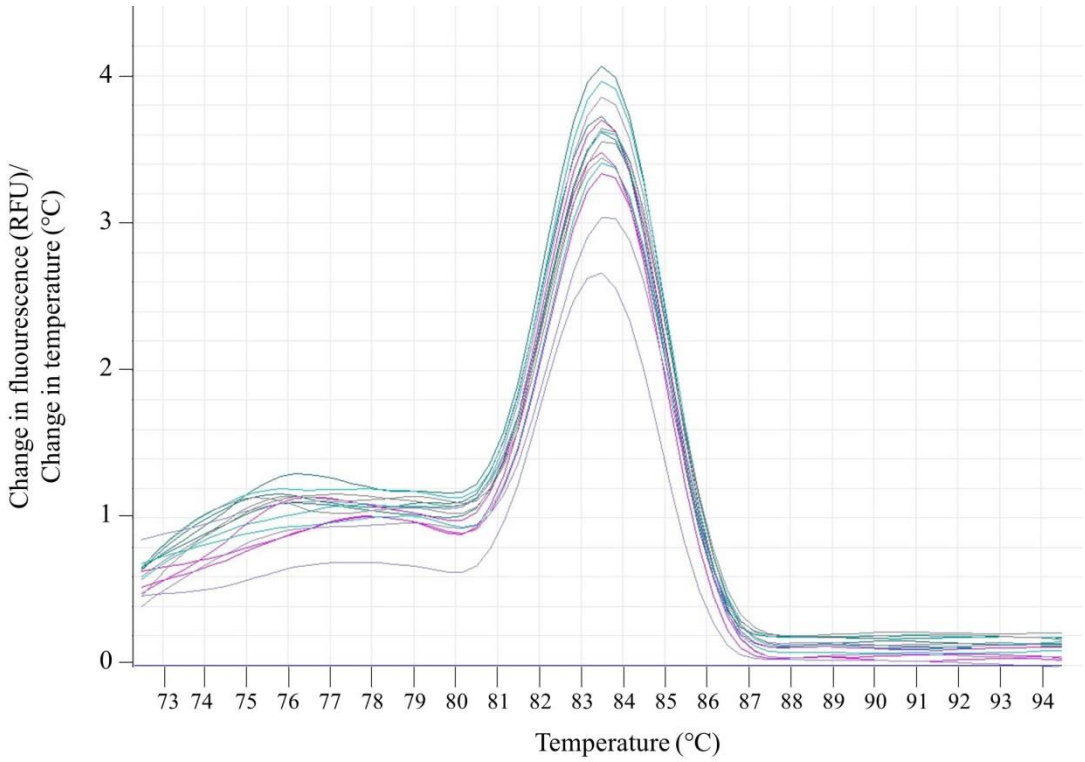


Figure 2.34: Melt curve for UCP2 amplicons produced using qRT-PCR. Peaks on the graph correspond to the dissociation of different double stranded DNA products. The graph shows a single dominant peak at approximately 83.5°C, which shows that a single dominant product was produced by the qRT-PCR reaction.

2.6 Gene expression analyses using qRT-PCR

2.6.1 Supplementation of the SBT-E1 cells with PUFA

The cells were cultured under the experimental cell culture conditions (Section 2.2) in culture medium containing either 10% (v/v), 5% (v/v) or 2% (v/v) FBS. The cells were supplemented with 125 µM vitamin E and for treatments containing PUFA, a final concentration 10 µM of 18:3n-3, 18:2n-6, 20:5n-3, 20:4n-6 or 22:6n-3 was added as described in Section 2.4.1. Cells were then cultured at 25°C for 1 day (cells supplemented with 10% (v/v) FBS or 2% (v/v) FBS with and without PUFA) or 3 days (cells supplemented with 5% (v/v) FBS with and without PUFA). At the conclusion of the experiment, the cells were harvested according to the protocol described in Section 2.2.4.

2.6.2 Harvest of SBT tissues

2.6.2.1 Harvest of tissues from farmed SBT

Tissue samples from farmed SBT were collected during a commercial harvest from a sea cage off the coast of Port Lincoln, South Australia in June 2014. Three fish were sampled with fork lengths of 120.7 ± 4.8 cm and gilled and gutted weights of 40.0 ± 6.1 kg. Samples of liver, muscle (tail cut, Fig. 2.35), heart and gill were collected. All tissues were placed in RNA*later*® at ambient temperature before the tissue was transported back to Flinders University and stored at -20°C.

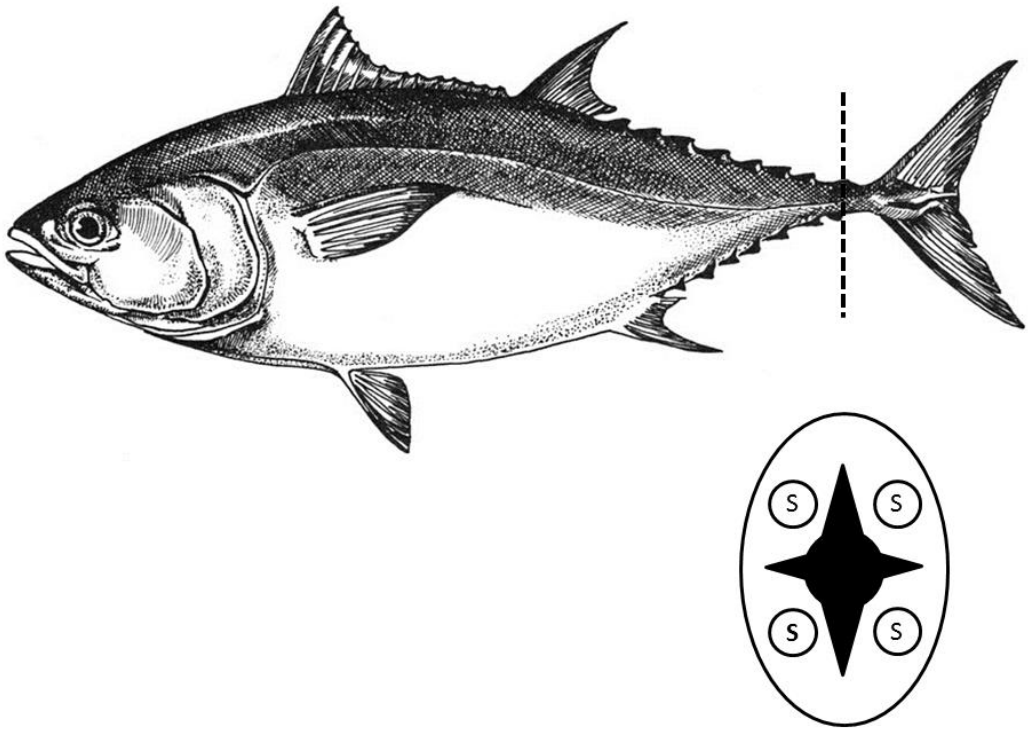


Figure 2.35: Locations of samples taken from the muscle of a farmed SBT specimen. The tail was removed during the harvest process and small cores of muscle tissue were obtained from the tail cut from the four areas around the spine as indicated by the (S) symbols. The tuna image was obtained from http://mvgazette.com/

2.6.2.2 Harvest of tissues from wild-caught SBT

Tissue samples from wild-caught SBT were collected by Dr Alistair Hobday (Commonwealth Scientific and Industrial Research Organisation, Marine and Atmospheric Research) in December 2014. The three fish had fork lengths of 72.3 ± 0.9 cm and were captured in the Great Australian Bight (Fig. 2.36) using a pole and line. Samples of liver, white muscle and red muscle were collected and placed in RNA*later*®. Samples were kept for >1 hour at room temperature before being frozen at -20°C on board the boat before they were transported to Flinders University and stored at -20°C.

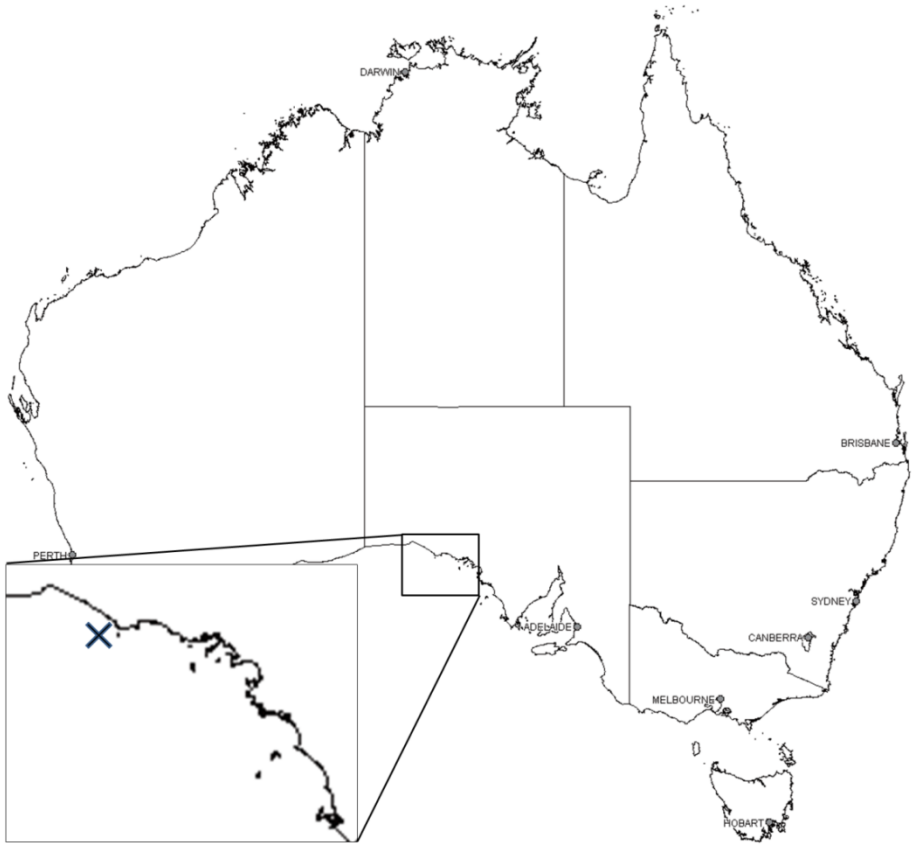


Figure 2.36: The sampling site for wild-caught SBT in the Great Australian Bight. Map adapted from image obtained from http://www.lib.unimelb.edu.au/

2.6.3 RNA extraction from the SBT-E1 cells

RNA was extracted from the SBT-E1 cells (Section 2.6.1) according to the protocol described in Section 2.5.4.

2.6.4 RNA extraction from the SBT tissues

The tissues from Section 2.6.2 were removed from the -20°C storage, taken out of RNA*later*® and were dabbed dry on a paper towel. The tissue was ground to a fine powder in liquid nitrogen in a mortar and pestle. Between 15 and 30 mg of each tissue was placed in a liquid nitrogen-cooled 1.5 mL tube after the liquid nitrogen had evaporated but before the tissue had thawed and RNA was extracted as described in Section 2.5.5. The concentration of the eluted RNA was estimated by using a NanoDrop 1000 spectrophotometer and the RNA was frozen at -80°C or used immediately for cDNA synthesis.

2.6.5 cDNA synthesis

A total of 1 μg of RNA was reverse transcribed using the protocol described in Section 2.5.6.

2.6.6 qRT-PCR

Gene expression analysis by qRT-PCR was conducted using a Platinum® SYBR® Green qPCR SuperMix-UDG kit (Invitrogen) according to the manufacturer's protocol except that a half volume (25 μl) reaction size was used. Each reaction contained the cDNA reverse-transcribed from 50 ng of RNA and 125 nM (each) of one of the pairs of primers listed in Table 2.2. The qRT-PCR reactions were performed and the resulting data were analysed using a Rotorgene 3000 thermal cycler (Corbett Research). The Ct values produced from the fluorescence curves

were used to determine the relative quantities of the transcripts from each of the target genes. The specific method of quantification is described in the appropriate results chapters.

2.7 Cloning of the PGC-1α cDNA

2.7.1 Design of primers

A search of the NCBI Nucleotide database was conducted for PGC-1α sequences from fish. The sequences were aligned using ClustalX2 (Larkin *et al.* 2007) and annotated using GeneDoc (Nicholas *et al.* 1997). Primers were designed using the Primer3 software (Koressaar and Remm 2007, Untergasser *et al.* 2012). Each primer set was further analysed using the OligoAnalyser® 3.1 software (Integrated DNA Technologies) to ensure the selected primer pairs annealed to DNA with a similar melting temperature with minimal potential to form primer dimers within the reaction mixtures. Following analysis, primer sets designed to anneal to selected regions of high conservation within the PGC-1α cDNA were chosen. The primers were then synthesised by GeneWorks (Thebarton, South Australia). A summary of the primers used to clone various fragments of the PGC-1α cDNA is shown in Table 2.5.

Table 2.5: A summary of the primer sets used to determine the sequence of the PGC-1α cDNA from SBT. Degenerate bases are indicated by R (A/G), Y (C/T) or W (A/T).

Primer name	**Direction**	**Sequence (5' → 3')**
PGC1A_5P_F1	Forward	ATGGCGTGGGACAGGTGTA
PGC1A_5P_F2	Forward	AGGACTCGGTGTGGAGAGAA
PGC1A_5P_F3	Forward	CTGCCTTGGTTGGTGAAGAC
PGC1A_C_F1	Forward	CCTCGTTTTCCTCCCTCTCC
PGC1A_C_F2	Forward	ACAARGGATCWCCGTTTGAG
PGC1A_C_F3	Forward	CATGAGTACAGCAGCGGAAA
PGC1A_3P_F1	Forward	CCCTTGARAAYGGACACACC
PGC1A_5P_R1	Reverse	AGTCGCTGACATCCAGCTCT
PGC1A_5P_R2	Reverse	CTGTAAGCACTGCCAGCAAG
PGC1A_C_R1	Reverse	GCTGCTGCTGTTGTTGTTGT
PGC1A_C_R2	Reverse	GATCCYTTGTGGTCATTTG
PGC1A_C_R3	Reverse	CTGCTTAGCCAAAGGACCAG
PGC1A_3P_R1	Reverse	AGTCGTCAGAATGGGAGTCC
PGC1A_3P_R2	Reverse	GTGGAGGCTGGATCAAAGTC

2.7.2 RNA extraction

Total RNA was extracted from SBT liver tissue using a RNeasy® Mini kit (Qiagen) according to the manufacturer's protocol. Optional steps in the protocol were completed as described in Section 2.5.4. The quantity of RNA in the eluate was estimated using a NanoDrop 1000 spectrophotometer (Thermo Scientific).

2.7.3 cDNA synthesis

A total of 4 μg of RNA was reverse transcribed to create cDNA as described in Section 2.5.6.

2.7.4 PCR amplification and gel electrophoresis

The PCR reactions required to amplify the target genes from the cDNA were conducted in 200 μl PCR tubes (Qiagen) for each primer set using a Phusion® high fidelity PCR kit (NEB) according to the manufacturer's instructions. The PCR cycling conditions are described in detail in Chapter 6. At the conclusion of each run, a 5 μl subsample of the PCR reaction mixture was analysed using gel electrophoresis and photographed as described in Section 2.3.2.2.

2.7.5 PCR product purification, sequencing and analysis

The PCR products from Section 2.7.4 were purified and sequenced according to the protocol described in Sections 2.3.2.3-2.3.2.4. The sequence obtained using each of the reverse primers was converted to the reverse complement as described in Section 2.3.2.5. Overlapping regions of the sequences obtained using each of the primer pairs were aligned to create the complete PGC-1α cDNA sequence.

2.8 Statistical analyses

Statistical analyses were conducted using the statistical package PASW Statistics 18 (SPSS Inc., Chicago). Specific details of the statistical analyses are included in the relevant chapters. Significant differences are reported when $p < 0.05$.

Chapter 3 – Effects of PUFA and vitamin E supplementation on cell proliferation and PUFA incorporation into cellular lipids in the SBT-E1 cell line

A significant portion of this chapter has been published[1] and a copy of the published paper can be found in Appendix A.

[1]Reference: Scholefield, A. M., Schuller, K. A., 2014, Cell proliferation and long chain polyunsaturated fatty acid metabolism in a cell line from southern bluefin tuna (*Thunnus maccoyii*), *Lipids*, 49(7), 703-714. The final publication is available at Springer via http://dx.doi.org/10.1007/s11745-014-3910-y.

3.1 Aims and background

The aim of this chapter was to investigate the effects of different fatty acid supplements on the cell proliferation and fatty acid incorporation into cellular lipids in the SBT-E1 cell line.

Previous studies have shown that supplementation of serum-deprived Atlantic salmon or turbot cell lines (i.e. cells cultured in reduced concentrations of FBS) with 18:3n-3 and 18:2n-6 can increase the proliferation of the cells (Tocher and Dick 1991). In cell lines from rainbow trout and turbot, the supplementation of the cells with 18:3n-3 and 18:2n-6 dramatically increased the concentrations of these fatty acids in their cellular lipids (Tocher *et al.* 1989). Furthermore, there are substantial differences in the capacity for fish cell lines to desaturate and elongate fatty acids (e.g. the desaturation and elongation of 18:3n-3 to the LC-PUFA 20:5n-3 and 22:6n-3) (Ghioni *et al.* 1999, Tocher and Ghioni 1999, Stubhaug *et al.* 2005). These differences appear to be dependent on trophic level of the species from which the cell line originated (i.e. higher trophic level fish have lower capacity for fatty acid desaturation and elongation). However, this has not yet been investigated in tunas. The work described in this chapter utilised a recently developed SBT cell line, SBT-E1. This is the first continuous cell line for any tuna species. Thus, it presents a unique opportunity as a model for investigating fatty acid metabolism and its regulation in tunas.

It was hypothesised that:

1. The proliferation of the SBT-E1 cells would be reduced under culture conditions deprived of fatty acids (i.e. reduced FBS concentration)

2. Addition of the fatty acid supplements to cells deprived of fatty acids would increase proliferation of the cells
3. The fatty acid profiles of the culture media would be reflected in the fatty acid profiles of the cells.
4. The individually supplied fatty acid supplements would be incorporated into the cells

3.2 Materials and Methods

3.2.1 Cell line and culture medium

The SBT-E1 cells were routinely cultured and maintained as described in Section 2.1.

3.2.2 Verification of the neutral red uptake (NRU) assay of cell proliferation

To quantify the effects of different fatty acids on cell proliferation, a rapid technique for viable cell estimation were required. The neutral red uptake (NRU) assay as described by Repetto *et al.* (2008) was chosen as a suitable method. Briefly, cells were detached from a >80% confluent 25 cm^2 or 75 cm^2 flask and resuspended in standard cell culture medium except with the FBS concentration reduced to 2% (v/v). Viable cell counts were conducted using Trypan blue dye as described in Section 2.2.2. Cells were seeded into a 96 well plate in triplicate wells for concentrations ranging from 0-250,000 cells/cm^2 (0-80,000 cells/well) to create a standard curve. After 4 hours, the medium was decanted and replaced with NRU assay medium (standard culture medium but with the FBS concentration reduced to 5% (v/v) and 10 μg/mL of Neutral Red (Sigma-Aldrich) added). The cells were then incubated for two hours at 25°C before being the monolayer was washed twice with 100 μl of PBS. Finally, the incorporated stain was solubilised with 100 μl of de-stain solution (50%

(v/v) ethanol, 5% (v/v) acetic acid) with shaking for 5 min and the absorbance was measured at 540 nm using a plate reader. A standard curve with linear regression fit was created using the known cell concentrations and unknown cell numbers were calculated using the equation of the line.

3.2.3 Cell proliferation experiments – testing the effects of different concentrations of FBS

Cells from stock culture flasks were seeded into 96-well plates in 100 µl of the standard culture medium but with the FBS concentration reduced to 2% (v/v) as described in Section 2.2. After the cells had adhered, the medium was removed and replaced with 100 µl per well of the standard cell culture medium but with the FBS concentration set at 1, 2, 5 or 10% (v/v). There were 10 plates with 3 replicate wells per plate for each treatment. One plate was sacrificed each day for 10 days to estimate cell proliferation using the NRU assay (Repetto *et al.* 2008) as described in Section 3.2.2.

3.2.4 Cell proliferation experiments – testing the effects of different PUFA

Cells from stock culture flasks were seeded into 96-well plates and incubated at 25°C to allow the cells to adhere to the wells as described in Section 2.2. The medium was then removed and replaced with 100 µl per well of the standard culture medium but with the FBS concentration reduced to 2%. For treatments containing fatty acids 2.5-20 µM 18:3n-3, 18:2n-6, 20:5n-3, 20:4n-6 or 22:6n-3 added as described in Section 2.4.1. There were 8 plates with 3 replicate wells per plate for each treatment. One plate was sacrificed each day for 8 days to estimate cell proliferation using the NRU assay as described in Section 3.2.2.

3.2.5 Cell proliferation experiments – testing the effects of different PUFA in the presence of vitamin E

Cells from stock culture flasks were seeded into 96-well plates in culture medium containing 2% (v/v) FBS as described in Section 2.2. After the cells had adhered to the wells, the medium was decanted and replaced with 100 μl per well of the standard culture medium (10% (v/v) FBS control) or standard culture medium but with the FBS concentration reduced to 2% (v/v). For treatments containing fatty acids 2.5-20 μM 20:5n-3, 20:4n-6 or 22:6n-3 added as described in Section 2.4.1. For each treatment there were 6 wells per plate that contained the FBS/fatty acid supplement. Three of these wells contained no vitamin E and the remaining 3 wells had vitamin E added at a final concentration of 2 mM as described in Section 2.4.1. A total of 10 identical plates were prepared and one plate was sacrificed each day for 10 days to estimate cell proliferation using the NRU assay as described in Section 3.2.2.

3.2.6 Vitamin E titration experiments

To determine the optimum concentration of vitamin E to prevent the apparent oxidative stress caused by 22:6n-3 (see Results and Discussion), a range of vitamin E concentrations from 0-2 mM was tested, in treatments with and without 22:6n-3. To do this, cells from stock culture flasks were seeded into 24-well plates (Nunc™) in the standard culture medium but with the FBS concentration reduced to 2% (v/v). Once the cells had adhered to the wells (after 4 hours), the medium was removed and replaced with 600 μl of the standard culture medium but with the FBS concentration reduced to 2% (v/v) and various concentrations of vitamin E added with or without 20 μM 22:6n-3 as described in Section 2.4.1. There were 3 replicate wells for each treatment. The cells were then cultured for 4 days before they were counted using the

NRU assay as described in Section 3.2.2, or harvested and counted using Trypan blue dye as described in Section 2.2.2.

3.2.7 Fatty acid profiling: The effects of vitamin E

Cells from stock culture flasks were seeded into 6-well plates in the standard culture medium but with the FBS concentration reduced to 2% (v/v). Once the cells had adhered (after 4 hours), the medium was removed and replaced with 3 mL of the standard culture medium but with the following modifications. The FBS concentration was reduced to 2% (v/v) in all treatments. For treatments containing PUFA, 10 μM 22:6n-3 was added and for treatments containing Vitamin E, either 125 μM or 500 μM was added as described in 2.4.1. The cells were allowed to proliferate for 4 days before they were harvested as described in Section 2.2.4. For each treatment, 3 wells were combined to provide sufficient cells for lipid extraction. This resulted in 3 replicates for each treatment. A 20 μl subsample of each cell suspension was taken to estimate viable cell number using Trypan blue dye as described in Section 2.2.2 and the remainder was used for fatty acid analysis. Total lipid was extracted and fatty acid methyl esters (FAME) were produced and analysed as described in Section 2.4.2.

3.2.8 Fatty acid profiling: The effects of different PUFA

Cells from stock culture flasks were seeded into 6-well plates and left to adhere as described in Section 3.2.7. Vitamin E (125 μM) was added to all treatments and for the PUFA treatments, the FBS concentration was reduced to 2% (v/v). The PUFA (18:3n-3, 18:2n-6, 20:5n-3, 20:4n-6 or 22:6n-3) were added at a concentration of 10 μM as described in Section 2.4.1. The cells were left to proliferate before they were harvested as described in Section 2.2.4. Fatty acids were extracted and FAME were

produced and analysed as described in Section 2.4.2. The peroxidation index was calculated from the fatty acid data using the formula of Hulbert et al. (Hulbert *et al.* 2007) as described in the footnote to Table 3.2.

3.2.9 Statistical analyses

Statistical analyses were conducted as described in Section 2.8. Specifically, significant differences in the data were determined using one-way analysis of variance (ANOVA) followed by *post-hoc* multiple comparisons using the Bonferroni method to correct for multiple-comparison bias (Abdi 2007). Specific details of the statistical analyses are included in the legends to the tables and figures.

3.3 Results

3.3.1 Validation of the NRU assay

A representative standard curve obtained using the NRU assay is shown in Figure 3.1. A line of best fit was added with the equation $y = (8 \times 10^{-6})x + 0.015$ where x is million cells and y is the absorbance at 540 nm. The number of cells correlated well with the absorbance at 540 nm ($r^2 = 0.99$) and showed low levels of variation from the mean up to a cell concentration of 30,000 cells/well (90,000 cells cm^{-2}).

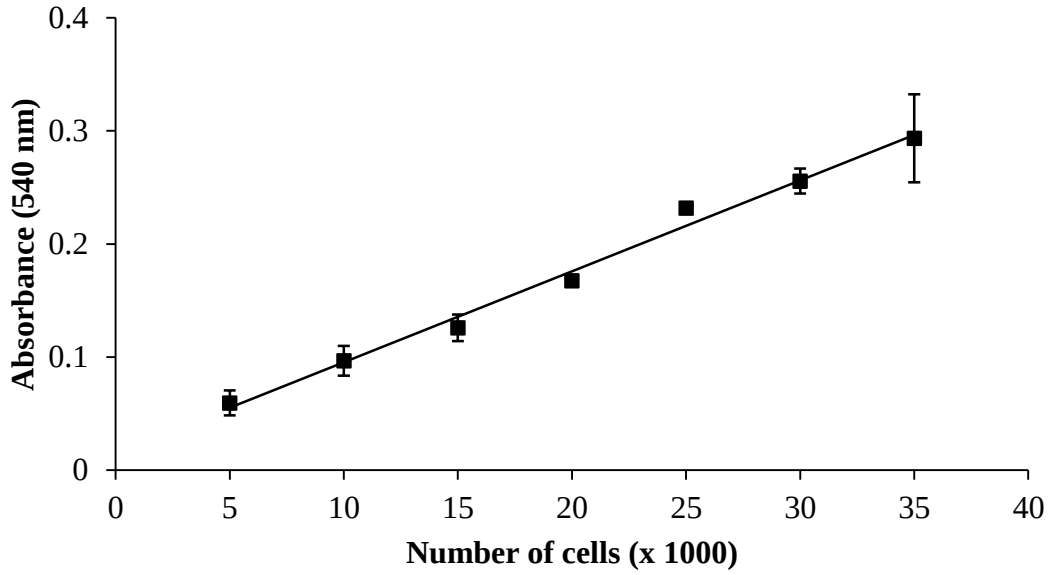


Figure 3.1: A representative standard curve for the neutral red (NR) assay. Cells were seeded into a 96 well plate at densities of 5,000 – 35,000 cells/well. The cells were seeded into the wells in standard cell culture medium but with the FBS concentration reduced to 2% (v/v). After the cells had adhered (approximately 4 h), the culture medium was replaced with the NR assay medium. The cells were washed and the dye was solubilised according to the methods in Section 3.2.2. The absorbance at 540 nm was read using a plate reader and plotted against cell number. Values represent mean +/- the standard error of the mean (n = 3). The equation of the linear regression was $y = (8 \times 10^{-6})x + 0.015$ ($R^2 = 0.99$).

3.3.2 Effects of FBS concentration on cell proliferation

The SBT-E1 cells cultured at FBS concentrations ≥ 2% (v/v) showed higher cell numbers at the end of the experiment compared with the beginning ($p < 0.01$; Fig. 3.2). The 2% (v/v) FBS treatment showed significantly reduced cell proliferation compared to the 5% (v/v) and 10% (v/v) FBS treatments at day 9 ($p < 0.01$). Therefore, 2% (v/v) FBS was chosen as a suitable intermediate concentration for the subsequent PUFA supplementation experiments so that both positive and negative effects of the PUFA on cell proliferation could be seen.

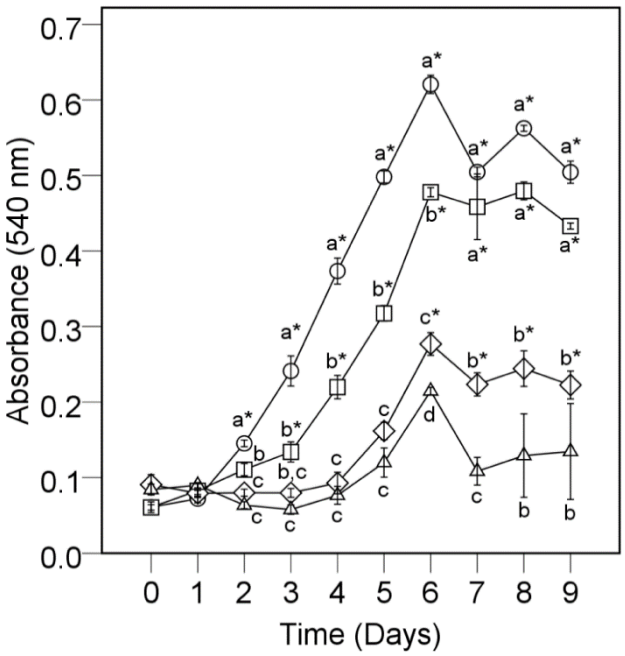


Figure 3.2: The effects of FBS concentration on the proliferation of the SBT-E1 cells in culture. Cells were cultured in 96-well plates at 25°C in 100 µl/well of the standard cell culture medium (unfiled circles), standard cell culture medium but with the FBS concentration reduced to 5% (v/v) (unfilled squares), 2% (v/v) (unfilled diamonds) or 1% (v/v) FBS (unfilled triangles) for up to 9 days. Data are the mean of 3 replicate wells. Error bars show ± standard error of the mean. The data were analysed using two separate one-way ANOVA followed by Bonferroni *post-hoc* comparisons. Significant differences between different FBS concentrations on a particular day are indicated by different letters. Significant differences between a particular day and day zero for a particular FBS concentration are indicated by asterisks.

3.3.3 Effects of PUFA supplementation on cell proliferation

The SBT-E1 cells proliferated well at all fatty acid concentrations for the 18:3n-3 and 18:2 n-6 treatments (Fig. 3.3A, B). The higher concentrations of both 18:3n-3 (20 μM) and 18:2 n-6 (≥10 μM) showed lower proliferation compared to the control during the early stages of the experiment ($p < 0.05$). However this effect was not retained and there were no significant differences to the control at the conclusion of the experiment (Fig. 3.3A, B). The lower concentrations (≤ 10 μM) of the 20:5n-3 and 20:4n-3 treatments also showed good proliferation throughout the course of the experiment (Fig. 3.3C, D). In contrast, the 20 μM treatment for both 20:5n-3 and 20:4n-3 showed good proliferation for the first 4 days but exhibited significantly lower proliferation than the control from day 6 onwards for 20:5n-3 ($p < 0.01$) and day 5 onwards for 20:4n-6 ($p < 0.05$). The 22:6n-3 treatments showed a negative dose-dependent response with the highest rate of cell proliferation observed in the 2.5 μM treatment and complete inhibition of cell proliferation observed in both the 20 μM and 10 μM treatments by days 1 and 6, respectively (Fig. 3.3E).

The cell proliferation experiment was repeated and the results are shown in Fig. 3.4. In the repeated experiment, the SBT-E1 cells showed a similar pattern of proliferation when supplied with 18:3n-3 and 18:2n-6 when compared to the original experiment and appeared to stop proliferating at day 6 (Fig. 3.4A, B). There were few significant differences during the experiment between the PUFA supplemented cells and the control for either 18:3n-3 or 18:2n-6 and none of the significant differences were maintained past day 7. However, the cells supplied 20:5n-3 showed significant inhibition of proliferation at all treatment concentrations with complete inhibition observed for all treatments from day 5 onwards ($p < 0.001$, Fig. 3.4C). This was in contrast to the cells supplied 20:5n-3 in the first proliferation experiment

which only showed inhibition of proliferation in the 20 μM treatment (Fig. 3.4C). A similar, but less severe trend was observed in the cells supplied with 20:4n-6. In the repeat experiment, there was complete inhibition of proliferation observed in cells supplied ≥10 μM 20:4n-6 from day 6 onwards ($p < 0.001$, Fig. 3.4D). The proliferation of cells supplied with 22:6n-3 was severely inhibited at all concentrations with significantly lower proliferation at all concentrations from day 2 onwards ($p \leq 0.004$, Fig. 3.4E) and complete inhibition observed from day 3 onwards ($p < 0.001$).

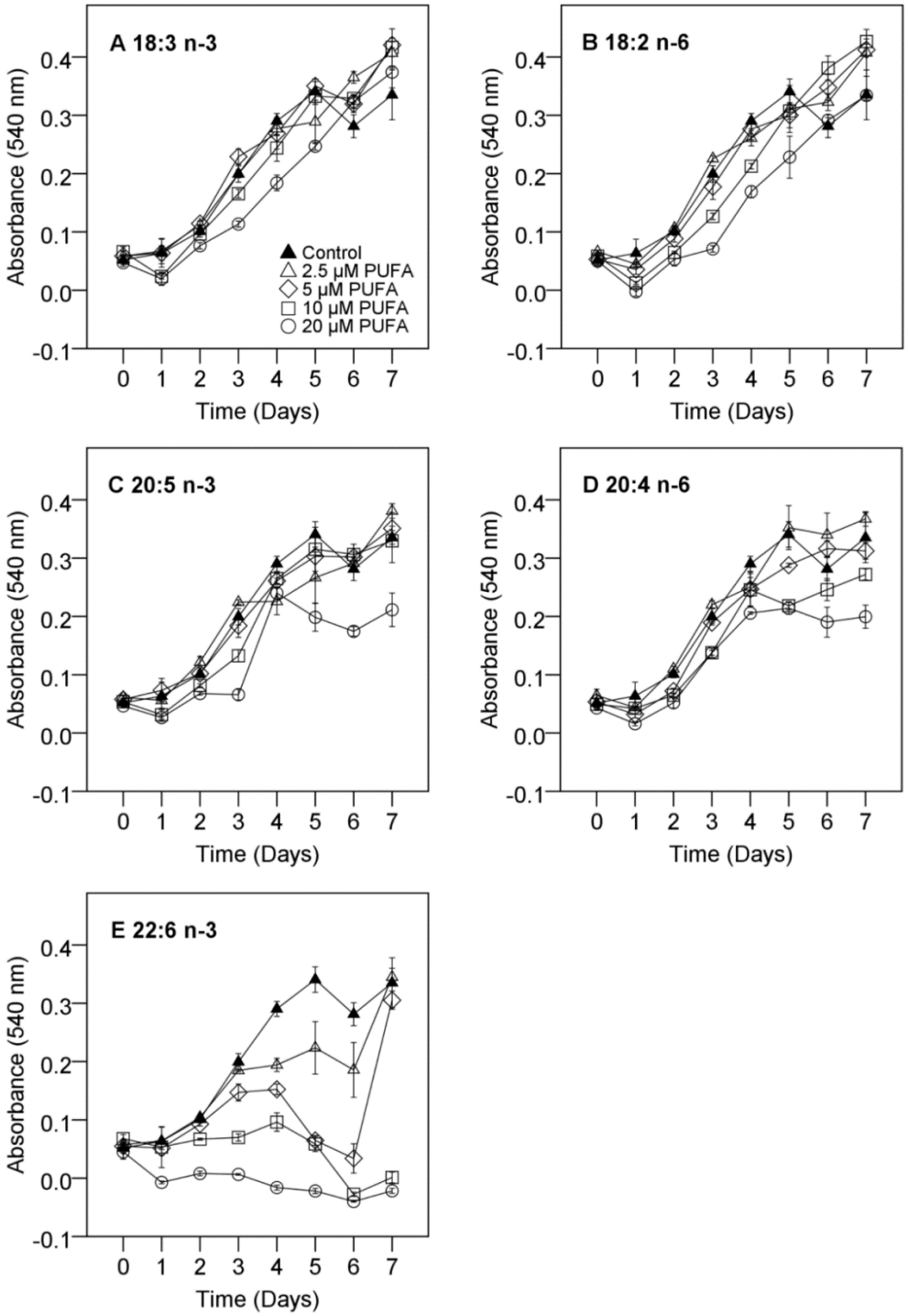


Figure 3.3: Effects of PUFA supplements on the proliferation of SBT-E1 cells in culture. Cells were cultured in 96-well plates at 25°C in 100 µl/well of the standard cell culture medium but with the FBS concentration reduced to 2% (v/v), supplemented with either 18:3n-3 (A), 18:2 n-6 (B), 20:5n-3 (C), 20:4 n-6 (D) or 22:6n-3 (E) at final concentrations of 2.5 µM (unfilled triangles), 5 µM (unfilled diamonds), 10 µM (unfilled squares) or 20 µM (unfilled circles). Control cells were cultured in the standard cell culture medium but with the FBS concentration reduced to 2% (v/v) with no fatty acid supplements (filled triangles). Data are the mean of 3 replicate wells and error bars show ± standard error of the mean. Significant differences between different fatty acid concentrations on a particular day or between a particular day and day zero for a particular fatty acid concentration were determined using two separate one-way analyses of variance followed by Bonferroni *post-hoc* comparisons. The results of the statistical analyses are given in the text.

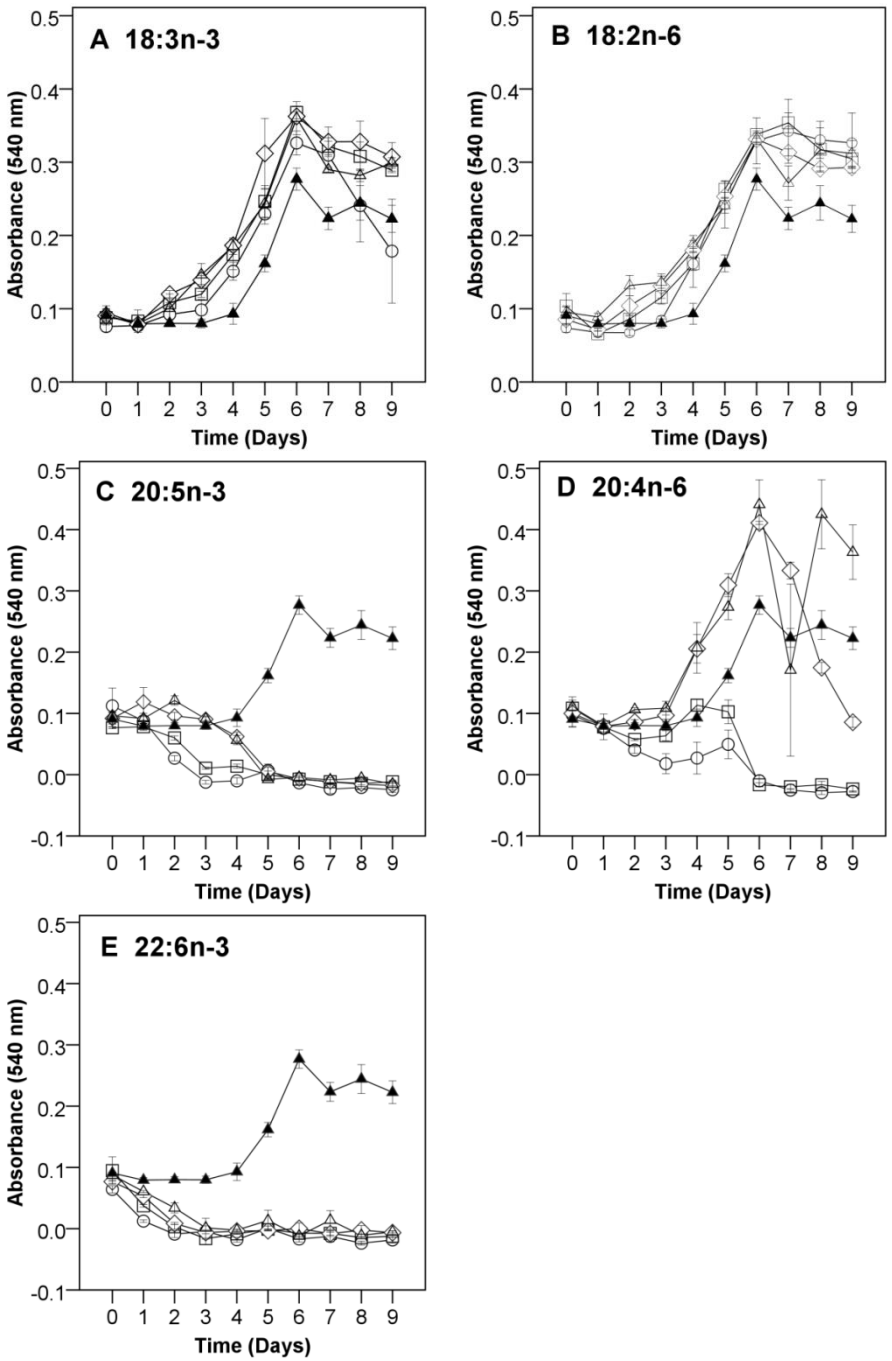


Figure 3.4: A repeat experiment investigating the effects of PUFA supplements on the proliferation of SBT-E1 cells in culture. Cells were cultured in 96-well plates at 25°C in 100 µl/well of the standard cell culture medium but with the FBS concentration reduced to 2% (v/v), supplemented with either 18:3n-3 (A), 18:2 n-6 (B), 20:5n-3 (C), 20:4 n-6 (D) or 22:6n-3 (E) at final concentrations of 2.5 µM (unfilled triangles), 5 µM (unfilled diamonds), 10 µM (unfilled squares) or 20 µM (unfilled circles). Control cells were cultured in the standard cell culture medium but with the FBS concentration reduced to 2% (v/v) with no fatty acid supplements (filled triangles). Data are the mean of 3 replicate wells and error bars show ± standard error of the mean. Significant differences between different fatty acid concentrations on a particular day or between a particular day and day zero for a particular fatty acid concentration were determined using two separate one-way analyses of variance followed by Bonferroni *post-hoc* comparisons. The results of the statistical analyses are given in the text.

3.3.4 Effects of vitamin E concentration on cell proliferation: Neutral red assay

Vitamin E appeared to have very little effect on the proliferation of cells supplied with either 10% (v/v) or 2% (v/v) FBS with no significant differences between the treatments with or without vitamin E from day 5 onwards (Fig. 3.5A, $p \geq 0.056$). The addition of 20:5n-3, 20:4n-6 or 22:6n-3 without vitamin E inhibited cell proliferation compared to the 2% (v/v) FBS control by the conclusion of the experiment at all concentrations ($p \leq 0.009$). Despite the addition of vitamin E alone having no significant effect on the proliferation of the cells, the combination of vitamin E and PUFA together showed greater changes. In all cases, cells supplied with PUFA and vitamin E together showed greater proliferation at the conclusion of the experiment than cells supplied PUFA alone with the exception of the 10 μM 22:6n-3 treatment ($p \leq 0.007$ and $p = 0.052$, respectively). The time point with the greatest number of cells was typically day 4 (Fig. 3.5). The combination of vitamin E and 10 or 20 μM 20:4n-6 at this time point resulted in greater cell proliferation compared to the cells supplied with vitamin E alone ($p < 0.001$ Fig. 3.5B) but the difference was not retained for the remainder of the experiment ($p \geq 0.087$). A similar trend was observed with cells supplied with 10 or 20 μM 20:5n-3 with greater proliferation at day 4 compared to cells supplied with vitamin E alone ($p = 0.002$ and < 0.001, respectively). The cells supplied 20 μM 20:5n-3 retained the higher levels of proliferation until the conclusion of the experiment ($p = 0.028$) but the cells supplied 10 μM 20:5n-3 did not ($p > 0.99$). There were no significant differences between the cells supplied 10 or 20 μM 22:6n-3 with vitamin E compared to the cells supplied vitamin E alone except for the 20 μM treatment at days 3 and 4. However the addition of vitamin E did appear to reverse the inhibition of proliferation that was seen in the cells supplied 22:6n-3 alone ($p < 0.001$).

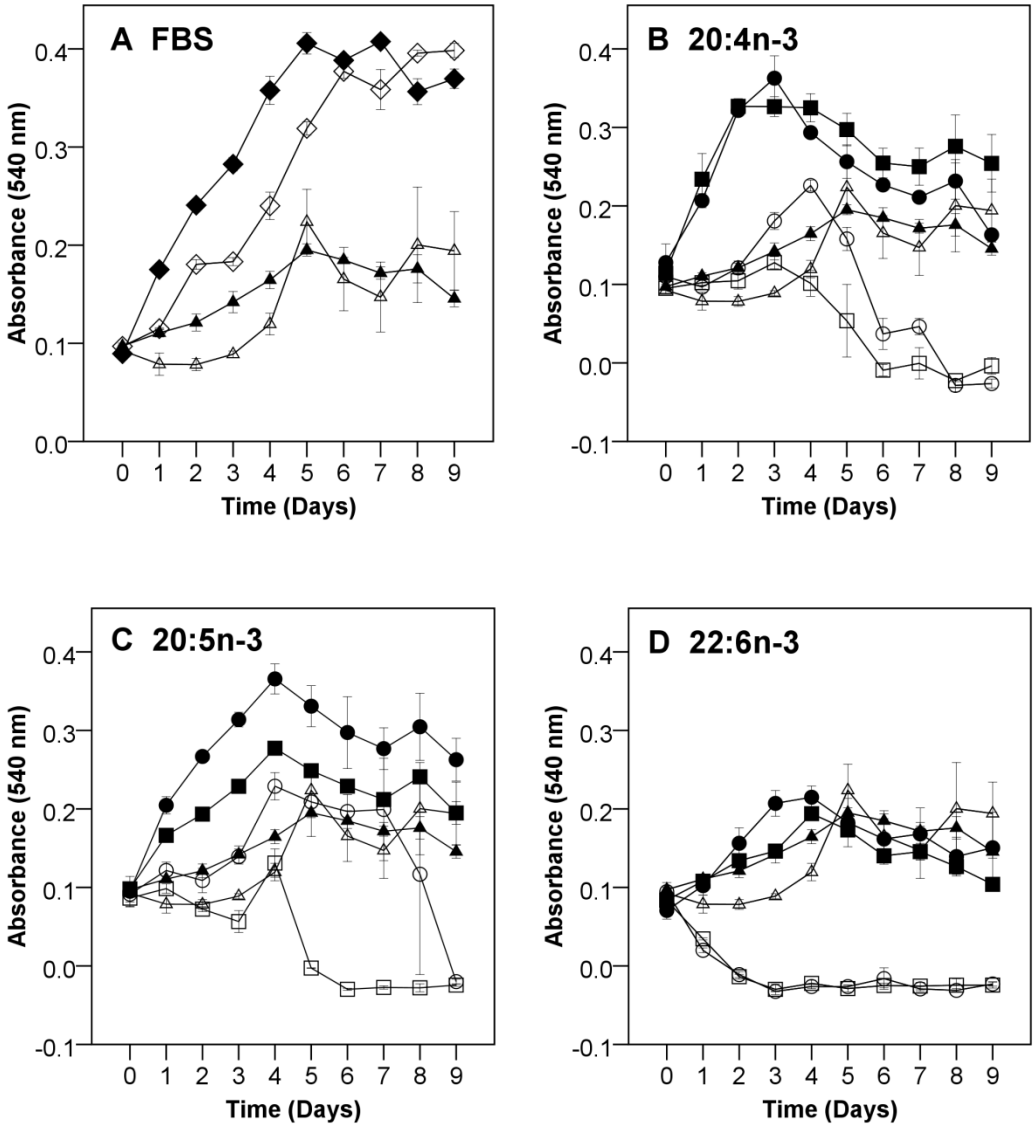


Figure 3.5: Neutral red absorbance growth curves of SBT-E1 cells cultured at 10 µM or 20 µM concentrations of different fatty acids ± 125 µM vitamin E. Cells were cultured in 96-well plates at 25°C in 100 µl/well of the standard cell culture medium (Panel A, diamonds) or the standard cell culture medium but with the FBS concentration reduced to 2% (v/v) (Panel A, triangles). Panels B-D show the results for cells cultured in standard cell culture medium but with the FBS concentration reduced to 2% (v/v) FBS and either 20:5n-3 (B), 20:4 n-6 (C) or 22:6n-3 (D) at final concentrations of 10 µM (squares) or 20 µM (circles). Control cells were cultured in standard cell culture medium but with the FBS concentration reduced to 2% (v/v) FBS with no fatty acid supplements (triangles). The filled data points indicate treatments where 2 mM vitamin E was added and the unfilled data points indicate fatty acid/FBS supplementation alone. Data are the mean of 3 replicate wells and error bars show ± standard error of the mean. Significant differences between different fatty acid concentrations on a particular day or between a particular day and day zero for a particular fatty acid concentration were determined using two separate one-way analyses of variance followed by Bonferroni *post-hoc* comparisons. The results of the statistical analyses are given in the text.

3.3.5 Effects of vitamin E concentration on cell proliferation: Vitamin E titration

To determine the optimal concentration of vitamin E supplementation, the cells were cultured with various concentrations of Vitamin E in the presence or absence of 20 μM 22:6n-3. In the presence of 22:6n-3, vitamin E supplementation positively affected cell proliferation at all vitamin E concentrations tested (Fig. 3.6, $p < 0.01$). The highest number of cells was observed in the 250 μM vitamin E treatment but this was not significantly different from any of the other vitamin E treatments except for the 0 and 2000 μM treatments which both gave significantly lower cell numbers ($p < 0.01$). In contrast, in the absence of 22:6n-3, concentrations of vitamin E above 125 μM had significant negative effects on cell number ($p \leq 0.001$). Based on the results above, a supplementation period of 4 days and concentrations of 10 μM fatty acid and 125 μM vitamin E were chosen for analysis of fatty acid incorporation into the cellular lipids.

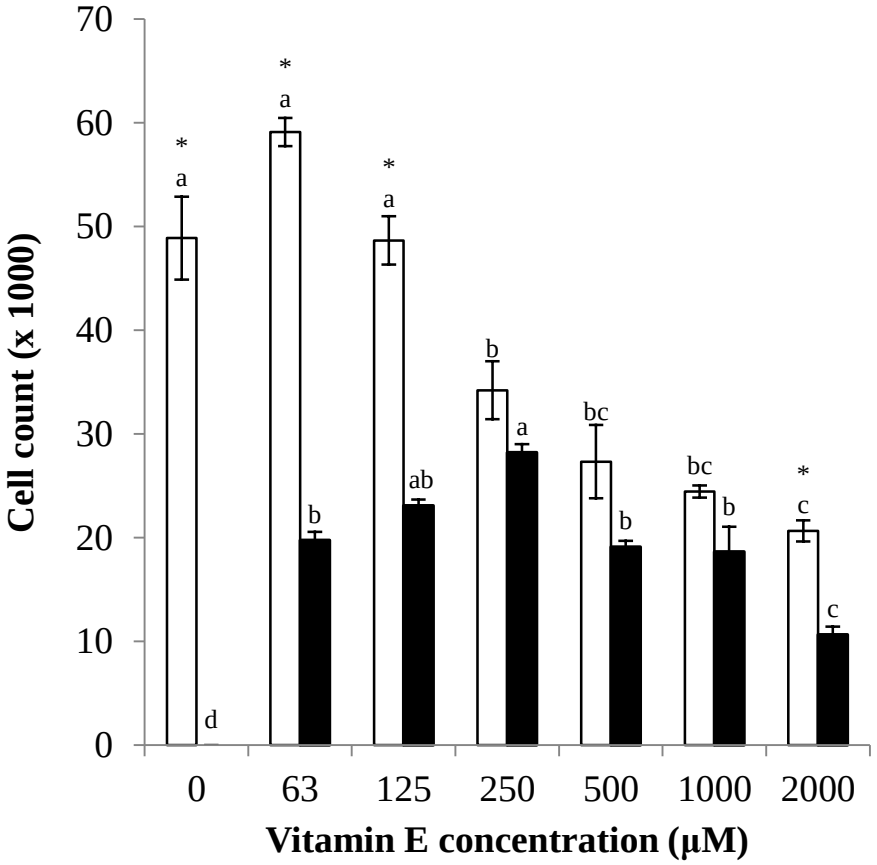


Figure 3.6: Effects of different vitamin E concentrations on cell number after 4 days of culture of SBT-E1 cells with or without 20 µM 22:6n-3. The cells were cultured in 24-well plates at 25°C in 600 µl of the standard cell culture medium but with the FBS concentration reduced to 2% (v/v) (unfilled columns), or the standard cell culture medium but with the FBS concentration reduced to 2% (v/v) FBS + 20 µM 22:6n-3 (filled columns). Vitamin E was added at a final concentration of 63-2000 µM. Control cells were not treated with vitamin E. Data are the mean of 3 replicate wells and error bars show ± standard error of the mean. Different letters denote significant differences within either the 2% FBS or 2% FBS + 20 µM 22:6n-3 treatment and asterisks denote significant differences between the plus and minus 22:6n-3 treatments at a particular vitamin E concentration as determined using two separate one-way analyses of variance followed by Bonferroni *post-hoc* comparisons.

3.3.6 Effects of vitamin E and 22:6n-3 on cellular fatty acid profile

Despite the changes in cell proliferation observed with the addition of vitamin E, there were no significant effects of vitamin E on cellular fatty acid composition in cells supplied either 2% (v/v) FBS or 2% (v/v) FBS plus 22:6n-3 (Table 3.1). The only significant differences in the profiles between the treatments were a significant increase in 22:6n-3 and total n-3 fatty acids ($p < 0.001$) and a significant decrease in total n-6 fatty acids ($p \leq 0.041$) in cells supplied 22:6n-3. Based on the vitamin E titration and fatty acid profiling results, a vitamin E concentration of 125 μM was chosen for the investigation of the effects of PUFA supplementation on the fatty acid profiles of the cells.

Table 3.1: The effect of different concentrations of vitamin E on the fatty acid profile of the total lipid extracted from the SBT-E1 cells. supplied with either 2% (v/v) FBS or 2% (v/v) FBS plus 10 μM 22:6n-3. The cells were cultured for 4 days in standard cell culture medium but with the FBS concentration reduced to 2% (v/v) (control) with or without vitamin E or 10 μM 22:6n-3. The data are expressed as % of total fatty acids and are shown as the mean +/- the standard error of the mean (n = 3). Values in the same row with different superscript letters are significantly different from one another ($p < 0.05$).

Fatty Acid	Cells				
	Control	+ 125 μM Vit. E	+ 500 μM Vit. E	+ 10 μM 22:6n-3, 125 μM Vit.	+ 10 μM 22:6n-3, 500 μM Vit.
14:0	1.0 ± 0.2	0.9 ± 0.2	1.0 ± 0.1	1.0 ± 0.1	1.2 ± 0.2
16:0	10.5 ± 1.2	12.1 ± 1.2	13.2 ± 0.2	11.5 ± 2.0	11.6 ± 1.4
18:0	6.9 ± 1.3	8.5 ± 0.9	9.3 ± 0.6	10.7 ± 1.9	10.9 ± 1.7
Total SFA	23.4 ± 3.0	26.4 ± 2.2	30.8 ± 1.8	27.7 ± 5.0	28.3 ± 4.2
16:1n-7	3.3 ± 0.9	3.4 ± 0.8	3.2 ± 0.6	1.2 ± 0.2	1.0 ± 0.1
18:1n-9	29.3 ± 11.5	19.3 ± 4.4	20.8 ± 4.0	11.2 ± 3.0	11.3 ± 2.5
18:1n-7	5.2 ± 1.5	5.3 ± 1.4	4.9 ± 0.8	2.7 ± 0.7	2.8 ± 0.6
Total MUFA	39.6 ± 11.3	30.2 ± 6.4	31.2 ± 5.0	15.8 ± 3.7	16.0 ± 2.9
18:2n-6	5.0 ± 1.4	3.9 ± 2.7	0.9 ± 0.89	0.5 ± 0.5	0.6 ± 0.6
18:3n-6	0.5 ± 0.1	0.1 ± 0.1	0.2 ± 0.2	0.1 ± 0.1	0.1 ± 0.1
20:2n-6	0.7 ± 0.4	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
20:4n-6	5.5 ± 1.8	7.4 ± 1.0	7.9 ± 0.8	4.4 ± 1.2	4.0 ± 1.0
22:4n-6/22:3n-3	0.7 ± 0.0	1.1 ± 0.1	1.0 ± 0.2	1.0 ± 0.2	0.3 ± 0.0
Total n-6	15.2 ± 1.6^{a}	15.3 ± 3.1^{a}	13.2 ± 0.4^{ab}	6.6 ± 0.8^{b}	6.5 ± 0.7^{b}
18:3n-3	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
20:3n-3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
20:5n-3	0.8 ± 0.2^{b}	1.0 ± 0.1^{ab}	1.1 ± 0.1^{ab}	1.9 ± 0.3^{a}	1.7 ± 0.2^{ab}
22:5n-3	4.1 ± 1.4^{ab}	5.3 ± 0.7^{ab}	5.9 ± 0.7^{a}	1.6 ± 0.3^{b}	1.5 ± 0.2^{b}
22:6n-3	5.3 ± 1.7^{b}	7.3 ± 0.9^{b}	8.0 ± 0.7^{b}	35.6 ± 0.7^{a}	38.4 ± 2.3^{a}
Total n-3	10.3 ± 3.2^{b}	13.6 ± 1.6^{b}	14.9 ± 1.3^{b}	39.2 ± 0.4^{a}	41.6 ± 2.0^{a}

3.3.7 Effects of PUFA supplementation on cellular fatty acid profile

In all cases, the cells contained different proportions of fatty acids to those observed in the culture medium which was the standard culture medium (see Materials and Methods) but with the FBS concentration reduced to 5% (v/v) (Table 3.2). Notably, there were significantly lower levels of saturated fatty acids in the cells (≤ 36.7% of total fatty acids) compared with the medium (54.0%, $p < 0.001$) and greater proportions of PUFA in the cells (≥ 27.9%) compared with the medium (10.4%, $p < 0.001$). Interestingly, 22:6n-3 showed a significantly higher level in the cells than in the medium when the cells were supplied with either 2% (v/v) or 10% (v/v) FBS. Specifically, there was an increase from 1.7% 22:6n-3 in the medium to 6.6% 22:6n-3 in the cells supplied with 2% (v/v) FBS and 8.2% 22:6n-3 in the cells supplied with 10% (v/v) FBS ($p <0.001$). Supplementation with either n-3 or n-6 PUFA significantly increased the levels of the respective PUFA in the cells in all cases ($p < 0.001$). These increases, however, did not necessarily translate into higher levels of products further along the LC-PUFA biosynthesis pathway. For example, addition of 18:3n-3 led to increases in cellular 18:3n-3 from trace amounts to 23% of the total fatty acids ($p < 0.001$). The GC trace did not contain a standard for 18:4n-3 but there were no detectable changes in the region where 18:4n-3 was expected to elute from the column (data not shown). Therefore, there was no detectable desaturation of 18:3n-3. In contrast, the cells fed 18:3n-3 showed apparent $C_{18} – C_{20}$ elongation with an increase in 20:3n-3 from undetectable levels to 1.7% of the total fatty acids (Table 3.2). There was no detectable desaturation of 18:2n-6 to 18:3 n-6 (Table 3.2), and despite an apparent increase in the elongation product, 20:2 n-6, from 0.4% to 1.1% of total fatty acids, this change was not statistically significant ($p = 0.109$) (Table 3.2). A substantial amount of $C_{20} – C_{22}$ elongation was seen in cells supplied 20:5n-3 with significant increases in both 20:5n-3 and 22:5n-3 (the elongation product of

20:5n-3) (p <0.001; Table 3.2). Separate elution of 22:3n-3 and 22:4n-6 was not possible but the combined GC trace for these fatty acids supported substantial elongation of 20:4n-6 with 22:3n-3 + 22:4n-6 increasing from 1.1% of the total fatty acids in the control cells to 11.0% in cells supplied with 20:4n-6 (p <0.01; Table 3.2). Therefore the cells exhibited high rates of $C_{20} - C_{22}$ elongation but only low rates of $C_{18} - C_{20}$ elongation. In contrast, there were no significant increases in 22:6n-3 concentration when the cells were supplied with either 18:3n-3 or 20:5n-3. Therefore, the enzymatic steps involved in the synthesis of 22:6n-3 from 20:5n-3 seemed to have limited activity.

The different concentrations of FBS, despite altering the proliferation of the cells (Fig. 3.2), did not have any significant effects on the proportions of most fatty acids in the cells (Table 3.2). The only exceptions were 14:0, which increased from 0.6% in the 10% (v/v) FBS treatment to 1.1% in the 2% (v/v) FBS treatment, and 20:3n-3 which decreased from 0.2% in the 10% (v/v) FBS treatment to undetectable in the 2% (v/v) FBS treatment (p <0.05, Table 3.2).

A high peroxidation index value of 292.5 was seen in the cells supplied 10 μM 22:6n-3 (Table 3.2). This value was significantly higher than the values for the other PUFA treatments and also for the 2% (v/v) and 10% (v/v) FBS controls (p <0.001; Table 3.2). In fact, the peroxidation index value for the cells treated with 22:6n-3 was approximately twice that for either the control cells, or the cells treated with either 18:3n-3 or 18:2n-6.

Table 3.2: The effect of fatty acid supplementation on the fatty acid profile of the total lipid extracted from the SBT-E1 cells. The cells were cultured for 4 days in standard cell culture medium (10% FBS), standard cell culture medium but with the FBS concentration reduced to 2% (v/v) (2% FBS) or the standard cell culture medium but with the FBS concentration reduced to 2% (v/v) plus 10 µM 18:3n-3, 18:2n-6, 20:5n-3, 20:4n-6 or 22:6n-3. The data are expressed as % of total fatty acids and are the mean ± the standard error of the mean (n=3). Values in the same row with different superscript letters are significantly different from one another ($p < 0.05$).

Fatty acid	Culture medium	Cells						
		2% FBS	2% FBS + 18:3n-3	2% FBS + 18:2n-6	2% FBS + 20:5n-3	2% FBS + 20:4n-6	2% FBS + 22:6n-3	10% FBS
14:0	2.1 ± 0.4^{a}	1.1 ± 0.1^{b}	0.9 ± 0.1^{bc}	0.9 ± 0.1^{bc}	1.2 ± 0.0^{ab}	1.4 ± 0.1^{ab}	1.4 ± 0.0^{ab}	0.6 ± 0.0^{c}
16:0	24.0 ± 0.9^{a}	16.1 ± 1.8^{b}	13.7 ± 0.6^{b}	13.7 ± 1.0^{b}	16.7 ± 0.5^{b}	15.9 ± 0.8^{b}	15.3 ± 2.4^{b}	14.2 ± 0.3^{b}
18:0	7.5 ± 0.4^{b}	13.0 ± 2.0^{a}	12.0 ± 0.1^{a}	10.8 ± 0.5^{ab}	12.0 ± 0.6^{a}	10.8 ± 0.6^{ab}	12.5 ± 0.1^{a}	11.0 ± 0.1^{ab}
Total SFA[1]	57.1 ± 1.5^{a}	34.8 ± 2.4^{b}	29.9 ± 0.7^{b}	29.6 ± 1.4^{b}	33.9 ± 0.9^{b}	31.8 ± 1.2^{b}	33.6 ± 3.4^{b}	31.6 ± 0.4^{b}
16:1n-7	2.4 ± 0.3^{ab}	3.8 ± 0.8^{a}	2.2 ± 0.9^{ab}	1.1 ± 0.2^{b}	2.5 ± 0.5^{ab}	1.4 ± 0.1^{ab}	1.4 ± 0.2^{ab}	3.2 ± 0.1^{ab}
18:1n-9	13.6 ± 3.5^{abc}	17.7 ± 1.9^{ab}	12.7 ± 3.6^{abc}	7.7 ± 0.6^{c}	12.4 ± 0.4^{abc}	10.0 ± 0.6^{bc}	10.3 ± 0.3^{bc}	19.9 ± 0.3^{a}
18:1n-7	2.8 ± 0.2^{ab}	5.1 ± 0.6^{a}	3.4 ± 1.3^{ab}	2.1 ± 0.2^{b}	3.3 ± 0.2^{ab}	2.6 ± 0.1^{ab}	2.8 ± 0.2^{ab}	5.0 ± 0.1^{ab}
Total MUFA[2]	22.6 ± 3.6^{ab}	30.3 ± 3.1^{a}	19.6 ± 5.7^{ab}	12.3 ± 1.1^{b}	20.1 ± 0.7^{ab}	15.5 ± 0.7^{b}	16.2 ± 0.6^{b}	31.5 ± 0.3^{a}
18:2n-6	2.3 ± 0.4^{b}	4.0 ± 1.1^{b}	1.2 ± 0.6^{b}	36.3 ± 4.5^{a}	2.5 ± 0.5^{b}	2.3 ± 0.4^{b}	2.6 ± 0.5^{b}	3.3 ± 0.1^{b}
18:3n-6	0.0 ± 0.0^{b}	0.3 ± 0.2^{ab}	0.3 ± 0.1^{ab}	0.2 ± 0.1^{ab}	0.4 ± 0.0^{ab}	0.7 ± 0.1^{a}	0.6 ± 0.2^{ab}	0.6 ± 0.0^{ab}
20:2n-6	0.0 ± 0.0^{b}	0.4 ± 0.4^{ab}	0.0 ± 0.0^{b}	1.2 ± 0.2^{a}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.1 ± 0.0^{b}
20:4n-6	2.7 ± 0.1^{d}	7.9 ± 0.2^{b}	6.5 ± 0.6^{bc}	5.2 ± 0.4^{c}	4.8 ± 0.6^{cd}	23.3 ± 0.8^{a}	4.3 ± 0.1^{cd}	9.0 ± 0.1^{b}
22:4n-6/22:3n-3	0.0 ± 0.0^{d}	1.2 ± 0.0^{bc}	1.4 ± 0.0^{bc}	1.1 ± 0.1^{bc}	1.2 ± 0.1^{bc}	12.6 ± 0.3^{a}	0.8 ± 0.1^{c}	1.8 ± 0.1^{b}
Total n-6[3]	5.7 ± 0.4^{c}	16.0 ± 1.5^{b}	10.8 ± 0.2^{bc}	45.9 ± 3.6^{a}	9.8 ± 1.3^{bc}	40.3 ± 0.8^{a}	9.3 ± 0.7^{bc}	17.5 ± 0.1^{b}
18:3n-3	0.2 ± 0.1^{b}	0.0 ± 0.0^{b}	22.7 ± 4.7^{a}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.1 ± 0.1^{b}	0.0 ± 0.0^{b}	0.1 ± 0.0^{b}
20:3n-3	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}	1.8 ± 0.1^{a}	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}	0.2 ± 0.0^{b}
20:5n-3	0.6 ± 0.1^{cd}	1.2 ± 0.2^{bc}	0.9 ± 0.1^{cd}	0.6 ± 0.1^{cd}	12.7 ± 0.2^{a}	0.3 ± 0.0^{d}	1.8 ± 0.3^{b}	1.2 ± 0.1^{bc}
22:5n-3	1.0 ± 0.1^{e}	5.3 ± 0.2^{bc}	4.6 ± 0.5^{bcd}	3.7 ± 0.3^{cd}	16.1 ± 0.9^{a}	3.0 ± 0.3^{de}	1.5 ± 0.1^{e}	6.3 ± 0.1^{b}
22:6n-3	2.0 ± 0.2^{d}	7.6 ± 0.2^{bc}	6.2 ± 0.6^{bc}	4.9 ± 0.6^{cd}	5.1 ± 0.3^{cd}	3.8 ± 0.1^{cd}	34.6 ± 1.7^{a}	9.2 ± 0.1^{b}
Totaln-3[4]	3.9 ± 0.5^{d}	14.1 ± 0.4^{bc}	36.2 ± 3.7^{a}	9.2 ± 0.9^{bcd}	33.9 ± 0.6^{a}	7.4 ± 0.4^{cd}	37.8 ± 1.9^{a}	17.2 ± 0.2^{b}
Total PUFA[5]	9.8 ± 0.5^{d}	31.3 ± 2.5^{c}	47.7 ± 3.9^{ab}	55.4 ± 2.9^{a}	43.8 ± 0.7^{b}	48.5 ± 1.0^{ab}	47.1 ± 1.2^{ab}	35.1 ± 0.2^{c}
PI[6]	41.7 ± 2.9^{f}	147.1 ±	169.7 ± 4.5^{cd}	132.0 ± 5.3^{e}	243.3 ± 0.7^{b}	201.4 ± 1.8^{c}	322.8 ± 13.9^{a}	175.4 ± 3.8^{cd}

Abbreviations: SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; PI, peroxidation index. [1]Also includes 8:0, 9:0, 12:0, 17:0 20:0 22:0 and 24:0. [2]Also includes 16:1 n-9, 17:1, 20:1 n-12, 20:1 n-11, 20:1 n-9, 22:1 n-11, 22:1 n-9 and 24:1. [3]Also includes 20:3 n-6, 22:2 n-6 and 22:5 n-6. [4]Also includes 16:2n-3. [5]Also includes 18:2 n-9, 20:2 n-9, 20:3 n-9.20:3 n-6, 22:2 n-6, 22:5 n-6 and 16:2n-3. [6] The peroxidation index was calculated using the formula: 0.025 x (% monoenoics) + 1 x (% dienoics) + 2 x (% trienoics) + 4 x (% tetraenoics) + 6 x (% pentaenoics) + 8 x (% hexaenoics) from Hulbert et al. (Hulbert *et al.* 2007)

3.4 Discussion

3.4.1 Validation of the NRU assay

The low degree of error and the high level of correlation obtained from the NRU assay absorbance curve show that the NRU assay is a reliable method to rapidly estimate viable cell abundance in the SBT-E1 cell line.

3.4.2 Effects of FBS concentration on cell proliferation

The SBT-E1 cells showed a pattern of rapid proliferation during the early stages, before a plateau at day 6 for all concentrations of FBS (Fig. 3.2). Mostly likely, this was due either to an exhaustion of the available nutrients in the case of the low FBS concentrations or to a filling of the available growth area at the high FBS concentrations. The plateau at the low FBS concentrations was consistent with what has been reported for turbot (*Scophthalmus maximus*) and Atlantic salmon (*Salmo salar*) cells in culture (Tocher and Dick 1991). However the turbot and Atlantic salmon cells, despite being seeded at a similar density, appeared to show slower and more consistent proliferation over a longer period of time with cells continuing to proliferate after 10 days, compared to a plateau at 6 days for the SBT-E1 cells (Tocher and Dick 1991). Therefore, the higher initial rate of proliferation may have caused the SBT-E1 cells to fill the available growth area more rapidly. Furthermore, at high cell densities the SBT-E1 cells appeared to show alterations in morphology. For example, the cells formed more isolated colonies and cells within particular colonies packed closer together and became more uniform in shape causing an increase in the cell density within individual colonies. Evidence of contact inhibition was also apparent between adjacent colonies. These morphological changes could suggest cell differentiation. Differentiation of the cells could also explain the slower proliferation observed after several days without subculture.

3.4.3 Effects of PUFA supplementation on cell proliferation

The SBT-E1 cells showed good proliferation over the course of 7 days when supplemented with either 18:3n-3 or 18:2n-6 but the numbers for these cells were only moderately higher than those for the control cells at any time (Fig. 3.3A, 3.3B). The cell numbers for the 2.5 μM 18:3n-3 and 10 μM 18:2n-6 treatments were significantly higher than those for the control at day 6 (p = 0.012 and 0.024, respectively), but this difference was not observed by day 7. This result was similar to what was seen in cultured turbot and Atlantic salmon cells which showed modest increases in proliferation (relative to the control) when supplied 5-25 μM 18:3n-3 or 18:2n-6 (Tocher and Dick 1991). The proliferation of the SBT-E1 cells was inhibited at high concentrations of 20:5n-3 or 20:4n-6 and this effect was even more pronounced in the cells supplied with 22:6n-3. This sensitivity to high concentrations of LC-PUFA, especially 22:6n-3, appeared to be much greater in the SBT-E1 cells than in cell lines from either turbot or Atlantic salmon (Tocher and Dick 1991).

A repeat experiment was conducted to confirm the effects of PUFA supplementation on the proliferation of the SBT-E1 cells. Similar to the first proliferation experiment, the cells supplied with 18:3n-3 and 18:2n-6 in the repeat proliferation experiment proliferated well and were not different from the control cells from day 7 onwards (Fig. 3.4A, 3.4B). However, the cells supplied with 20:5n-3, 20:4n-6 or 22:6n-3 showed significant inhibition of proliferation (Fig. 3.4C, 3.4D, 3.4E) and the effect was seen earlier and at lower concentrations than the cells in the first proliferation experiment (Fig. 3.3C, 3.3D, 3.3E), The proliferation in the repeat experiment was substantially lower than what was observed in the first proliferation experiment. The fatty acids that are supplied to cells in culture are typically incorporated readily into

their cellular membranes these membranes are the main location where fatty acids are found (Tocher *et al.* 1989). This is in contrast to whole animals where fatty acids are commonly found stored as triacylglycerol in adipose tissue (Sprague *et al.* 2012). The large number of double bonds in PUFA mean that they are more prone to oxidative attack by reactive oxygen species (ROS) such as ·OH than less unsaturated fatty acids and this can lead to the production of lipid peroxyl radicals, which can, in turn, attack other lipids, leading to a lipid peroxidation chain reaction (Gutteridge and Halliwell 1990). When this occurs in cellular membranes, it can lead to membrane damage and apoptosis (Mourente *et al.* 2007). The severity of the inhibition of cell proliferation correlated with the number of double bonds (and therefore their susceptibility to oxidative attack) present in each of the fatty acids (Gutteridge 1995, Mazière *et al.* 1999). It was therefore hypothesised that the differences in proliferation may be due to oxidative stress, as the PUFA stock solutions had been opened and stored for approximately 1 month prior to conducting the repeat experiment.

The severity of the effects of oxidative stress on the SBT-E1 cells may also be elevated compared to what is seen in whole animal tissues as cells in culture often lack the antioxidant protection that is present in the whole organism (Halliwell 2007). Mazière et al. (1999) found that in cultured human fibroblasts supplied with 50 μM 20:4n-6, there was a 2-fold increase in intracellular ROS and a 4-fold increases in lipid peroxidation products (thiobarbituric acid reactive substances, TBARS). The effects on both ROS and TBARS were reversed by the addition of 50 μM vitamin E (Mazière *et al.* 1999). Similarly, Tocher and Dick (1991) found that turbot fin cells supplied with 25 μM 20:5n-3 showed improvements in proliferation when 1.8 mM vitamin E was included in the culture medium. Therefore, we decided

to test whether vitamin E could reverse the negative effects of PUFA supplementation on the proliferation of the SBT-E1 cells.

3.4.4 Effects of vitamin E concentration on cell proliferation: Neutral red assay

To test the effects of vitamin E on cell proliferation, two different assay methods were used. The first was the NRU assay and the second was cell counts using Trypan blue dye (described in Section 2.2.2). The results from the NRU assay showed that vitamin E had very little effect on the proliferation cells supplied with 10% (v/v) or 2% (v/v) FBS alone (Fig. 3.5A) but showed improvement in the proliferation of cells supplied with 2% (v/v) FBS and 10-20 μM 20:5n-3 (Fig. 3.5B), 20:4n-6 (Fig. 3.5C) or 22:6n-3 (Fig. 3.5D). However, at the conclusion of the experiment, visual observation of the plates appeared to show a very low number of cells in the treatments containing vitamin E. A possible explanation for this result is due to the fact that the neutral red dye works by binding to the membranes of the lysosomes within the cells. Wang and Quinn (1999) found that the concentration of vitamin E in the lysosome membranes was more than 10 times greater than other organelles. Thus large doses of vitamin E could change the structure of the lysosome membranes and thus affect the binding of the neutral red dye, leading to a greater absorbance despite the fact that a low number of cells were present. Therefore we resolved to quantify the effects of vitamin E on cell proliferation by direct cell counts rather than by the NRU assay.

3.4.5 Effects of vitamin E concentration on cell proliferation: Vitamin E titration

To define the optimal concentration of vitamin E supplementation for the SBT-E1 cells, the cell numbers were estimated using Trypan blue dye as described in Section 2.2.2 after supplementation with various concentrations of vitamin E in the presence

and absence of 22:6n-3. The results showed that vitamin E did indeed reverse the inhibitory effects of 22:6n-3 on cell proliferation (Fig. 3.6). Tocher and Dick (Tocher and Dick 1991) found that in turbot cells fed 25 μM 20:5n-3, cell proliferation could be improved with the addition of 1.8 mM vitamin E but the effects of vitamin E on cells supplied with 22:6n-3 were not investigated in that study. Our results showed that in the SBT-E1 cells, a 63 μM concentration of vitamin E was sufficient to reverse the negative effects of 20 μM 22:6n-3 on cell proliferation and the greatest benefit was seen in the presence of 250 μM vitamin E. Therefore, despite the SBT-E1 cells showing high sensitivity to apparent LC-PUFA-induced oxidative stress, this effect could readily be reversed by the addition of vitamin E.

Vitamin E improved the proliferation of the SBT-E1 cells both in the absence and in the presence of 22:6n-3 but the optimal vitamin E concentration was different; lower in the absence of 22:6n-3 than in the presence of 22:6n-3. This suggests, either that the basal cell culture medium was deficient in vitamin E or that the amount of vitamin E required by the cells varied according to the PUFA supplement. The high potential for 22:6n-3 to cause oxidative stress, as evidenced by the high peroxidation index value for the cells supplied 22:6n-3 (Table 3.2), may explain the higher optimum vitamin E concentration required in the presence of 22:6n-3 compared with in the absence of 22:6n-3. In other words the positive effects of oxidative protection outweighed the negative effects of additional vitamin E when the cells were cultured in the presence of 22:6n-3. The biochemical basis for this is not known but previous studies have shown that high concentrations of vitamin E (≥ 115 μM) inhibited cell proliferation in human mesangial and glomerular microvascular endothelial cells (Zhang *et al.* 2001). Furthermore, Jóźwik et al. (2005) found that when vitamin E

was supplied to mice in high doses, there was destabilisation of the cellular membranes, leading to membrane leakiness and ultimately, apoptosis.

3.4.6 Effects of vitamin E and 22:6n-3 on cell number and cellular fatty acid profile

There is a wealth of literature that indicates that vitamin E is a dietary antioxidant that defends both cell lines and whole organisms against oxidative stress and lipid peroxidation (e.g. Wang and Quinn 1999, Mourente *et al.* 2000, Guerriero and Ciarcia 2001, Mourente *et al.* 2002a, Gao *et al.* 2010, Gao *et al.* 2012). To our knowledge, however, there are no studies directly reporting the effects of vitamin E on the fatty acid profiles of cultured cells. Nevertheless, there are some studies that report the effects of vitamin E on fatty acid profiles in whole animals. For example, Faizan et al. (2013) found that supplementing feeds for farmed Atlantic salmon with vitamin E changed the oxidative status of the membranes but did not affect the fatty acid composition. Conversely, Fritche et al. (1992) found that an increase in dietary vitamin E corresponded to a decrease in the levels of 20:4n-6 and 20:5n-3 but not 22:6n-3 in the blood serum of rats but there was no reporting on the effects on tissue fatty acid profiles. Likewise, Lebold et al. (2011) found that dietary vitamin E deficiency resulted in a decreased level of 20:4n-6 and a decreased 20:4n-6 to 18:2n-6 ratio in the viscera of zebrafish, but no full fatty acid profiles were reported. Therefore the role of vitamin E in protecting the cell membranes from oxidative attack is clear, but there is little information about the effect of vitamin E on the fatty acid profiles of those membranes. Our results show that the inclusion of vitamin E allowed survival of the SBT-E1 cells in the presence of 22:6n-3, but did not have any effect on the fatty acid profile of the cellular lipids. The high mortality of the SBT-

E1 cells treated with 22:6n-3 in the absence of vitamin E prevented the testing of the effects of 22:6n-3 alone on the fatty acid profile of the SBT-E1 cells.

3.4.7 Effects of PUFA supplementation on cellular fatty acid profile

Tuna lipids have unusually high 22:6n-3 to 20:5n-3 ratios but the molecular basis for this is not known (Mourente and Tocher 2009, Morais *et al.* 2011). The SBT-E1 cells cultured in the presence of 2% (v/v) FBS had fatty acid profiles containing 1.1% 20:5n-3 and 6.6% 22:6n-3 giving a 22:6n-3 to 20:5n-3 ratio of 6.0 (Table 3.2). Similarly, when the cells were cultured in the presence of 10% (v/v) FBS, their fatty acid profiles contained 1.1% 20:5n-3 and 8.2% 22:6n-3 giving a 22:6n-3 to 20:5n-3 ratio of 7.5. The high 22:6n-3 to 20:5n-3 ratios observed in the SBT-E1 cells have also been observed in the muscle lipids of SBT consuming its natural diet in the wild but not in other bluefin tuna species. For example, SBT muscle lipids contained 4.8% 20:5n-3 and 44.1% 22:6n-3 giving a 22:6n-3 to 20:5n-3 ratio of 9.2 (Nichols *et al.* 1998). For ABT, the values were 4.9% 20:5n-3 and 12.1% 22:6n-3 giving a 22:6n-3 to 20:5n-3 ratio of 2.5 (Sprague *et al.* 2012). For PBT, the values were 6.9% 20:5n-3 and 23.3% 22:6n-3 giving a 22:6n-3 to 20:5n-3 of 3.4 for dorsal skeletal muscle as well as 9.8% 20:5n-3 and 20.2% 22:6n-3 giving a 22:6n-3 to 20:5n-3 ratio of 2.1 for ventral skeletal muscle (Nakamura *et al.* 2007). Thus, the 22:6n-3 to 20:5n-3 ratio in the SBT-E1 cells approached that in the wild-caught SBT and exceeded that in the wild-caught ABT or PBT.

The cell culture medium contained only 1.7% of the total fatty acids as 22:6n-3 whereas the cells cultured with either 2% or 10% (v/v) FBS contained 6.6% and 8.2% of the total fatty acids as 22:6n-3. Thus there was apparent selective retention of 22:6n-3 in the cellular lipids. This was not seen with 20:5n-3 with the proportions

of 20:5n-3 being similar in the medium and in the cells. 22:6n-3 is difficult to oxidise because the Δ4 bond must be removed by peroxisomal β-oxidation (Tocher 2003) and this could explain the selective retention of 22:6n-3 reported here. Other fish species oxidise 22:6n-3 by the same mechanism and therefore also show selective retention of 22:6n-3. However, the levels of 22:6n-3 reported here in the SBT-E1 cells cultured with 10% (v/v) FBS were greater than those reported previously for other fish cells in culture (Tocher *et al.* 1989). Here we found 8.2% 22:6n-3 in the SBT-E1 cells cultured with 10% (v/v) FBS whereas previous studies have found only 1.3% 22:6n-3 in rainbow trout gonad cells and only 3.9% 22:6n-3 in turbot fin cells when these cells were cultured with 10% (v/v) FBS (Tocher *et al.* 1989). The greater apparent selective retention of 22:6n-3 in the SBT-E1 cells is consistent with the high 22:6n-3 to 20:5n-3 ratios observed in tuna tissues (Mourente and Tocher 2009, Morais *et al.* 2011).

Another explanation for the high 22:6n-3 to 20:5n-3 ratio in the SBT-E1 cells could be the apparently high level of conversion of 20:5n-3 to 22:5n-3. In particular, the cells supplied 20:5n-3 contained 16.1% of their total fatty acids as 22:5n-3, a significant increase from the 4.6% found in the control cells (Table 3.2). This indicated substantial $C_{20} - C_{22}$ elongation. In a previous study, the proportion of 22:5n-3 in SBT liver was reported as 5.33% of total fatty acids (Gregory *et al.* 2010), which is similar to that observed for the cells supplemented with 2% (v/v) FBS in the present study. However there are currently no data on the activity of any fatty acyl elongase enzymes in any SBT tissues and therefore further study to determine the capability of tuna to elongate 20:5n-3 to 22:5n-3 is needed. The apparently high level of elongation in the SBT-E1 cells could serve to remove 20:5n-3 from the cells,

lowering the proportion of 20:5n-3 relative to 22:6n-3 and increasing the 22:6n-3 to 20:5n-3 ratio.

20:4n-6 is the n-6 equivalent of 20:5n-3 and occurs at the same point in the LC-PUFA biosynthesis pathway (see Chapter 1, Section 1.3, Figure 1.1). In this study, the cells appeared to incorporate 20:4n-6 more readily than 20:5n-3, with cells supplemented with 20:4n-6 containing 22.3% of their fatty acids as 20:4n-6 (Table 3.2). There also seemed to be a similarly high level of elongation of 20:4n-6 in cells supplied 20:4n-6 as was seen in cells supplied 20:5n-3. In particular, the combined GC trace for 22:3n-3 and 22:4 n-6 indicated an increase from 1.1% in cells supplied 2% FBS to 11.0% in cells supplied 2% FBS and 10 μM 20:4n-6 (Table 3.2). The apparently substantial elongation of 20:5n-3 and 20:4n-6 to 22:5n-3 and 22:4n-6, respectively, is indicative of a high level of Elovl5 activity in the SBT-E1 cells. The data presented here show far less Elovl5 activity towards 18:3n-3 and 18:2n-6 compared with 20:5n-3 and 20:4n-6 suggesting a preference of the Elovl5 enzyme for C_{20} substrates over C_{18} substrates. Alternatively, there may be an Elovl2 or Elovl4 enzyme that is active in the SBT-E1 cells. One or both of these enzymes could show activity towards C_{20} and C_{22} substrates but not C_{18} substrates in the SBT cells and may catalyse the substantial elongation of 20:5n-3 and 20:4n-6 observed here.

One possible explanation for the apparently high rates of C_{20} PUFA elongation in the SBT-E1 cells could be the roles that 20:5n-3 and 20:4n-6 play in mediating the inflammatory response in vertebrates through the production of eicosanoids such as leukotrines and prostanoids (Simopoulos 2002). The majority of the eicosanoids that promote the inflammatory response are derived from 20:4n-6 whereas the down-

regulation of this pathway and the return of the tissue to homeostasis is due to metabolites of 20:5n-3 (Calder 2009, Chapkin *et al.* 2009). Therefore, the balance between 20:4n-6 and 20:5n-3 concentrations needs to be tightly regulated as an abundance of 20:4n-6 could lead to an exacerbated inflammatory response whereas an excess of 20:5n-3 could lead to the silencing of what may otherwise be a healthy inflammatory response (Chapkin *et al.* 2009). As a result, whilst the use of the LC-PUFA biosynthesis pathway in the production of 22:6n-3 may be redundant in predatory marine fish such as tunas, some of the enzymes involved, especially Elovl5, may still be utilised as regulators of these organisms' inflammatory response by elongating (and thereby removing) excess 20:4n-6 or 20:5n-3.

The low levels of 18:3n-3 and 18:2n-6 elongation may be because the Elovl5 enzyme, in its normal cellular environment, utilises 18:4n-3 and 18:3n-6 as its C_{18} substrates. Previous research with a cell line from Atlantic Salmon showed 5.6% elongation of 18:3n-3, compared with 23.6% elongation of 18:4n-3 (Ghioni *et al.* 1999). The corresponding figures for a cell line from turbot were 0.9% for 18:3n-3 and 4.4% for 18:4n-3 (Ghioni *et al.* 1999). Therefore, it is likely that there is competition between different C_{18} substrates for Elovl5-catalyzed elongation and the high concentrations of 18:3n-3 and 18:2n-6 used in this study provided a favourable environment for their elongation. Furthermore, and perhaps more importantly, it is likely that there is also competition between C_{18} and C_{20} substrates for Elovl5-catalyzed elongation. The functional characterisation of SBT Elovl5 recombinantly expressed in yeast showed that there was a strong preference for 18:4n-3 and 18:3n-6 over C_{20} substrates (Gregory *et al.* 2010). To investigate this further, the SBT-E1 cells could be supplied a combination of 18:3n-3 and 18:2n-6 or 18:4n-3 and 18:3n-6

along with 20:5n-3 and 20:4n-6 in a similar way as described by Gregory et al. (Gregory *et al.* 2010).

In contrast to the apparent elongation that was observed for 18:3n-3, 18:2n-6 and especially 20:5n-3 and 20:4n-6, there was very little evidence of desaturation in any of the treatments. These data are consistent with the lack of desaturase activity observed in attempts to functionally characterise a SBT Δ6Fads cDNA by heterologous expression in yeast (M.K. Gregory and K.A. Schuller, unpublished).

In summary, this chapter has characterised the effects of various PUFA on cell proliferation, PUFA incorporation into cellular lipids and PUFA metabolism in the SBT-E1 cell line. Studies on PUFA metabolism in SBT and other tuna species have been limited to date by the high commercial value of these species. This chapter has provided the first *in vitro* study of fatty acid metabolism in any tuna species and the results show that the SBT-E1 cell line is a useful model. Future investigations with the cell line could include analyses of the effects of fatty acid supplementation on the expression of the Fads and Elovl genes involved in the *de novo* synthesis of 20:5n-3 and 22:6n-3.

Chapter 4 – Dynamics of fatty acid metabolism in the SBT-E1 cell line and fatty acid profiling of SBT-E1 cells and SBT tissues

A significant portion of this chapter has been published[1] and a copy of the published paper can be found in Appendix B

[1]Reference: Scholefield, A. M., Tocher, D. R., Schuller, K.A., 2015, Dynamics of fatty acid metabolism in a cell line from southern bluefin tuna (*Thunnus maccoyii*), *Aquaculture, Special Issue: Proceedings of the 16th International Symposium on Fish Nutrition and Feeding*, 449, 58-68

4.1. Aims and background

The aims of this chapter were to use of the SBT-E1 cell line to investigate:

1. The esterification of individual fatty acids into the various lipid classes
2. The metabolism of fatty acids via desaturation, elongation and/or β-oxidation
3. The expression of the Δ6Fads and Elovl5 genes which catalyse fatty acid desaturation and elongation, respectively

Fatty acid uptake, esterification into cellular lipids, desaturation, elongation and β-oxidation has been extensively studied in fish cell lines (Ghioni *et al.* 1997, Torstensen and Stubhaug 2004, Mourente *et al.* 2005, Stubhaug *et al.* 2007). In Atlantic salmon, phosphatidylcholine (PC) has been shown to be the most abundant lipid class and most fatty acids are incorporated predominantly into PC or phosphatidylethanolamine (PE) (Tocher and Sargent 1990, Stubhaug *et al.* 2005). Fatty acid desaturation, elongation and β-oxidation have been investigated in cell lines and/or hepatocytes from many species including Atlantic salmon (Torstensen and Stubhaug 2004, Stubhaug *et al.* 2005), European seabass (Mourente *et al.* 2005) and rainbow trout (Ghioni *et al.* 1997) and turbot (Ghioni *et al.* 1999).

These studies have typically shown that predatory marine fish have a low capacity for LC-PUFA synthesis and a substantial capacity for n-3 fatty acid β-oxidation however this has not yet been investigated in tunas. Interestingly, tunas have a high 22:6n-3:20:5n-3 ratio compared to other fish species (Mourente and Tocher 2009, Morais *et al.* 2011). A high level of 22:6n-3 is normal in predatory marine fish due to high levels of this fatty acid in their diet. Furthermore, 22:6n-3 is more difficult to β-oxidise as the Δ4 double bond is removed in the peroxisomes, before the fatty acid is

exported to the mitochondria for the remainder of the β-oxidation process (Tocher 2003). This leads to conservation of 22:6n-3. However, neither of these points explains the disparity in the 22:6n-3:20:5n-3 ratio between tunas and other predatory fish. An additional explanation could be that tuna exhibit a greater preference towards utilising 20:5n-3 over 22:6n-3 for β-oxidation, thus contributing to the high 22:6n-3:20:5n-3 ratio, but this has not been tested.

The preceding chapter described the effects of different fatty acids on the proliferation and fatty acid composition of the SBT-E1 cells and this chapter builds upon this work to describe the dynamics of fatty acid metabolism in the SBT-E1 cells.

It was hypothesised that:

1. The fatty acid profiles of the various lipid classes would be similar between SBT-E1 cells and SBT tissues
2. The SBT-E1 cells would show different patterns of esterification into the different lipid classes
3. The SBT-E1 cells would show substantial elongation of C_{18} and C_{20} fatty acids (presumed to be catalysed by Elovl5), but low levels of C_{22} elongation (presumed to be catalysed by Elovl2)
4. The SBT-E1 cells would show some Δ6Fads-mediated fatty acid desaturation, but only minor amounts of Δ5Fads-mediated fatty acid desaturation
5. The SBT-E1 cells would show preferential β-oxidation of n-3 PUFA substrates compared with n-6 PUFA substrates

6. The SBT-E1 cells would show preferential β-oxidation of 20:5n-3 over 22:6n-3

4.2. Materials and methods

4.2.1 Cell culture

Aliquots of the SBT-E1 cell line was retrieved from cryostorage and the cells were cultured in the standard culture medium (Section 2.1.1). The retrieved cells were passaged as described in Section 2.1.3 until there were sufficient cells for the experiments described below.

4.2.2 Lipid class composition of the total lipid from the SBT-E1 cells

The SBT-E1 cells were cultured in the standard culture medium (Section 2.1.1) and total lipid was extracted as described in Section 2.4.3. Lipid classes were separated and analysed as described in Section 2.4.3.

4.2.3 Fatty acid profiles of the total lipid and the various lipid classes from the SBT-E1 cells

The SBT-E1 cells were cultured in the standard cell culture medium (Section 2.1.1.). Total lipid was extracted from the SBT-E1 cells the different classes of lipids were separated as described in Section 2.4.3. The fatty acid profiles of the separated lipid classes were produced as described in Section 2.4.4.

4.2.4 Fatty acid profiles of the total lipid and the various lipid classes from SBT tissues

Samples of tail cut muscle, liver, intestine and kidney were obtained from farmed SBT specimens as described in Section 2.4.5. Total lipid was extracted, polar and

neutral lipids were separated and FAME were produced and analysed as described in Section 2.4.5.

4.2.5 Incubation of the SBT-E1 cells with [1-^{14}C]-labelled fatty acids

The SBT-E1 cells were cultured under the standard cell culture conditions (Section 2.1) until there were enough for the experiment. Once there were enough, they were pelleted and then resuspended in the standard cell culture medium but with the FBS concentration reduced to 5% (v/v). The resuspended cells were counted and seeded into 75 cm^2 cell culture flasks in the standard cell culture medium but with the FBS concentration educed to 5% (v/v) (Section 2.2). Once the cells had adhered to the surface of the flasks (after approximately 4 h), 0.25 μCi (0.33 μM) of each [1-^{14}C]-labelled fatty acid complexed with FAF-BSA was added to the appropriate flasks (Section 2.4.6) and the cells were incubated at 25 ^{o}C for 72 h.

4.2.5 Esterification of [1-^{14}C]-labelled fatty acids into individual lipid classes

At the end of the incubation (see above), the culture medium was removed and the cells were harvested (Section 2.2.4). The esterification of the [1-^{14}C]-labelled fatty acids into individual lipid classes was analysed according to the protocol described in Section 2.4.6.

4.2.6 Metabolism of [1-^{14}C]-labelled fatty acids by desaturation and/or elongation

The cells were incubated with the [1-^{14}C]-labelled fatty acids and the desaturation and elongation of these fatty acids was conducted as described in Section 2.4.7.

4.2.7 β-oxidation of [1-^{14}C]-labelled fatty acids

The cells were incubated with [1-^{14}C]-labelled fatty acids and β-oxidation of these fatty acids was determined as described in Section 2.4.8.

4.2.8 Incubation of the SBT-E1 cells with unlabelled fatty acids

The SBT-E1 cells were seeded into 75 cm^2 culture flasks in the standard cell culture medium but with the FBS concentration set at 2, 5 or 10% (v/v) as described in Section 2.2. Once the cells had adhered to the flasks (after approximately 4 h), 125 μM vitamin E and 10 μM of each fatty acid were added to the appropriate flasks as described in Section 2.4.1. The cells were then incubated at 25 °C for either 24 or 72 h.

4.2.9 RNA extraction and first-strand cDNA synthesis

RNA was extracted from the SBT-E1 cells and cDNA was synthesised from 1 μg of the extracted RNA using the methods described in Sections 2.6.3 and 2.6.5, respectively.

4.2.10 Quantitative real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed according to the protocol described in Section 2.6.6. For each treatment and for each gene of interest, the fold-change in expression normalised to β-actin and relative to the control cells was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). This was done according to the equations below:

1. Ct (gene of interest) – Ct (β-actin) = ΔCt
2. ΔCt (treatment) – ΔCt (control treatment) = ΔΔCt
3. $2^{-\Delta\Delta Ct}$ = fold-change in expression

4.2.11 Statistical analyses

Statistical analyses were conducted as described in Section 2.8. Specifically, the lipid and fatty acid composition data were analysed using one-way ANOVA followed by Bonferroni *post-hoc* comparisons. The gene expression data were analysed using one-way ANOVA followed by Dunnett's 2-sided multiple comparison test.

Table 4.1: Primers used for qRT-PCR

Target gene	Forward primer sequence (5´→ 3´)	Reverse primer sequence (5´→ 3´)	Amplicon size (base pairs)	Efficiency
β-Actin	ACCCACACAGTGCCCATCTA	TCACGCACGATTTCCCTCT	155	0.91
Δ6Fads	CCGTGCACTGTGTGAGAAAC	CAGTGTAAGCGATAAAATCAGCTG	152	1.00
Elovl5	CCCACCAACATTTGCACTCA	GTCCTGGCAGTAGAAGTTGT	189	0.96

4.3 Results

4.3.1 Lipid class composition of the total lipid from the SBT-E1 cells

The lipid class composition of the total lipid from the SBT-E1 cells cultured in medium containing 5% (v/v) FBS is shown in Table 4.2. The total lipid consisted of approximately equal proportions of polar and neutral lipids. Amongst the polar lipids, PC was the most abundant (26.2% of total lipid) followed by PE (15.1% of total lipid). In terms of relative abundance, both PC and PE were significantly more abundant than any of the other polar lipid classes and PC was significantly more abundant than PE.

Table 4.2: Lipid class composition of the total lipid from SBT-E1 cells cultured in the standard cell culture medium but with the FBS concentration reduced to 5% (v/v). Data are the mean ± the standard error of the mean (n = 3). Different superscript letters indicate significant differences between the different lipid classes ($p < 0.05$)

Lipid class	Content (% of total lipid)
Phosphatidylcholine	26.2 ± 1.0^{b}
Phosphatidylethanolamine	15.1 ± 0.5^{c}
Phosphatidylserine	4.6 ± 0.4^{de}
Phosphatidylinositol	6.5 ± 0.3^{d}
Phosphatidic acid/cardiolipin	2.1 ± 0.1^{e}
Sphingomyelin	4.8 ± 0.6^{de}
Total neutral lipids	40.7 ± 1.1^{a}

4.3.2 Fatty acid composition of the total lipid and the various lipid classes from the SBT-E1 cells

Table 4.3 shows the fatty acid compositions of the total lipid and the various lipid classes from the SBT-E1 cells compared with the fatty acid composition of the total lipid from the culture medium. The culture medium was rich in saturated fatty acids (especially 16:0 and 18:0) and MUFA (especially 18:1n-9) but poor in PUFA (including 18:2n-6 and 18:3n-3) and LC-PUFA (including 20:4n-6, 20:5n-3, 22:5n-3 and 22:6n-3). This was as expected because foetal bovine serum (FBS) was the source of the fatty acids in the culture medium. The fatty acid composition of the total lipid from the SBT-E1 cells reflected that of the culture medium except that there was evidence for enrichment of the cells with 20:4n-6, 22:5n-3 and 22:6n-3. The various fatty acids were distributed differently between the different classes of lipids. Neutral lipids were rich in 16:0 whereas PC was rich in 18:1n-9. PE was rich in the most abundant LC-PUFA 20:4n-6, 22:5n-3 and 22:6n-3. PE also contained the majority of the 20:5n-3 but the level was very low compared with the other LC-PUFA.

Table 4.3: Fatty acid compositions of the culture medium, the total lipid from the SBT-E1 cells and the various lipid classes from the SBT-E1 cells. The data are the mean ± SE (n = 3) and are expressed as % of total fatty acids on a mass basis. Different superscript letters within a row indicate significant differences between the different lipid classes ($p < 0.05$). The data were arc-sin transformed prior to statistical analysis using a one-way analysis of variance (ANOVA) followed by Bonferroni *post-hoc* comparisons.

Fatty Acid	Culture[1] medium	Total lipid[2]	Lipid class[3]					
			PC	PE	PS	PI/PA/CL	SM	TN
14:0	2.1 ± 0.4	0.6 ± 0.0	0.8 ± 0.1^{b}	2.6 ± 1.1^{b}	2.2 ± 0.5^{b}	2.3 ± 0.9^{b}	2.1 ± 0.0^{b}	8.5 ± 0.2^{a}
16:0	24.0 ± 0.9	14.2 ± 0.3	12.9 ± 0.2^{b}	15.7 ± 6.0^{b}	16.2 ± 2.0^{ab}	14.9 ± 5.8^{b}	16.2 ± 3.8^{ab}	38.9 ± 1.4^{a}
18:0	7.5 ± 0.4	11.0 ± 0.1	6.1 ± 0.0^{c}	15.4 ± 0.6^{ab}	19.8 ± 2.6^{ab}	21.4 ± 0.3^{a}	8.2 ± 2.4^{bc}	12.0 ± 0.4^{bc}
Total SFA[4]	57.1 ± 1.5	31.6 ± 0.4	20.8 ± 0.3^{b}	38.9 ± 7.9^{b}	44.5 ± 1.2^{ab}	41.5 ± 6.5^{b}	36.0 ± 6.9^{b}	70.5 ± 1.6^{a}
16:1n-7	2.4 ± 0.3	3.2 ± 0.1	7.2 ± 0.1^{a}	1.1 ± 0.0^{c}	0.8 ± 0.1^{c}	0.8 ± 0.0^{c}	2.2 ± 0.4^{b}	1.4 ± 0.1^{bc}
18:1n-9	13.6 ± 3.5	19.9 ± 0.3	43.0 ± 0.3^{a}	9.4 ± 0.4^{b}	9.4 ± 1.2^{b}	10.5 ± 0.7^{b}	7.5 ± 2.2^{bc}	4.3 ± 0.4^{c}
18:1n-7	2.8 ± 0.2	5.0 ± 0.1	10.0 ± 0.1^{a}	5.2 ± 0.6^{b}	1.4 ± 0.2^{c}	2.7 ± 0.3^{c}	2.0 ± 0.6^{c}	1.1 ± 0.1^{c}
Total MUFA[5]	22.6 ± 3.6	31.5 ± 0.3	61.7 ± 0.3^{a}	17.0 ± 0.8^{b}	13.6 ± 0.8^{b}	14.8 ± 0.9^{b}	12.5 ± 2.2^{b}	11.4 ± 0.9^{b}
18:2n-6/19:0	2.3 ± 0.4	3.3 ± 0.1	2.9 ± 0.0^{a}	1.3 ± 0.0^{b}	1.0 ± 0.1^{b}	0.9 ± 0.2^{b}	0.0 ± 0.0^{c}	1.2 ± 0.2^{b}
18:3n-6	0.0 ± 0.0	0.6 ± 0.0	0.5 ± 0.0^{a}	0.2 ± 0.0^{ab}	0.0 ± 0.0^{c}	0.2 ± 0.1^{ab}	0.0 ± 0.0^{c}	0.1 ± 0.0^{b}
20:2n-6	0.0 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.1	0.4 ± 0.2	0.2 ± 0.2	1.0 ± 0.5	0.4 ± 0.2
20:4n-6	2.7 ± 0.1	9.0 ± 0.1	1.8 ± 0.1^{c}	11.0 ± 1.2^{b}	2.1 ± 0.3^{c}	16.1 ± 1.7^{a}	0.0 ± 0.0^{d}	1.2 ± 0.1^{c}
22:4n-6/22:3n-3	0.0 ± 0.0	1.8 ± 0.1	0.3 ± 0.0^{b}	0.9 ± 0.1^{a}	1.2 ± 0.2^{a}	0.2 ± 0.1^{bc}	0.0 ± 0.0^{c}	0.2 ± 0.0^{bc}
Total n-6 PUFA[6]	5.7 ± 0.4	17.5 ± 0.1	7.4 ± 0.2^{b}	16.0 ± 1.8^{a}	8.5 ± 1.6^{b}	23.1 ± 2.7^{a}	6.0 ± 0.7^{b}	4.4 ± 0.2^{b}
18:3n-3	0.2 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
20:3n-3	0.0 ± 0.0	0.2 ± 0.0	0.1 ± 0.0^{ab}	0.1 ± 0.1^{ab}	0.0 ± 0.0^{b}	0.2 ± 0.2^{ab}	0.0 ± 0.0^{b}	0.5 ± 0.0^{a}
20:5n-3	0.6 ± 0.1	1.2 ± 0.1	0.4 ± 0.0^{b}	1.6 ± 0.2^{a}	0.1 ± 0.1^{c}	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}
22:5n-3	1.0 ± 0.1	6.3 ± 0.1	1.3 ± 0.0^{b}	6.5 ± 0.7^{a}	6.0 ± 1.2^{a}	1.4 ± 0.1^{b}	0.0 ± 0.0^{c}	0.5 ± 0.1^{b}
22:6n-3	2.0 ± 0.2	9.2 ± 0.1	1.8 ± 0.0^{b}	11.3 ± 1.1^{a}	8.2 ± 0.9^{a}	3.2 ± 0.5^{b}	9.3 ± 1.3^{a}	1.2 ± 0.2^{b}
Total n-3 PUFA[7]	3.9 ± 0.5	17.2 ± 0.2	3.7 ± 0.1^{d}	19.5 ± 1.9^{a}	14.3 ± 2.1^{ab}	4.7 ± 0.5^{cd}	9.3 ± 1.3^{bc}	2.2 ± 0.3^{d}

[1]The culture medium contained 5% (v/v) FBS which was the source of the fatty acids. [2]The cells were cultured in the presence of 10% (v/v) FBS. [3]The cells were cultured in the presence of 5% (v/v) FBS. [4]Also includes 8:0, 9:0, 10:0, 11:0, 12:0, 13:0, 15:0, 17:0, 20:0, 22:0 and 24:0. [5]Also includes 11:1, 13:1, 14:1, 15:1, 16:1n-9, 17:1, 19:1, 22:1n-9 and 24:1. [6]Also includes 20:3 n-6, 22:2n-6 and 22:5n-6. [7]Also includes 18:4n-3.

4.3.4 Fatty acid composition of the total lipid and phospholipids from SBT tissues

The fatty acid profiles of the total lipid and the phospholipids from the SBT tissues are shown in Table 4.4. As in the cell line, 22:6n-3 was the most abundant PUFA in the total lipid and in the phospholipids followed by 20:5n-3 but the proportions of these fatty acids (as percent of total fatty acids) were greater in the tissues than in the cell line which presumably reflects the dearth of these fatty acids in the cell culture medium. In the tissues, 22:6n-3 was more abundant in the phospholipids than in the total lipid whereas 20:5n-5 was not. This suggested selective retention of 22:6n-3 in the phospholipids. As a result, the 22:6n-3 to 20:5n-3 ratios were greater in the phospholipids than in the total lipid.

Table 4.4: Fatty acid composition of total lipid and phospholipids from SBT liver, muscle, intestine and kidney. The data are the mean ± SE (n = 3) expressed as % of total fatty acids on a mass basis.

Fatty Acid	Total lipid				Phospholipids			
	Liver	Muscle	Intestine	Kidney	Liver	Muscle	Intestine	Kidney
14:0	2.54 ± 0.83	3.75 ± 0.39	3.95 ± 0.19	4.36 ± 0.16	1.22 ± 0.35	1.34 ± 0.09	1.33 ± 0.12	2.26 ± 0.56
16:0	19.26 ± 1.24	19.93 ± 0.97	19.54 ± 0.51	19.26 ± 0.27	22.34 ± 1.06	21.07 ± 1.05	18.44 ± 0.64	24.19 ± 0.58
18:0	8.24 ± 1.31	7.62 ± 1.04	7.49 ± 0.73	5.84 ± 0.49	12.76 ± 1.60	13.39 ± 0.83	18.33 ± 1.01	10.81 ± 0.66
Total SFA[1]	31.84 ± 1.63	33.37 ± 1.80	33.05 ± 0.77	31.31 ± 0.80	38.08 ± 0.43	37.31 ± 0.65	39.91 ± 0.68	39.32 ± 1.86
16:1n-7	2.54 ± 0.48	3.97 ± 0.35	4.56 ± 0.49	4.93 ± 0.44	1.01 ± 0.16	1.52 ± 0.08	1.43 ± 0.10	2.28 ± 0.70
18:1n-9	15.65 ± 6.38	13.87 ± 0.20	14.43 ± 1.30	15.50 ± 1.02	6.11 ± 2.10	8.20 ± 0.46	6.66 ± 0.34	9.94 ± 1.74
18:1n-7	3.38 ± 0.45	2.11 ± 1.02	3.30 ± 0.17	3.39 ± 0.16	2.05 ± 0.35	2.89 ± 0.13	2.48 ± 0.11	3.60 ± 0.38
Total MUFA[2]	34.42 ± 5.09	31.54 ± 4.18	30.88 ± 3.64	32.27 ± 2.66	14.69 ± 1.47	17.57 ± 0.93	16.27 ± 0.92	21.01 ± 2.02
18:2n-6/19:0	1.82 ± 0.24	1.92 ± 0.12	1.79 ± 0.19	1.77 ± 0.15	1.05 ± 0.04	1.69 ± 0.05	2.11 ± 0.17	1.45 ± 0.21
18:3n-6	0.24 ± 0.01	0.20 ± 0.01	0.02 ± 0.00	0.22 ± 0.00	0.09 ± 0.01	0.12 ± 0.00	0.18 ± 0.02	0.17 ± 0.01
20:2n-6	0.43 ± 0.06	0.34 ± 0.01	0.29 ± 0.01	0.30 ± 0.02	0.31 ± 0.05	0.35 ± 0.03	0.31 ± 0.03	0.34 ± 0.01
20:4n-6	1.48 ± 0.22	1.38 ± 0.18	1.44 ± 0.09	1.37 ± 0.10	3.67 ± 0.38	2.37 ± 0.07	3.15 ± 0.17	3.86 ± 0.49
22:4n-6/22:3n-3	0.13 ± 0.00	0.15 ± 0.02	0.15 ± 0.02	0.15 ± 0.02	0.09 ± 0.02	0.20 ± 0.01	0.22 ± 0.03	0.20 ± 0.02
Total n-6 PUFA[3]	4.85 ± 0.32	4.82 ± 0.44	4.61 ± 0.46	4.59 ± 0.26	6.02 ± 0.29	5.94 ± 0.10	7.16 ± 0.31	6.92 ± 0.38
18:3n-3	1.00 ± 0.16	0.96 ± 0.05	1.03 ± 0.16	1.14 ± 0.15	0.32 ± 0.03	0.41 ± 0.02	0.55 ± 0.06	0.53 ± 0.19
20:3n-3	0.20 ± 0.02	0.14 ± 0.00	0.13 ± 0.01	0.14 ± 0.01	0.13 ± 0.02	0.12 ± 0.01	0.10 ± 0.01	0.16 ± 0.00
20:5n-3	7.61 ± 1.33	6.99 ± 0.31	8.33 ± 0.34	9.09 ± 0.28	6.73 ± 1.06	6.41 ± 0.44	8.45 ± 0.15	7.66 ± 0.77
22:5n-3	2.14 ± 0.21	1.73 ± 0.05	1.49 ± 0.05	1.75 ± 0.01	1.30 ± 0.01	1.66 ± 0.09	1.23 ± 0.09	1.34 ± 0.06
22:6n-3	17.30 ± 1.91	20.14 ± 2.35	20.18 ± 2.54	18.93 ± 1.69	32.41 ± 0.44	30.43 ± 0.74	26.08 ± 1.23	22.82 ± 3.04
Total n-3 PUFA[4]	28.25 ± 3.34	29.96 ± 2.02	31.16 ± 2.81	31.05 ± 1.70	40.89 ± 1.33	38.99 ± 0.26	36.40 ± 1.22	32.51 ± 3.67
22:6n-3/20:5n-3 ratio	2.36 ± 0.31	2.92 ± 0.47	2.43 ± 0.30	2.09 ± 0.23	5.11 ± 0.93	4.81 ± 0.46	3.09 ± 0.17	2.96 ± 0.11

[1]Also includes 8:0, 9:0, 10:0, 11:0, 12:0, 13:0, 15:0, 17:0, 20:0, 22:0 and 24:0. [2]Also includes 11:1, 13:1, 14:1, 15:1, 16:1n-9, 17:1, 19:1, 22:1n-9 and 24:1. [3]Also includes 20:3 n-6, 22:2n-6 and 22:5n-6.
[4]Also includes 18:4n-3.

4.3.4 Esterification of [1-^{14}C]-labelled fatty acids into different classes of lipids

The results for the esterification of [1-^{14}C]-labelled fatty acids into different classes of lipids in the SBT-E1 cells are shown in Fig. 4.1. Most of the fatty acids, other than 20:5n-3, were incorporated predominantly into PC. In the case of 20:5n-3, there was approximately equal incorporation into PC and PE. The label recovered in PC ranged from approximately 50% to a little more than 60% of the total for all of the fatty acids except 20:5n-3. Nearly 80% of the label from 20:5n-3 was recovered in both PC and PE with approximately 40% in each. When the fatty acid esterification data were expressed relative to the lipid class abundance data, there was clear preferential incorporation of 16:0 into PC and sphingomyelin (SM), 18:1n-9 into PC and phosphatidylserine (PS) and 18:2n-6 into PC and phosphatidic acid/cardiolipin (PA/CL) (data not shown). The results for 18:3n-3 were similar to those for 18:2n-6 though not as pronounced. This way of expressing the data also showed preferential incorporation of 20:5n-3 into PE over PC.

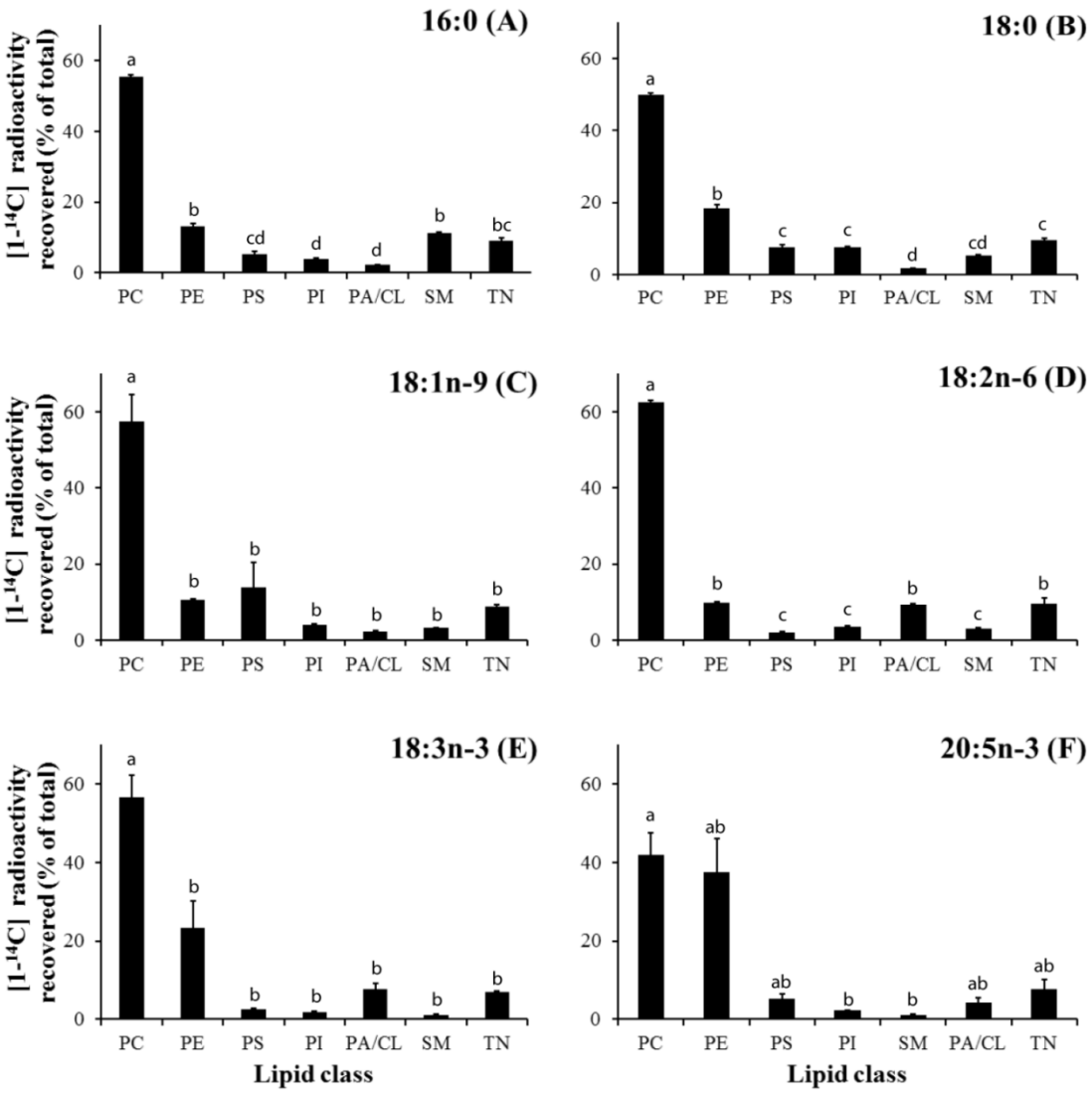


Figure 4.1: Incorporation of [1-^{14}C]-labelled fatty acids into different classes of lipids in the SBT-E1 cell line. The data are the mean ± standard error of the mean (n = 3). PC: Phosphatidylcholine, PE: Phosphatidylethanolamine, PS: Phosphatidylserine, PI: Phosphatidylinositol, PA/CL: Phophatidic acid/Cardiolipin, SM: Sphingomyelin, TN, Total neutral lipids. For each individual fatty acid, significant differences between different classes of lipids are indicated by different letters.

4.3.4 Metabolism of [1-^{14}C]-labelled PUFA by desaturation and/or elongation

The results for the desaturation/elongation of incorporated [1-^{14}C]-labelled PUFA by the SBT-E1 cells are shown in Table 4.5. Cells supplied with [1-^{14}C]18:3n-3 converted 12.8% of this substrate to desaturation/elongation products. The majority of this conversion was to the C_2 elongation product 20:3n-3 (9.4%) but there was also some Δ6 desaturation to yield 18:4n-3 (2.2%) and a small amount of Δ6 desaturation plus C_2 elongation to yield 20:4n-3 (1.2%). In contrast, the cells supplemented with [1-^{14}C]18:2n-6 showed approximately equal amounts of either C_2 elongation (2.8%) or Δ6 desaturation (2.5%) but no detectable Δ6 desaturation plus C_2 elongation and the total amount of substrate conversion (5.3%) was significantly less than for 18:3n-3. In contrast to the above, the cells supplied [1-^{14}C]20:5n-3 showed substantial C_2 elongation (16.8% substrate conversion). Thus, the cells exhibited appreciable fatty acyl elongase activity but only very limited Δ6Fads activity.

Table 4.5: Metabolism of [1-^{14}C] PUFA by desaturation and/or elongation in the SBT-E1 cells. The data are the mean ± standard error (n = 3) and are expressed as % of total radioactivity recovered for each of the PUFA supplied. n.d. = not detected. Asterisks indicate significant differences in conversion percentages within a particular treatment and different superscript letters indicate different conversion percentages with different supplied fatty acids determined using one-way ANOVA followed by Bonferroni post-hoc comparisons (p <0.05)

	[1-^{14}C] PUFA supplied		
Fatty acid	18:3n-3	18:2n-6	20:5n-3
18:3n-3	87.2 ± 1.0	n.d.	n.d.
18:4n-3	2.2 ± 0.1	n.d.	n.d.
20:3n-3	9.4 ± 0.8*	n.d.	n.d.
20:4n-3	1.2 ± 0.1	n.d.	n.d.
20:5n-3	n.d.	n.d.	83.2 ± 0.8
22:5n-3	n.d.	n.d.	16.8 ± 0.8
18:2n-6	n.d.	94.7 ± 0.3	n.d.
18:3n-6	n.d.	2.5 ± 0.3	n.d.
20:2n-6	n.d.	2.8 ± 0.1	n.d.
Total conversion	12.8 ± 1.0^{b}	5.3 ± 0.3^{c}	16.8 ± 0.8^{a}

4.3.5 β-oxidation of [1-^{14}C]-labelled fatty acids

The results for the β-oxidation of the incorporated [1-^{14}C]-labelled fatty acids by the SBT-E1 cells are shown in Fig. 4.2. The rates obtained for 20:5n-3 and 18:3n-3 were approximately 2- and 4-fold greater, respectively, than those obtained for 18:0, 18:1n-9 or 18:2n-6. Thus, the cells showed high rates of β-oxidation of n-3 PUFA, especially 18:3n-3.

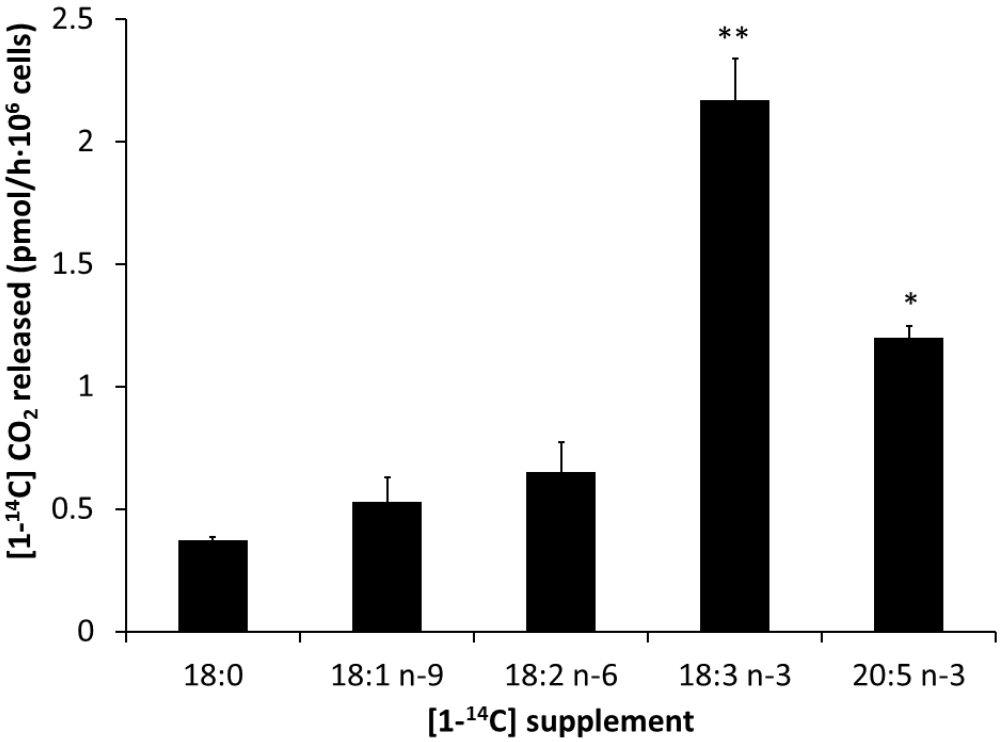


Figure 4.2: β-oxidation of [1-^{14}C]-labelled fatty acids by the SBT-E1 cell line. The data are the mean ± standard error of the mean (n = 3). Significant differences between the different fatty acids are indicated by an asterisk.

4.3.6 Effects of FBS concentration and fatty acid supplementation on Δ6Fads and Elovl5 gene expression in the SBT-E1 cell line

In cell culture, the main source of fatty acids to support cell proliferation is the FBS in the culture medium (Tocher *et al.* 1988). Fig. 4.3A shows the effects of different PUFA supplements on Δ6Fads and Elovl5 gene expression in the SBT-E1 cells cultured in the presence of 5% (v/v) FBS for 72 h. Addition of the PUFA to the culture medium significantly reduced the expression of Δ6Fads but not Elovl5. Fig. 4.3B shows the effects of different PUFA supplements on Δ6Fads and Elovl5 gene expression in the SBT-E1 cells cultured either in the presence of 10% (v/v) FBS or with or without various PUFA in the presence of 2% (v/v) FBS. In this case, reducing the FBS concentration from 10% to 2% (v/v) significantly increased the expression of Δ6Fads and supplementation of the culture medium with the various PUFA in the presence of 2% (v/v) FBS returned the expression of Δ6Fads to the low level seen in the presence of 10% (v/v) FBS. Thus, fatty acid deprivation (i.e. reduction of the FBS concentration from 10% to 2% (v/v)) increased the expression of Δ6Fads but this was reversed when the cells were supplied with various PUFA. Similarly, Elovl5 gene expression appeared to be higher in cells cultured in 2% (v/v) FBS compared to 10% (v/v) FBS but there were no significant differences between the different treatments in the case of Elovl5. In Fig. 4.3C, the relative expression (gene of interest transcript copy number relative to β-actin transcript copy number) of Δ6Fads is compared with that of Elovl5. Interestingly, expression of the Δ6Fads gene was more than 2 orders of magnitude greater than that of the Elovl5 gene.

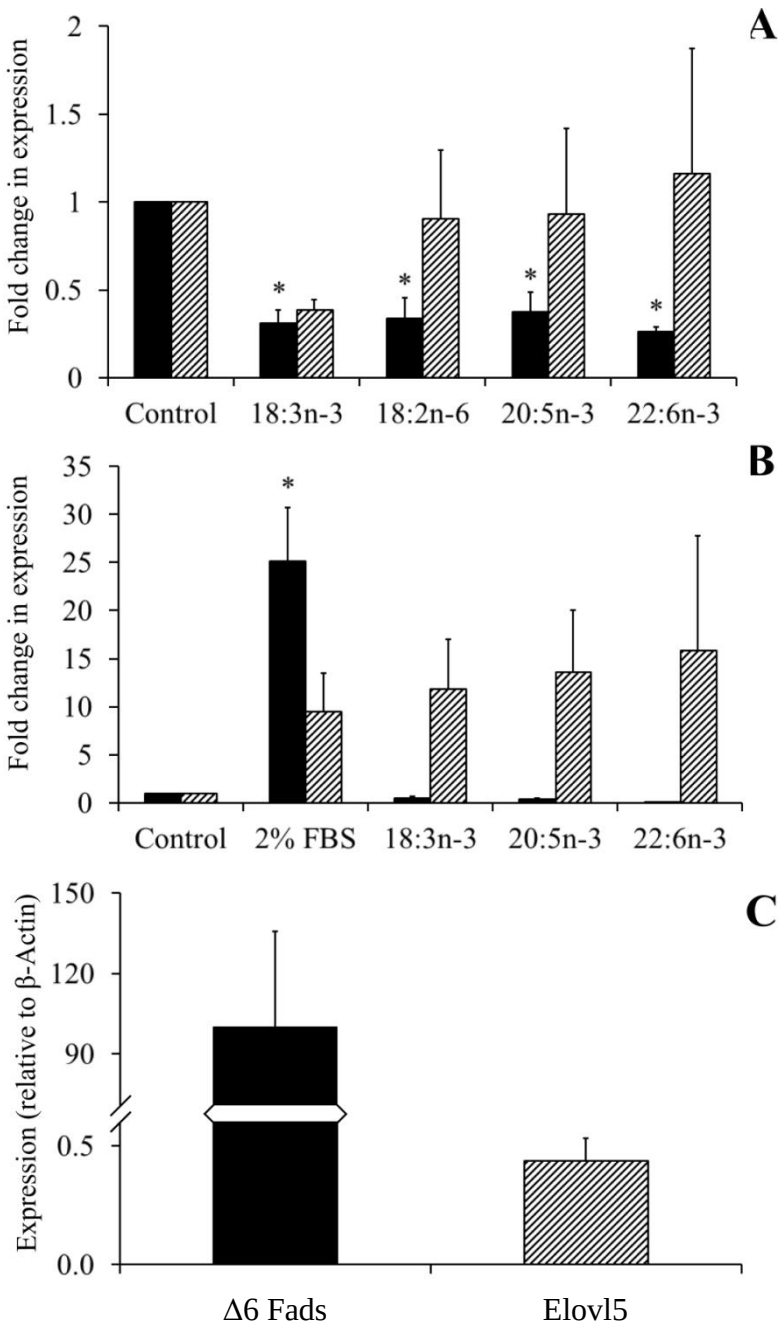


Figure 4.3: The effects of FBS concentration and individual fatty acid supplementation on Δ6Fads (filled columns) and Elovl5 (cross-hatched columns) gene expression in the SBT-E1 cell line. In Panel A, the cells were incubated under the different conditions for 3 days and the fatty acids were supplied at a concentration of 10 μM. In Panel B, the cells were incubated under the different conditions for 24 h and the fatty acids were supplied at a concentration of 10 μM. In Panel C, the cells were maintained in the standard cell culture medium containing 10% (v/v) FBS. In Panels A and B, the data have been normalised using β-actin and are expressed relative to the control, arbitrarily set at a value of 1.0. In Panel C, the transcript abundances for Δ6Fads and Elovl5 are expressed relative to the transcript abundance for β-actin. The data are the mean ± SE (n = 3). Significant differences between the treatments and the control are indicated by an asterisk.

4.4 Discussion

4.4.1 Lipid class composition of the total lipid from the SBT-E1 cells

In established cell lines from a range of freshwater, anadromous and marine fish species, polar lipids (mostly phospholipids) accounted for 40.5-76.1% of the total lipid and the most abundant phospholipid was PC (19.4-40.8% of total lipid) followed by PE (17.3-23.7% of total lipid) (Tocher *et al.* 1988, Tocher *et al.* 1989, Tocher and Dick 1999, Tocher and Ghioni 1999). Similarly, in the SBT-E1 cell line, phospholipids accounted for 59.3% of the total lipid and the most abundant phospholipid was PC (26.2% of total lipid) followed by PE (15.1% of total lipid). Interestingly, PE was less abundant in the SBT-E1 cells compared with the other cells. Studies with fish tissues have shown that PC is almost always the most abundant phospholipid, followed by PE (Tocher 1995, Morais *et al.* 2011, Sprague *et al.* 2012). Phospholipids, predominantly found in cell membranes, are important in maintaining structural integrity and fluidity and are not found in high concentrations elsewhere in the cell. Cold acclimation is associated with increased proportions of PE and decreased proportions of PC in fish lipids (Tocher 1995). The SBT-E1 cells were cultured at a higher temperature (25^{o}C) than the other cells (22^{o}C) (Tocher *et al.* 1988, Tocher *et al.* 1989, Tocher and Dick 1999, Tocher and Ghioni 1999). This could perhaps explain the lower proportion of PE in the SBT-E1 cells. Alternatively, it could be due to the fact that tunas are partially endothermic (Graham and Dickson 2004) with less need to adjust the fluidity of their membranes to cope with lower water temperatures.

The lipid class compositions of tuna tissues have not been studied in detail except in longtail tuna (*Thunnus tonggol*) and Atlantic bluefin tuna (*Thunnus thynnus*) (Mourente *et al.* 2002b, Saito *et al.* 2005, Sprague *et al.* 2012). In “ordinary muscle”,

"dark muscle" and liver from longtail tuna, the relative amounts of polar and neutral lipids varied depending upon the tissue but the most abundant polar lipid class was almost always PC (10.5-24.3% of total lipid) followed by PE (7.5- 25% of total lipid) (Saito *et al.* 2005). Similar results were found for red muscle, white muscle, liver and ovary from female Atlantic bluefin tuna during their reproductive migration to their spawning grounds in the Mediterranean Sea (Mourente *et al.* 2002b, Sprague *et al.* 2012). Thus, broadly speaking, the SBT-E1 cells retained the characteristics of their species of origin with respect to the phospholipid composition of their total lipid.

4.4.2 Fatty acid composition of the total lipid and the various lipid classes from the SBT-E1 cells and SBT tissues

The most abundant fatty acids in the total lipid of the SBT-E1 cell line were 16:0, 18:0, 18:1n-9, 20:4n-6, 22:5n-3 and 22:6n-3. This was similar to cell lines from other marine fish species, namely turbot (*Scophthalmus maximus*) and gilthead seabream (*Sparus aurata*) (Ghioni *et al.* 1999, Tocher and Ghioni 1999) and also to cell lines from freshwater and anadromous fish species (Tocher *et al.* 1988, Tocher *et al.* 1989, Tocher and Dick 1999). However, there was one major difference and this was the appreciably greater abundance of 22:6n-3 in the tuna cell line (9.2% of total fatty acids) compared with freshwater or anadromous fish cell lines (1.1-3.8% of total fatty acids) and also with marine fish cell lines (3.9- 5.5% of total fatty acids). This suggested selective retention of 22:6n-3 by the tuna cell line because the amount of 22:6n-3 supplied in the culture medium was very low (only 2% of total fatty acids). It has been observed that tunas caught from the wild have unusually high proportions of 22:6n-3 in their lipids and higher ratios of 22:6n-3 to 20:5n-3 than in their prey (Murase and Saito 1996, Saito *et al.* 1996). For example, muscle tissues from yellowfin tuna (*Thunnus albacares*) caught off the Pacific coast of Japan contained

3.3- 5.0 % and 22.0-36.0 % of their total fatty acids as 20:5n-3 and 22:6n-3, respectively, giving 22:6n-3 to 20:5n-3 ratios of 6.7-8.6 (Saito *et al.* 1996). This compared with 8.4% and 22.7% of their total fatty acids as 20:5n-3 and 22:6n-3, respectively, and a 22:6n-3 to 20:5n-3 ratio of only 2.7 for their stomach contents. Similar results have been found for Albacore (*Thunnus alalunga*) and longtail tuna (*Thunnus tonggol*) (Murase and Saito 1996, Saito *et al.* 2005). This led to the proposition that tunas selectively retain 22:6n-3 in their tissues. Selective retention could be due to selective esterification into cellular lipids or to sparing from β-oxidation (Tocher 2003). Similarly, high 22:6n-3 to 20:5n-3 ratios could be due to selective metabolism of 20:5n-3 either via elongation to 22:5n-3 or via β-oxidation (Tocher 2003). The initial reactions in the β-oxidation of 22:6n-3 in mammals involve the peroxisomes and this, presumably, is also true for fish (Madsen *et al.* 1999, Tocher 2003). Thus, selective retention of 22:6n-3 could be due to inefficient oxidation as it involves partial oxidation in the peroxisomes followed by transfer to the mitochondria for completion. In the present study we found 22:6n-3 to 20:5n-3 ratios ranging from 2.1 to 2.9 in the total lipid and 3.0 to 5.1 in the phospholipids of various tissues from farmed SBT. These values are somewhat lower than those observed for yellowfin tuna (see above). This is due to the higher proportions of 20:5n-3 in the SBT total lipid (7.0- 9.1% of total fatty acids) as compared with the yellowfin tuna total lipid (3.3-5.0% of total fatty acids). This may reflect the differences between the diets of wild tunas and farmed tunas (Saito *et al.* 2005). Overall though, the SBT-E1 cell line appears to have retained the traits of selective retention of 22:6n-3 and high ratios of 22:6n-3 to 20:5n-3 which are characteristic of tuna tissues.

The main fatty acids in the total lipid of the SBT-E1 cells showed different distributions between the different lipid classes. For example, neutral lipids contained a high proportion of 16:0, almost 40% of the total fatty acids. This compared with a median value of only 25% in other fish cell lines (Tocher *et al.* 1988). Similarly, PC contained a high proportion of 18:1n-9, approximately 43%. This compared with a median value of only 28% in other fish cell lines (Tocher *et al.* 1988). The important n-6 LC-PUFA, 20:4n-6, was found predominantly in PE and the combined PI/PA/CL fraction in the SBT-E1 cells. This was similar to several other fish cell lines (Tocher *et al.* 1988). In contrast, the important n-3 LC-PUFA, 22:6n-3, was found predominantly in PE, PS and SM. This was similar to a cell line from turbot, another marine fish species, but different to cell lines from freshwater and anadromous fish species. In general, LC-PUFA were incorporated predominantly into PE both in the SBT-E1 cell line and in other fish cell lines.

Although it was not particularly abundant, it is important to consider the distribution of 20:5n-3 because of its widely recognized human health benefits as the precursor to eicosanoids which are less pro-inflammatory than those derived from the equivalent n-6 LC-PUFA, 20:4n-6 (Yates *et al.* 2014). The only lipid class that contained significant amounts of 20:5n-3 was PE. In this lipid class, the ratio of 22:6n-3 to 20:5n-3 was 7.0, similar to the ratio of 7.9 found for the total lipid. Again this suggests selective incorporation of 22:6n-3 into the cellular lipids and/or selective metabolism of 20:5n-3.

In tuna tissues, the distribution of different fatty acids between different classes of lipids has not been investigated except to some extent in longtail tuna (Saito *et al.* 2005). In this species, the distribution of various fatty acids between triacylglycerols

(neutral lipids), PC and PE (the two most abundant classes of polar lipids) in "light muscle", "dark muscle", liver, pyloric ceca and orbital oil was studied. In all of these tissues 16:0 and 18:1n-9 were most abundant in the triacylglycerol fraction. This was different from the SBT-E1 cells in which 18:1n-9 was most abundant in PC. In the longtail tuna tissues, 20:4n-6 was in roughly equal amounts in PC and PE but in reduced amounts in the triacylglycerol fraction. This was different to what was seen in the SBT-E1 cells in which 20:4n-6 was significantly more abundant in PE than in PC. In the tissues of longtail tuna, 22:6n-3 was most abundant in PC (26.4-49.5% of total fatty acids) followed closely by PE (20.6-45.4% of total fatty acids) and then triacylglycerols (9.0-9.5% of total fatty acids). This was quite different from the SBT-E1 cells. In the SBT-E1 cells, 22:6n-3 was most abundant in PE, PS and SM and very little was found in either PC or triacylglycerols. In general it can be said that LC-PUFA are more abundant in PE than PC in fish cell lines including the SBT-E1 cell line studied here (Tocher *et al.* 1988) but this is not the case in tuna tissues in which the predominant LC-PUFA, 22:6n-3, is abundant in both PC and PE (Saito *et al.* 2005). Thus it appears that the fatty acid profile of the various lipid classes of the SBT-E1 cells show some differences to those of tuna tissues. The fatty acid compositions of the diets of fish and the media used to culture fish cells may influence the distributions of individual fatty acids between the different classes of lipids.

4.4.3 Esterification of [1-^{14}C] labelled fatty acids into different classes of lipids

In cell lines from Atlantic salmon, rainbow trout and gilthead sea bream, the vast majority (~ 40-65%) of the label from 18:2n-6 and 18:3n-3 was recovered in PC (Tocher and Sargent 1990, Tocher and Ghioni 1999). This was similar to what was found in the SBT-E1 cell line. In contrast, the results for 20:5n-3 were much more

variable. In the SBT-E1 cells, approximately equal amounts of the label (~ 40%) from 20:5n-3 were recovered in PC and PE. This compared with ~ 30% in PC and ~ 60% in PE in Atlantic salmon and gilthead sea bream cell lines as opposed to ~ 60% in PC and ~ 30% in PE in rainbow trout cells. Despite this variation, all three PUFA were incorporated predominantly into either PC or PE (Tocher and Sargent 1990, Tocher and Dick 1999, Tocher and Ghioni 1999). In hepatocytes from Atlantic salmon fed diets containing either fish oil or a vegetable oil blend, a similar trend was observed (Stubhaug *et al.* 2005). In the polar lipid fraction, label from 16:0, 18:1n-9, 18:2n-6, 18:3n-3, 20:5n-3 and 22:6n-3 was recovered predominantly in PC followed by PE, except in the case of 20:5n-3 where PC was followed by PI. Thus, the cell lines, which have been long established, behaved similarly to primary cultures recently isolated from tissues. Studies of fish tissues have shown higher levels of 16:0 and lower levels of PUFA incorporation into PC as compared with PE and PS (Tocher 1995). Conversely, they have shown high levels of PUFA (mostly C_{20} and C_{22}) incorporation into PE. The SBT-E1 cell line showed these characteristics as well, especially with respect to the incorporation of 20:5n-3 into PE, thus supporting the hypothesis that the SBT-E1 cells would show different patterns of esterification into the different lipid classes.

4.4.4 Metabolism of [1-^{14}C] labelled PUFA by desaturation and/or elongation

Studies with cell lines from Atlantic salmon, rainbow trout, turbot (*Scophthalmus maximus*) and gilthead seabream (*Sparus aurata*) have shown negligible conversion of 18:3n-3 to 22:6n-3 but some conversion of 18:3n-3 to 20:5n-3 (Tocher and Sargent 1990, Ghioni *et al.* 1999, Tocher and Ghioni 1999). The conversion of 18:3 to 20:5n-3 was greater in freshwater (rainbow trout) and anadromous (Atlantic salmon) fish species than in marine fish species (turbot and gilthead sea bream). In

the marine species, label from [1-^{14}C]18:3n-3 accumulated in 18:4n-3 suggesting significant Δ6Fads activity but limited Elovl5 and/or Δ5Fads activity. In gilthead seabream, the limitation was found to lie at the Δ5Fads catalysed desaturation of 20:4n-3 to 20:5n-3 rather than at the Elovl5 catalysed elongation of 18:4n-3 to 20:4n-3 (Tocher and Ghioni 1999). Clearly there are differences between different fish species depending on their environment and their diet.

The SBT-E1 cell line showed very limited capacity to desaturate 18:3n-3 to 18:4n-3 or 18:2n-6 to 18:3n-6. The apparent rates of conversion were less than 3%. This indicated very limited Δ6Fads activity. Previous studies, under similar conditions, have shown apparent conversion rates for 18:3n-3 to 18:4n-3 ranging from 12.2 to 64.2% in cell lines from Atlantic salmon, rainbow trout, turbot and gilthead sea bream (Tocher and Sargent 1990, Ghioni *et al.* 1999, Tocher and Ghioni 1999). Thus, the SBT-E1 cell line had by far the lowest conversion rates of any fish cell line studied to date. In contrast, the conversion rate for 20:5n-3 to 22:5n-3 (16.8%) in the SBT-E1 cells was, as hypothesised, quite substantial. This rate was towards the upper end of the range (8.1 to 17.5%) found for cell lines from Atlantic salmon, rainbow trout, turbot and gilthead sea bream (Tocher and Sargent 1990, Ghioni *et al.* 1999, Tocher and Ghioni 1999). Thus, the SBT-E1 cell line exhibited substantial fatty acyl elongase activity but very limited Δ6Fads activity. The substantial fatty acyl elongase activity of the SBT-E1 cells can be seen as at least part of the explanation for the low steady state levels of 20:5n-3 and high steady state levels of 22:5n-3 in the SBT-E1 cellular lipids (discussed above). This high elongase activity coupled with high rates of β-oxidation for 20:5n-3 (discussed below) presumably explains the high 22:6n-3 to 20:5n-3 ratios in the SBT-E1 cells. High 22:6n-3 to 20:5n-3 ratios appear to be

characteristic of tuna tissues in general (Mourente and Tocher 2009, Morais *et al.* 2011).

The results presented here indicate that, contrary to the proposed hypotheses, the SBT-E1 cell line was essentially completely lacking in Δ6Fads activity. Previous studies have shown that cell lines from Atlantic salmon and rainbow trout (anadromous and freshwater species) had substantial Δ6 and Δ5 Fads activities but lacked Δ4 Fads activity whereas cell lines from turbot and gilthead sea bream (marine species) had substantial Δ6Fads activity but a low 18:4n-3 to 20:5n-3 conversion rate (Tocher *et al.* 1989, Tocher and Sargent 1990). The low 18:4n-3 to 20:5n-3 conversion rate in the gilthead sea bream cell line was found to be due to low Δ5 Fads activity rather than low Elovl5 activity (Tocher and Ghioni 1999). Although we have only tested 18:3n-3, 18:2n-6 and 20:5n-3, it appears as if *de novo* LC-PUFA biosynthesis in the SBT-E1 cell line is not limited by Elovl5 activity. This makes it similar to Atlantic salmon and gilthead sea bream. Thus, there is no straightforward distinction between freshwater, anadromous and marine species. This is also clear when we consider that the cell lines from Atlantic salmon, turbot and gilthead sea bream all exhibited substantial Δ6Fads activity whereas the SBT-E1 cell line did not. Thus, the SBT-E1 cell line had the least capacity of all of these cell lines for *de novo* synthesis of LC-PUFA from C_{18} PUFA. This result supports the proposed hypothesis that tunas naturally have a limited capacity for LC-PUFA biosynthesis, consistent with their high trophic level in marine food webs (Mourente and Tocher 2009). However, this result could also suggest that the SBT-E1 cell line originated from a tissue other than liver or intestine, and thus could reflect the limited capacity for LC-PUFA biosynthesis of the tissue of origin.

4.4.5 β-oxidation of [1-^{14}C]-labelled fatty acids

The SBT-E1 cell line showed a high rate of β-oxidation with 20:5n-3, higher than with 18:0, 18:1n-9 or 18:2n-6. This high rate of β-oxidation of 20:5n-3 along with the high rate of elongation of this fatty acid to 22:5n-3 (discussed above) may explain the high 22:6n-3 to 20:5n-3 ratios observed in the SBT-E1 cells and also in tuna tissues (Mourente *et al.* 2002b, Saito *et al.* 2005, Mourente and Tocher 2009). This is important because it suggests that selective metabolism of 20:5n-3 rather than, or perhaps in conjunction with, selective esterification of 22:6n-3 into cellular lipids explains the high 22:6n-3 to 20:5n-3 ratios observed in tuna tissues.

In the SBT-E1 cell line, 18:3n-3 was oxidised at nearly twice the rate of 20:5n-3 and at more than 3-fold the rate of 18:0, 18:1n-9 or 18:2n-6. This showed a preference for 18:3n-3 over 20:5n-3 and for these two fatty acids over the other fatty acids tested. As predicted, the n-3 substrates were preferentially β-oxidised over the n-6 substrates. In a previous study, 18:3n-3 was oxidised at 5.1- to 8.0-fold the rate of 20:5n-3 in hepatocytes and at 3.5- to 4.8-fold the rate of 20:5n-3 in enterocytes from European sea bass (*Dicentrarchus labrax*) (Mourente *et al.* 2005). In contrast, in Atlantic salmon hepatocytes, 18:3n-3 and 20:5n-3 were oxidised at similar rates to one another and those rates were significantly lower than for 18:1n-9 and 18:2n-6 (Stubhaug *et al.* 2005). Thus, it appears as if inclusion of vegetable oils rich in 18:3n-3 at the expense of fish oils in feeds for farmed bluefin tunas will be successful in terms of supporting the energy requirements of the animals. However, caution must be exercised when extrapolating from *in vitro* studies to metabolism in whole fish. For example, feeding studies with juvenile Atlantic salmon have shown that dietary excess of a given fatty acid leads to increased catabolism of that particular fatty acid except in the case of 22:6n-3 (Bell *et al.* 2001, Bell *et al.* 2002, Torstensen and

Stubhaug 2004, Stubhaug *et al.* 2007). Thus, in the intact organism, fatty acid metabolism may be regulated at the level of uptake and incorporation into cellular lipids rather than at the level of β-oxidation. This remains to be investigated in tunas.

4.4.6 Δ6Fads and Elovl5 gene expression in the SBT-E1 cell line

It is generally accepted that large predatory fishes, such as tunas, have limited capacity for *de novo* synthesis of LC-PUFA from their C_{18} precursors 18:3n-3 and 18:2n-6 but recently it was reported that unfed Atlantic bluefin tuna (*Thunnus thynnus*) larvae had increasing Δ6Fads and Elovl5 gene expression with time post hatch (Tocher 2003, Mourente *et al.* 2005, Morais *et al.* 2011). This suggested that nutritional deprivation increases expression of these genes. This is consistent with the results reported here for the SBT-E1 cell line in that reducing the FBS concentration in the cell culture medium from 10% to 2% (v/v) significantly increased the expression of Δ6Fads. Another interesting observation was that Δ6Fads gene expression was much greater (20-fold) than Elovl5 gene expression in the SBT-E1 cell line. This was surprising given that the ^{14}C-labelling experiments had shown that Δ6Fads enzyme activity was much greater than fatty acyl elongase activity towards 20:5n-3 in the SBT-E1 cell line. Functional characterisation of a SBT Elovl5 gene in yeast has shown that the gene product has substantial activity towards 20:5n-3 (Gregory *et al.* 2010). Thus Elovl5 was believed to be the main enzyme responsible for the elongation of 20:5n-3 in SBT. The results of the current study, however, suggest that a different fatty acyl elongase enzyme may be involved. Indeed functional characterisation of an Elovl2 from rainbow trout (*Oncorhynchus mykiss*) has shown that this enzyme has similar levels of activity towards 20:5n-3 as Elovl5 enzymes (Gregory and James 2014). Further work is therefore required to determine whether SBT has other Elovl enzymes.

4.4.7 Conclusions

In summary, the SBT-E1 cell line was similar to other fish cell lines and tissues in that by far the most abundant phospholipid was PC and quantitatively important saturated fatty acids (16:0, 18:0) and monounsaturated fatty acids (18:1n-9) as well as essential PUFA (18:3n-3 and 18:2n-6) were incorporated predominantly into this lipid. Unlike cell lines from other fish species but similar to tuna tissues, the SBT-E1 cell line exhibited apparent selective retention of 22:6n-3 and a high ratio of 22:6n-3 to 20:5n-3. Major contributors to this were the substantial fatty acyl elongase activity converting 20:5n-3 to 22:5n-3 and the high rate of β-oxidation of 20:5n-3. It is proposed that these factors also contribute to the high 22:6n-3 to 20:5n-3 ratios found in tuna tissues. The SBT-E1 cell line exhibited negligible Δ6Fads activity towards either 18:3n-3 or 18:2n-6. The low flux of substrates along the n-3 LC-PUFA synthesis pathway is consistent with the expectation that large carnivorous marine fish such as tunas have limited capacity for *de novo* synthesis of n-3 LC-PUFA from their C_{18} precursor presumably related to the abundance of n-3 LC-PUFA in marine food webs. However, an interesting and unexpected finding was that the SBT-E1 cell line had very high levels of expression of a Δ6Fads gene and low levels of expression of an Elovl5 gene. This was the reverse of what was expected from the ^{14}C-labelling experiments and suggests that there may be other genes involved in *de novo* LC-PUFA biosynthesis in tunas.

Chapter 5 – Analysis of gene expression in the SBT-E1 cell line

5.1 Aims and background

The aims of this chapter were to investigate the effects of mild or severe serum deprivation coupled with supplementation with a range of different fatty acids on PGC-1α, PPARγ, CS, COX1 and UCP2 gene expression in the SBT-E1 cells.

The primary target for the gene expression analyses described in this thesis was PGC-1α. This enzyme acts as a potent co-activator of a range of transcription factors in mammals (Lin *et al.* 2005). There has not been extensive work conducted on PGC-1α and its co-activation targets in fish, but in mammals, it plays a central role in controlling mitochondrial biogenesis and, by extension, respiratory capacity (Moyes 2003, Lin *et al.* 2005, Liu and Lin 2011). Tunas require a high metabolic rate to fuel the continual contraction and relaxation of their red muscle, which powers their constant swimming. This constant swimming is necessary (a) to counteract their negative buoyancy and (b) because they are obligate ram ventilators. As a byproduct, the continual contraction and relaxation of their red muscle provides the heat for their red muscle endothermy, an unusual characteristic amongst fish (Moyes *et al.* 1992, Dickson and Graham 2004, Graham and Dickson 2004). It is currently unknown whether PGC-1α and the transcription factors it co-activates play a role in endothermy in tunas. PGC-1α co-activates members of the PPAR family, and two representatives, PPARα and PPARγ were chosen for the gene expression analysis in this thesis due to their contrasting roles in fatty acid metabolism. PPARα is involved in stimulating fatty acid β-oxidation through stimulation of the gene expression of CPT-1, which is involved in transporting fatty acids into the mitochondria to undergo β-oxidation (Baldán *et al.* 2004). In contrast, PPARγ is involved in adipogenesis and stimulates the transport of fatty acids to adipocytes to form triglycerides (Schoonjans *et al.* 1996, Guan *et al.* 2005). PPARγ has also been shown to bind fatty acids as

natural ligands and thus fatty acids can stimulate triglyceride synthesis via their interactions with PPARγ (Barrera *et al.* 2008). Primers for qRT-PCR were designed for both PPARα and PPARγ, however, due to a lack of conserved regions in the alignments between PPARα sequences of other fish and mammalian species, the primers designed for PPARα did not allow the amplification of a PCR product. Therefore, PPARγ gene expression was investigated alone as the representative co-activation target of PGC-1α. The CS and COX1 genes encode proteins that localise to the mitochondrial matrix, and the inner mitochondrial membrane, respectively (Alp *et al.* 1976, Wiegand and Remington 1986, Khalimonchuk and Rödel 2005). They can therefore be used as indicators of mitochondrial abundance and/or respiratory capacity (Moyes *et al.* 1992, Wikström *et al.* 1997, Khalimonchuk and Rödel 2005). UCP2 gene expression has been shown to increase in rats fed fish oil and in rat intestinal epithelial cells (IEC-6) treated with LC-PUFA in a PPARα-dependent manner (Murase *et al.* 2001). UCP2 was chosen as a representative downstream target of PGC-1α and it may be expected that UCP2 gene expression would increase in response to PUFA supplementation.

In cell culture, the main source of fatty acids is FBS (Tocher and Dick 1991), and thus, to elucidate the effects of individual fatty acids on gene expression, it was necessary to deprive the cells of FBS. It was hypothesised that

1. Serum depravation would decrease the expression of each of the target genes due to (a) a reduction in mitochondrial abundance, and thus fatty acid β-oxidation (PGC-1α, CS and COX1), (b) a reduction in fatty acids binding as ligands (PPARγ) and (c) a reduction in uncoupling (UCP2) in order to preserve fuel for essential cellular processes

2. Supplementation of the serum deprived cells with different fatty acids would increase the expression of all of the target genes due to a reversal of the processes described in hypothesis 1.
3. PGC-1α gene expression would correlate with PPARγ gene expression, thus indicating potential co-activation of PPARγ by PGC-1α
4. PGC-1α gene expression would correlate with CS and COX1 gene expression, thus indicating a role for PGC-1α in determining mitochondrial abundance

5.2 Materials and methods

5.2.1 Culture and harvest of the SBT-E1 cells

Cells were routinely cultured and maintained as described in Section 2.1. Cells from stock cultures were harvested from 80-90% confluent 75 cm^2 flasks according to the protocol described in Section 2.6.

5.2.2 PUFA supplementation

Fatty acid and vitamin E stock solutions were prepared as described in Section 2.4.1. The cells were cultured, supplemented with fatty acids, incubated at 25°C for either 24 or 72 h and then harvested according to the protocol described in Section 2.6.1.

5.2.3 RNA extraction

RNA was extracted from the harvested cells according to the protocol described in Section 2.6.3.

5.2.4 cDNA synthesis

A total of 1 μg of RNA was used to synthesise cDNA according to the protocol described in Section 2.6.5.

5.2.5 Design, synthesis and validation of primer sets

Primers were designed, synthesised and validated according to the protocol described in Section 2.5.

5.2.6 Analysis of gene expression by qRT-PCR

Analysis of gene expression by qRT-PCR was conducted and Ct values for each sample were calculated according to the protocol described in Section 2.6.6. The fold change in expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001), normalising to the expression of the housekeeping gene, β-actin and to a designated control treatment. In some experiments, the cells cultured in the standard culture medium but with the FBS concentration reduced to 5% (v/v) was the control whereas in other experiments it was the cells culture in the standard culture medium containing 10% (v/v) FBS. To do this, the following equations were used.

4. Ct (gene of interest) – Ct (β-actin) = ΔCt
5. ΔCt (treatment) – ΔCt (control) = ΔΔCt
6. $2^{-\Delta\Delta Ct}$ = fold change in expression

5.2.7 Statistical analyses

The data were analysed as described in Section 2.8. Briefly, significant differences in the data were determined by one-way ANOVA using Dunnett's multiple comparison test to compare changes in the treatments relative to the control. Significant differences are reported when $p < 0.05$.

5.3 Results

5.3.1 Gene expression of the transcriptional regulators PGC-1α and PPARγ

Under the standard cell culture conditions, i.e., in the presence of 10% (v/v) FBS, the PGC-1α gene was expressed at very low levels compared with the other genes investigated (Table 5.1). There were no significant differences in the expression of PGC-1α in response to addition of PUFA after 3 days (Fig. 5.1A). When the cells were cultured in the presence of either 10% (v/v) or 2% (v/v) FBS for 24 h, there was a 3-fold increase in PGC-1α gene expression in the cells culture with 2% (v/v) FBS compared with the cells cultured with 10% (v/v) FBS (Fig. 5.1B). Thus, serum deprivation increased the expression of PGC-1α. The addition of individual fatty acids to the culture medium had no additional effect. In other words, individual fatty acids could not reverse the stimulatory effect of serum deprivation on PGC-1α gene expression. The gene expression of PPARγ was also low under standard culture conditions (Table 5.1). There were no significant differences in the gene expression of PPARγ in response to serum concentration or PUFA supplementation after 3 days (Fig. 5.2 A) or after 24 h (Fig. 5.2B).

Table 5.1: The expression levels of various genes in the SBT-E1 cells under the standard culture conditions (i.e. in the presence of 10% (v/v) FBS), relative to a β-actin housekeeping gene. Values were calculated using the Ct values from the fluorescence curves and normalised to β-actin using the equation $2^{-\Delta Ct}$ (Section 5.2.6). The gene expression data are the mean of three replicate flasks of cells. The corresponding standard error of the mean is shown in the adjacent column (n = 3).

Gene	Gene expression ($2^{-\Delta Ct}$)	SEM
β-actin	1.0	0.0
PGC-1α	3.2×10^{-6}	1.0×10^{-6}
PPARγ	6.6×10^{-6}	1.1×10^{-6}
CS	3.6×10^{-2}	0.1×10^{-2}
Cox1	1.7	0.2
UCP2	2.9×10^{-3}	0.3×10^{-3}

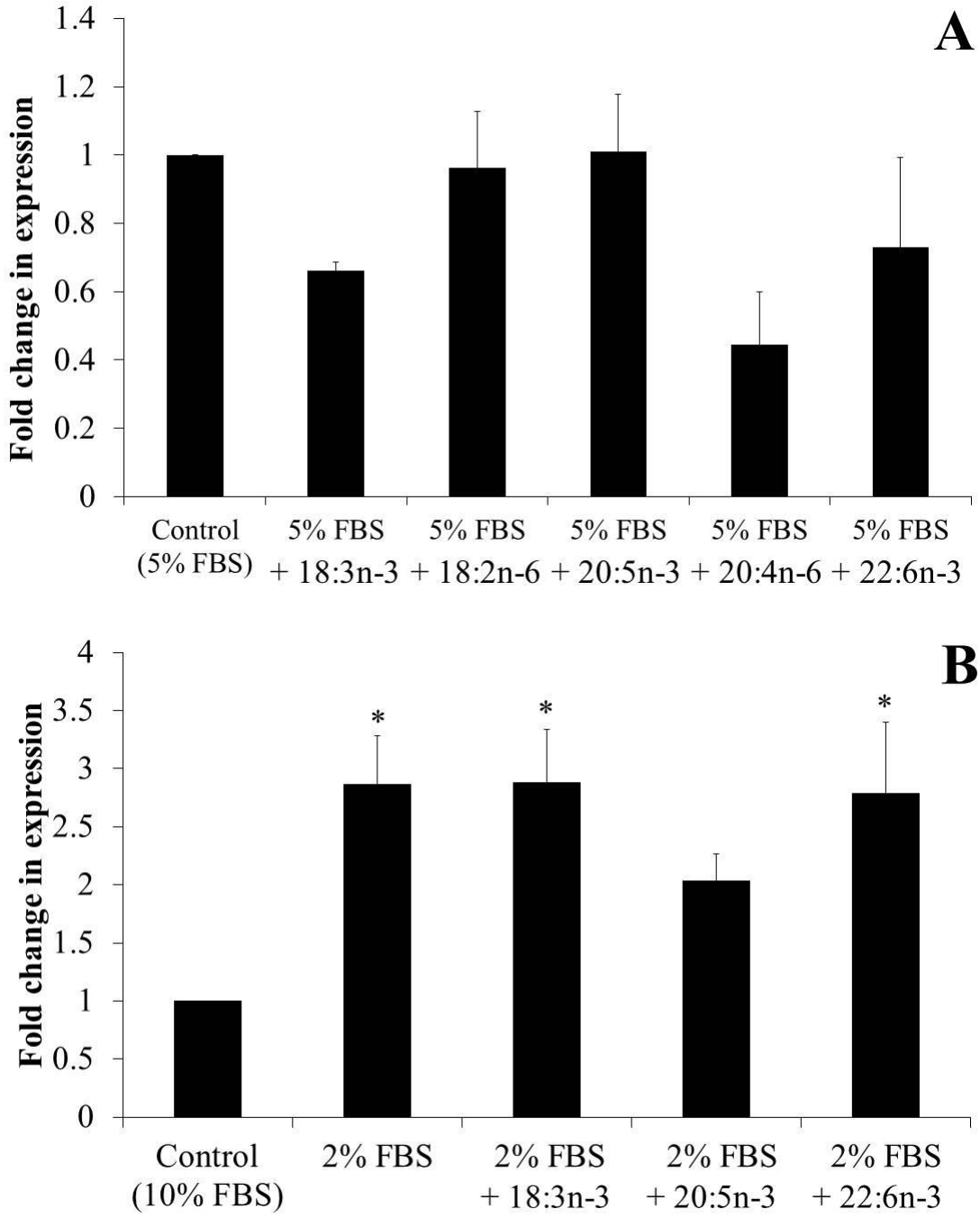


Figure 5.1: The effects of FBS concentration and individual fatty acid supplementation on PGC-1α gene expression in the SBT-E1 cells. Panel A shows PGC-1α gene expression in the cells incubated under the different conditions for 3 days. Panel B shows PGC-1α expression in the cells incubated under the different conditions for 24 h. The data have been normalised using β-actin and are expressed relative to the control, arbitrarily set at a value of 1.0. The data are the mean ± SE (n = 3). Significant differences between the treatments and the control are indicated by an asterisk.

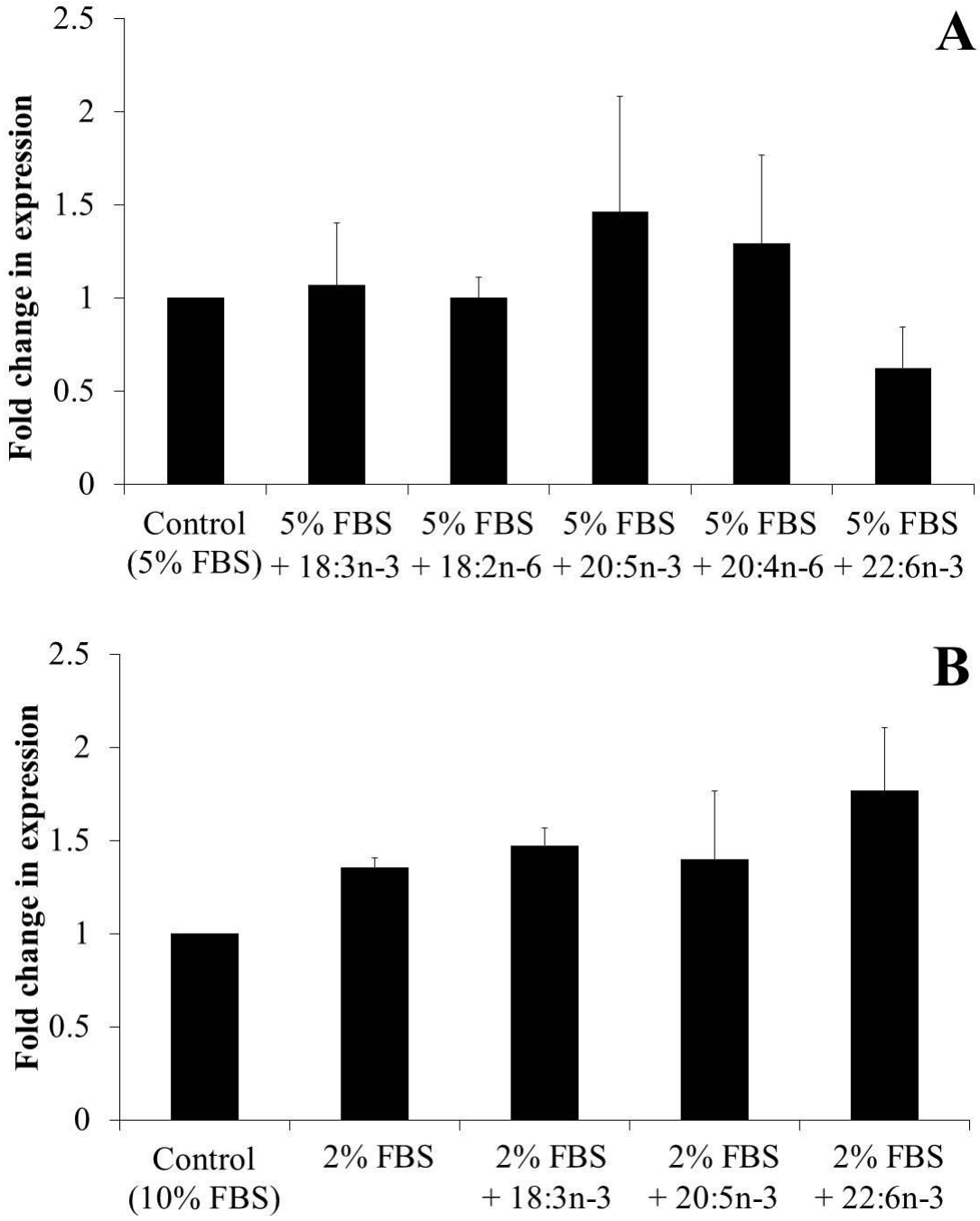


Figure 5.2: The effects of FBS concentration and individual fatty acid supplementation on PPARγ gene expression in the SBT-E1 cells. Panel A shows PPARγ gene expression in the cells incubated under the different conditions for 3 days. Panel B shows PPARγ gene expression in the cells incubated under the different conditions for 24 h. The data have been normalised using β-actin and are expressed relative to the control, arbitrarily set at a value of 1.0. The data are the mean ± SE (n = 3).

5.3.2 Gene expression of the mitochondrial markers CS and COX1

The transcripts of the mitochondrial markers CS and COX1 were both highly abundant in the SBT-E1 cells under the standard cell culture conditions (i.e. in the presence of 10% (v/v) FBS) with expression levels relative to β-Actin of 3.6 x 10^{-2} and 1.7 respectively (Table 5.1). However, there were no significant changes in the expression of CS in the cells subjected to culture in medium containing 5% (v/v) FBS for 3 days (Fig. 5.3A) or 2% (v/v) FBS for 24 hours with or without various individual fatty acids (Fig. 5.3B). In the cells cultured in the medium containing 5% (v/v) FBS for 3 days, COX1 gene expression was significantly reduced in the cells supplied with 18:3n-3, 18:2n-6, 20:4n-6 and 22:6n-3, compared with the control (Fig 5.4A p <0.05). The cells supplied with 20:5n-3 showed no difference to the control. The cells cultured in the medium containing 2% (v/v) FBS for 24 h showed approximately 20% higher COX1 gene expression compared to the cells under standard culture conditions (i.e. in the presence of 10% (v/v) FBS) (Fig. 5.4B, p < 0.05). The cells supplied with 18:3n-3, 20:5n-3 and 22:6n-3 showed no significant changes to the control cells subjected to the standard culture conditions.

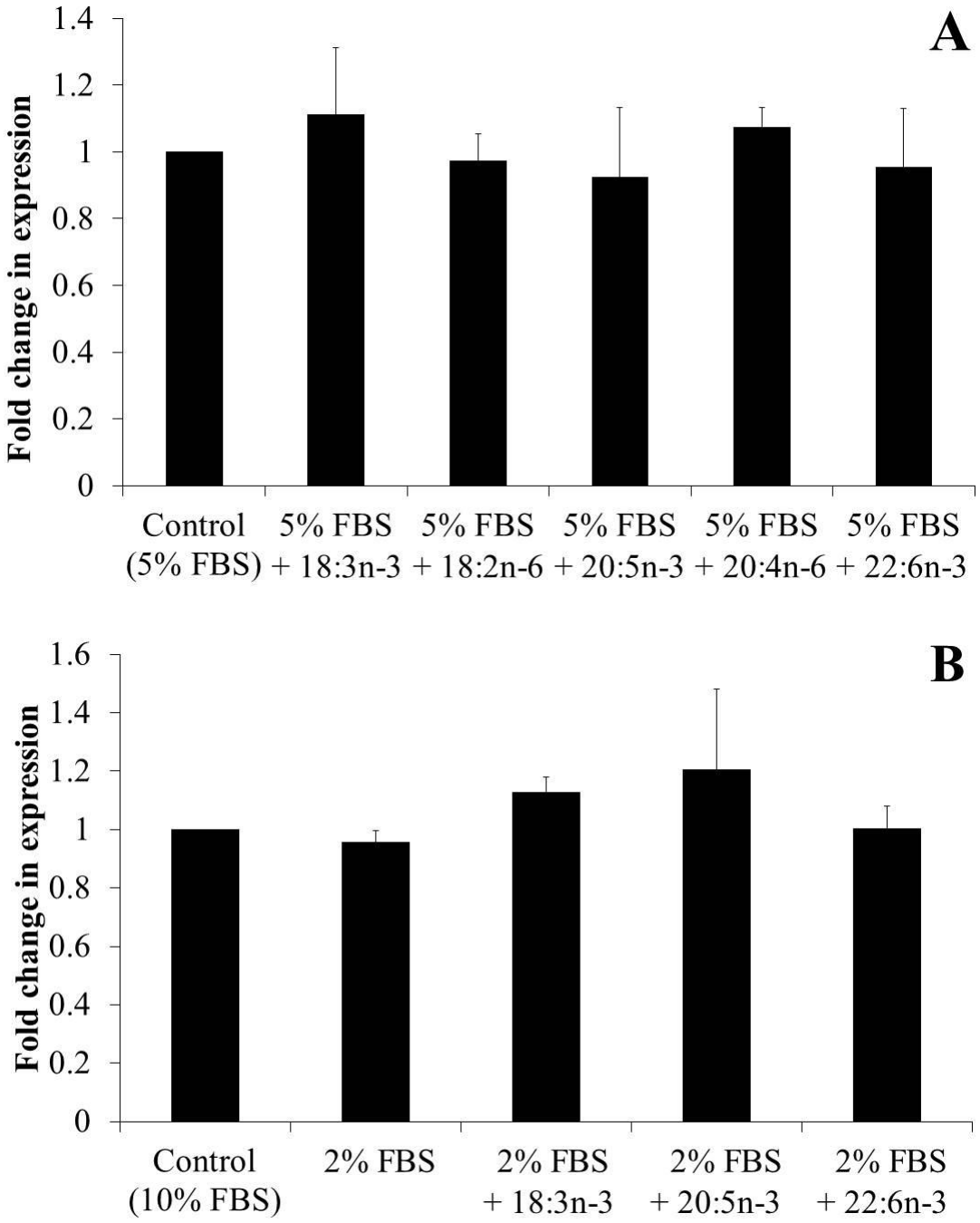


Figure 5.3: The effects of FBS concentration and individual fatty acid supplementation on CS gene expression in the SBT-E1 cells. Panel A shows CS gene expression in the cells incubated under the different conditions for 3 days. Panel B shows CS expression in the cells incubated under the different conditions for 24 h. The data have been normalised using β-actin and are expressed relative to the control, arbitrarily set at a value of 1.0. The data are the mean ± SE (n = 3).

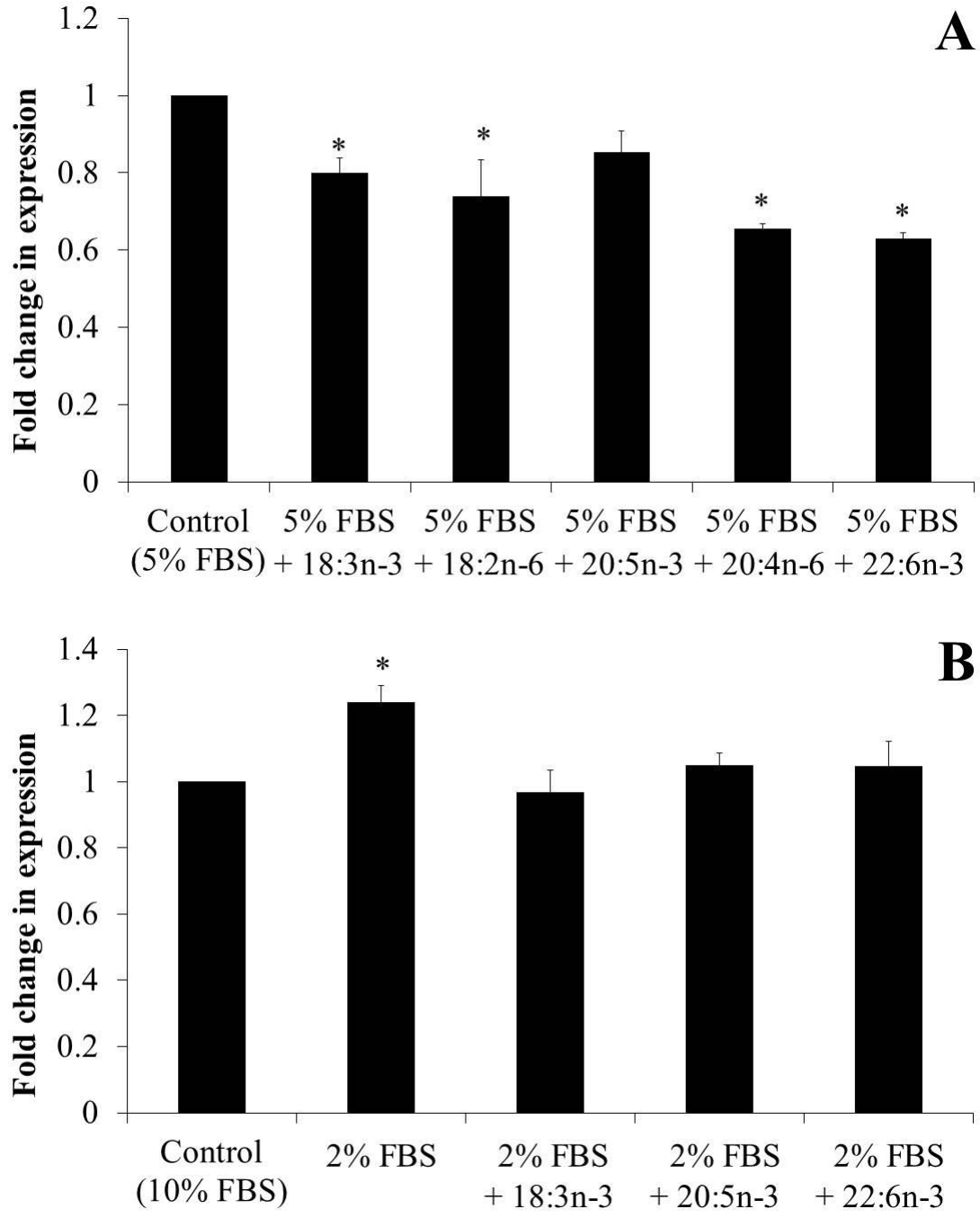


Figure 5.4: The effects of FBS concentration and individual fatty acid supplementation on COX1 gene expression in the SBT-E1 cells. Panel A shows COX1 gene expression in the cells incubated under the different conditions for 3 days. Panel B shows COX1 gene expression in the cells incubated under the different conditions for 24 h. The data have been normalised using β-actin and are expressed relative to the control, arbitrarily set at a value of 1.0. The data are the mean ± SE (n = 3). Significant differences between the treatments and the control are indicated by an asterisk.

5.3.3 Gene expression of UCP2

The expression of UCP2 was more than 100-fold greater than that of either PPARγ or PGC-1α but it was still considerably lower than that of β-Actin with a difference of 2.9 x 10^{-3}-fold (Table 5.1). The expression of the UCP2 gene was unaffected by individual fatty acid supplementation in the cells cultured in the medium containing 5% (v/v) FBS for 3 days (Fig. 5.5A). The cells cultured in the medium containing 2% (v/v) FBS for 24 h appeared to show higher levels of expression than the cells cultured under the standard culture conditions (i.e. in the presence of 10% (v/v) FBS) but the expression of UCP2 was highly variable between replicates (Fig. 5.5B). Therefore, despite the average expression in the cells supplied 2% FBS (v/v) ± various individual fatty acids being almost 4-fold higher than in that of the control cells supplied 10% (v/v) FBS, there were no significant differences between any of the treatments.

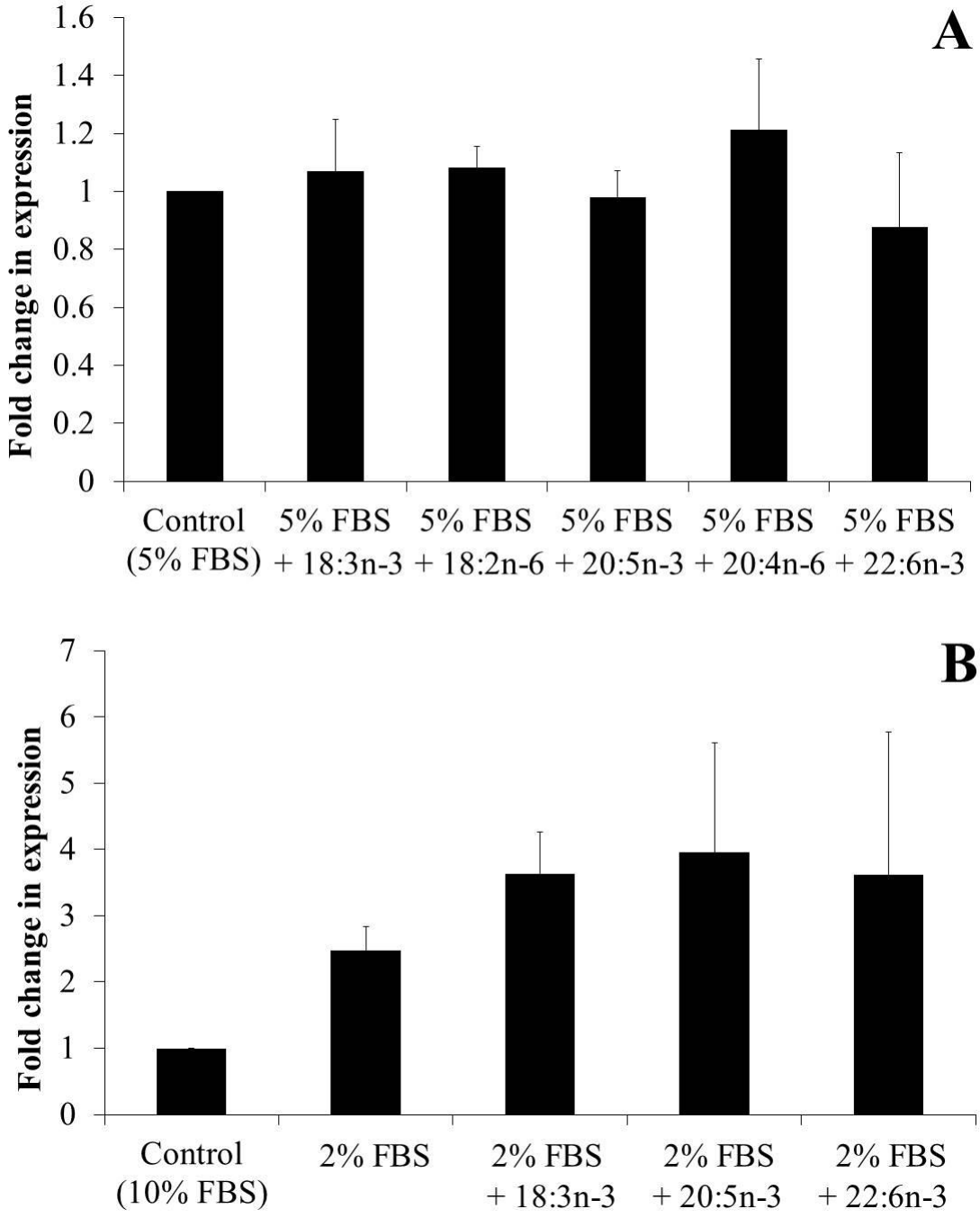


Figure 5.5: The effects of FBS concentration and individual fatty acid supplementation on UCP2 gene expression in the SBT-E1 cells. Panel A shows UCP2 gene expression in the cells incubated under the different conditions for 3 days. Panel B shows UCP2 gene expression in the cells incubated under the different conditions for 24 h. The data have been normalised using β-actin and are expressed relative to the control, arbitrarily set at a value of 1.0. The data are the mean ± SE (n = 3).

5.4 Discussion

5.4.1 Gene expression of the transcriptional regulators PGC-1α and PPARγ

PGC-1α is considered an important regulator of metabolism (Liu and Lin 2011). Thus it was expected that changes in nutrient availability and supplementation with fatty acids would have substantial effects on the expression of the PGC-1α gene. Contrary to what was hypothesised, the reduction of FBS concentration to 2% (v/v) for 24 h induced a 3-fold increase in the abundance of the PGC-1α transcript. Also contrary to the proposed hypotheses, the addition of fatty acids to the cells supplied the reduced FBS concentration had no additional effect on PGC-1α gene expression, with the exception of 20:5n-3, which reduced the PGC-1α gene expression to a point that was not significantly different to the control. These results suggest that 20:5n-3 was able to at least partially overcome the effects of reduced serum concentration, but it is likely that multiple fatty acids of importance were being supplied by the FBS.

Given that there were only small changes in the expression of the PGC-1α gene, it is not surprising that the gene expression of PPARγ was unchanged under the different cell culture conditions presented in this chapter. The lack of significant changes in gene expression for both PPARγ and PGC-1α make it difficult to define if there is a correlation between the expression of these two genes.

5.4.2 Gene expression of CS and COX1

The CS and COX1 genes were both expressed to a high level, with COX1 transcripts 1.7 times more abundant than those for β-Actin. This could indicate that the SBT-E1 cells had a high abundance of mitochondria and therefore may show high rates of

aerobic metabolism. Herzig *et al.* (2000) found that stimulation of mouse BALB/3T3 cells with 20% (v/v) FBS after a prolonged period of starvation caused increases in COX1 protein abundance. In fish, gene transcripts for the different subunits of the COX enzyme were found to be reduced between 2.2-4.3-fold in the liver of rainbow trout that were subjected to starvation for 3 weeks (Salem *et al.* 2007). In several tuna species, it has been shown that tissues that rely on aerobic metabolism (e.g. red muscle) show higher expression of both COX and CS enzymes compared to tissues that rely on more anaerobic (glycolytic) metabolism (e.g. white muscle) (Dalziel *et al.* 2005). Interestingly, the results presented in this chapter showed minimal changes in the gene expression of CS and COX1 transcripts under serum deprivation and fatty acid supplementation conditions with the only significant change in expression being a very modest increase in COX1 transcript abundance in serum deprived cells. The level of COX1 gene expression that occurred in the cells subjected to culture in medium with the FBS concentration reduced to 2% (v/v) was only 20% higher than the level in the control cells and is unlikely to be biologically significant. Thus it appears that the reduction in FBS concentration and the individual fatty acid supplements at the concentrations used here were not sufficient to induce changes in mitochondrial abundance. Further work using different concentrations of fatty acids and different combinations of fatty acids may be required to determine the conditions needed to alter mitochondrial abundance in the SBT-E1 cells.

5.4.3 Gene expression of UCP2

UCP2 gene expression has been found to be significantly higher in the liver in mice fed a fish oil based diet compared with a vegetable oil based diet (Mori *et al.* 2007). In contrast, in Atlantic salmon, Torstensen *et al.* (2009) found no differences in

UCP2 gene expression in liver, heart, red muscle or white adipose tissue in fish fed a diet containing vegetable oil compared to those fed a diet containing fish oil. The results presented here are more consistent with the latter of these studies and showed no significant changes in UCP2 gene expression in the SBT-E1 cells that were supplied a variety of different fatty acids. Thus it appears that fatty acids have minimal effects on the metabolic pathways that involve UCP2, at least in the SBT-E1 cells. It is unknown if other members of the UCP family may be affected by the supplementation of the SBT-E1 cells with different fatty acids and this could be an subject for future research.

In conclusion, contrary to the proposed hypotheses, there were minimal changes in the gene expression of the mitochondrial indicators (CS and COX1) in the SBT-E1 cells under culture conditions of reduced serum concentration and fatty acid supplementation. The up-regulation of PGC-1α in cells cultured in medium with the FBS concentration reduced to 2% (v/v) for 24 h may indicate a role of PGC-1α in the cellular response to reduced fatty acid deprivation. Interestingly, the cells supplied with reduced serum concentration and 20:5n-3 did not exhibit significantly increased PGC-1α gene expression indicating that 20:5n-3 was able to overcome some of the effects of reduced serum concentration.

Chapter 6 – Cloning of a PGC-1α cDNA from SBT liver tissue

6.1 Aims and background

The aim of this chapter was to clone a PGC-1α cDNA from SBT. The PGC-1α gene encodes a transcriptional co-activator which in mammals plays a significant role in mitochondrial biogenesis, and, by extension, fatty acid β-oxidation (Lin *et al.* 2005, Bremer and Moyes 2011). However, few studies have investigated the role of PGC-1α in fish. A review of the evolution of PGC-1α in vertebrates revealed that the fish and mammalian PGC-1α proteins shared all the major functional domains (LeMoine *et al.* 2010b). However, the fish PGC-1α proteins contained serine and glutamine-rich insertions within the domain responsible for binding to NRF-1 and NRF-2 and also exhibited a higher rate of evolution than mammalian PGC-1α (LeMoine *et al.* 2010b). It appears, therefore, that there may be some differences in the role of PGC-1α in fish, compared to mammals. Tunas have higher CS and COX enzyme activities in their red muscle than their white muscle (Dalziel *et al.* 2005) and this indicates that they have more mitochondria in their red muscle than their white. The greater numbers of mitochondria have been associated with red muscle endothermy because mitochondria are an important source of heat (Dickson and Graham 2004, Graham and Dickson 2004). Thus, it was predicted that PGC-1α gene expression would be greater in tuna red muscle than white. However, no PGC-1 genes have yet been cloned from any tuna species. Therefore, the work in this chapter was conducted with the aim of cloning a PGC-1α cDNA from SBT in preparation for investigating the expression of PGC-1α in various tissues of SBT. It was hypothesised that (a) SBT would express a PGC-1α gene similar in sequence to mammalian and other fish PGC-1α genes and (b) the nucleotide sequence of the SBT PGC-1α cDNA would include the characteristic features previously described for PGC-1α cDNAs from mammals and other fish.

6.2 Materials and methods

6.2.1 Primer design

Primers for PCR were designed based on conserved regions identified in a multiple sequence alignment of PGC-1α cDNAs from mammals and fish as described in Section 2.7.1. The primers were designed to anneal to regions of the SBT PGC-1α cDNA which would allow amplification of overlapping fragments and are shown in Table 6.1.

Table 6.1: A summary of the primer sets used to determine the sequence of the PGC-1α cDNA from SBT. Degenerate bases are indicated by R (A/G), Y (C/T) or W (A/T). The primer annealing location is based on the finally assembled SBT PGC-1α cDNA sequence (Fig. 6.2).

Primer name	**Direction**	**Sequence (5' → 3')**	**Primer annealing location (bp)**
PGC1A_5P_F1	Forward	ATGGCGTGGGACAGGTGTA	1-19
PGC1A_5P_F2	Forward	AGGACTCGGTGTGGAGAGAA	23-42
PGC1A_5P_F3	Forward	CTGCCTTGGTTGGTGAAGAC	53-72
PGC1A_C_F1	Forward	CCTCGTTTTCCTCCCTCTCC	782-801
PGC1A_C_F2	Forward	ACAARGGATCWCCGTTTGAG	974-993
PGC1A_C_F3	Forward	CATGAGTACAGCAGCGGAAA	1440-1459
PGC1A_3P_F1	Forward	CCCTTGARAAYGGACACACC	2666-2685
PGC1A_5P_R1	Reverse	AGTCGCTGACATCCAGCTCT	111-130
PGC1A_5P_R2	Reverse	CTGTAAGCACTGCCAGCAAG	255-274
PGC1A_C_R1	Reverse	GCTGCTGCTGTTGTTGTTGT	864-883
PGC1A_C_R2	Reverse	GATCCYTTGTGGTCATTTG	965-983
PGC1A_C_R3	Reverse	CTGCTTAGCCAAAGGACCAG	1601-1620
PGC1A_3P_R1	Reverse	AGTCGTCAGAATGGGAGTCC	2766-2785
PGC1A_3P_R2	Reverse	GTGGAGGCTGGATCAAAGTC	2782-2801

6.2.1 Harvest of tissue

Liver tissue was collected from a single SBT specimen with a fork length of 130 cm and a gilled and gutted weight of 52 kg (Section 2.6.2.1).

6.2.2 RNA extraction

Total RNA was extracted from the SBT liver tissue according to the protocol described in Section 2.7.2

6.2.3 cDNA synthesis

A total of 4 μg of RNA was used to synthesise cDNA according to the protocol described in Section 2.7.3.

6.2.5 Standard PCR amplification and gel electrophoresis

The PCR reactions required to amplify the PGC-1α cDNA and the subsequent separation of those products by agarose gel electrophoresis were conducted according to the protocol in Section 2.7.4. The PCR cycling conditions for short products (<300 bp) were 98°C for 30 s followed by 30 cycles of 98°C for 10 s, 63°C for 30 s and 72°C for 30s, followed by a final elongation step at 72°C for 10 min. The PCR cycling conditions for medium length (500-1500 bp) and long (>1500 bp) products were the same as described above except that the time for the elongation step was increased from 30 s to 1 min and 2 min, respectively. The longer time was required to allow for elongation of the longer PCR products.

6.2.6 Nested PCR

When the PCR reactions resulted in the amplification of multiple products, nested PCR was conducted to improve the specificity of the amplification. Briefly, the 45 µl of the PCR reaction mixture that remained after gel electrophoresis was purified using a Wizard® SV gel and PCR clean-up system (Promega) according to the manufacturer's instructions. Two µl of the purified product was then used in a second round of PCR amplification using primers 'nested' within the amplicon according to the cycling conditions described in Section 6.2.5.

6.2.7 Sequencing of PCR amplicons and sequence analysis

PCR amplicons were purified and sequenced according to the protocol described in Section 2.7.5. The sequence data analysed were using the basic local alignment search tool (BLAST). Overlapping sequences from multiple sequencing runs were combined and aligned to construct the consensus SBT PGC-1α sequence. The consensus sequence was then aligned to other fish PGC-1α sequences.

6.2.8 Determination and analysis of the predicted SBT PGC-1α amino acid sequence

The nucleotide sequence of the SBT PGC-1α cDNA was used to predict the corresponding amino acid sequence using the translation tool from the ExPASy bioinformatics resource portal (www.expasy.org). The cDNA sequences from various other fish and mammalian species were concurrently translated and the resulting amino acid sequences were aligned using ClustalX 2.1 (Larkin *et al.* 2007). The accession numbers for the individual sequences are stated in the appropriate figure captions. The alignment was annotated using existing literature and an

annotated rat PGC-1α sequence (NM_031347) to indicate regions that are characteristic of or important to the function of PGC-1α. The assembled SBT PGC-1α sequence, along with predicted PGC-1α amino acid sequences for various fish, mammalian and avian species were subjected to phylogenetic analysis using the molecular evolutionary genetic analysis (MEGA) program version 5 (Tamura *et al.* 2011). The phylogenetic tree was constructed using the maximum parsimony method to produce a consensus tree after 1000 bootstrap iterations.

6.3 Results

6.3.1 Determining the SBT PGC-1α cDNA sequence

A 2,799 base pair SBT PGC-1α cDNA sequence was obtained by cloning overlapping fragments within the coding region of the gene. Each fragment was sequenced a minimum of three times using either forward or reverse primers and a representative diagram of the fragments used to construct the full PGC-1α cDNA is shown in Fig. 6.1. The first 3 bp of the cDNA coded for a methionine residue and was the putative start codon. The cDNA sequence therefore represented the first 2799 bp of the open reading frame (ORF). There was a small amount of cDNA from the 3' end that was unable to be cloned; therefore the cDNA did not contain an in-frame stop codon. The SBT PGC-1α cDNA sequence aligned to a range of fish species is shown in Fig. 6.2. There was some sequence divergence between the different fish species within the first 50 bp of the alignment. Apart from this divergence, the first 600 bp of the alignment showed substantial conservation between the different species. The central region (600-2450 bp) of the nucleotide sequence showed a lack of conservation between the different species with some species showing insertions of 1-50 bp that were not present in other fish species. The

most prominent regions to contain insertions and/or deletions were between bases 600 and 940 and between bases 1,200 and 2,450. The 3' end of the nucleotide sequence from bases 2,450 until the end was well conserved between the different fish species.

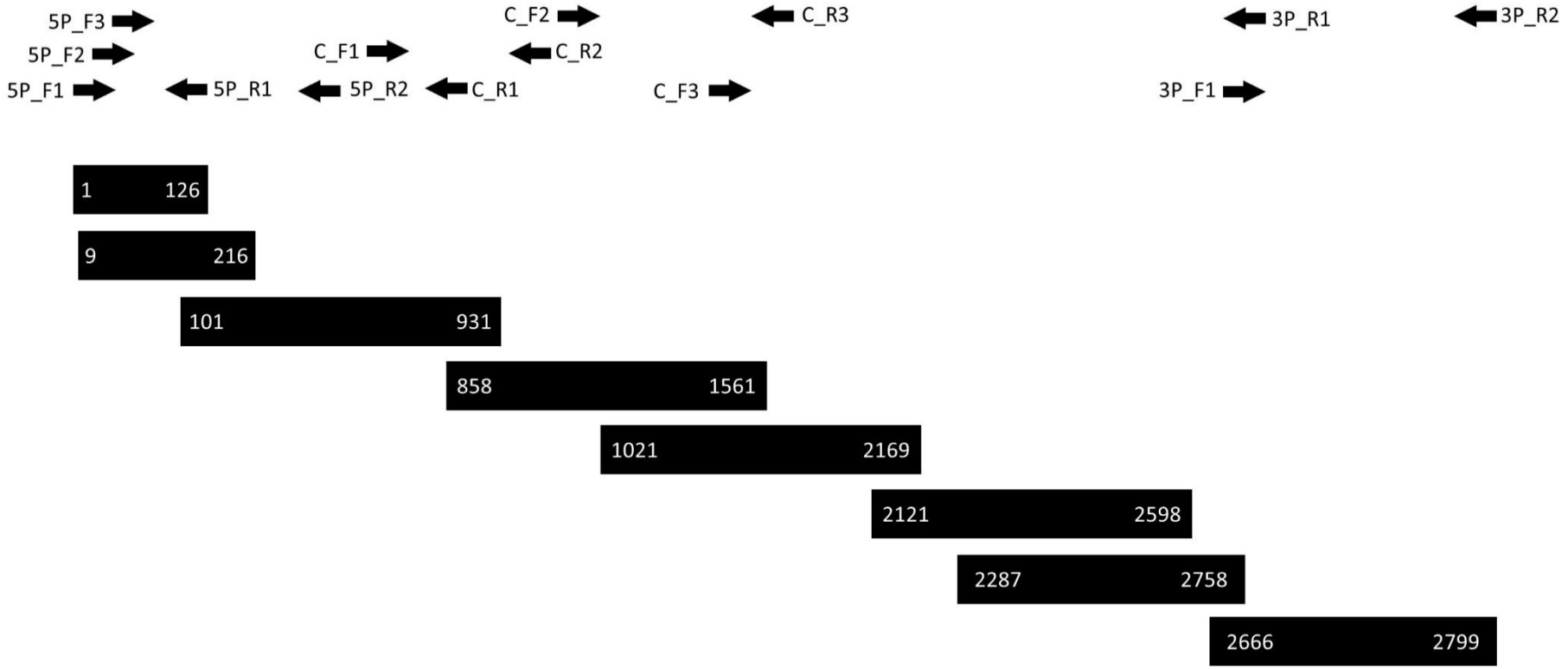
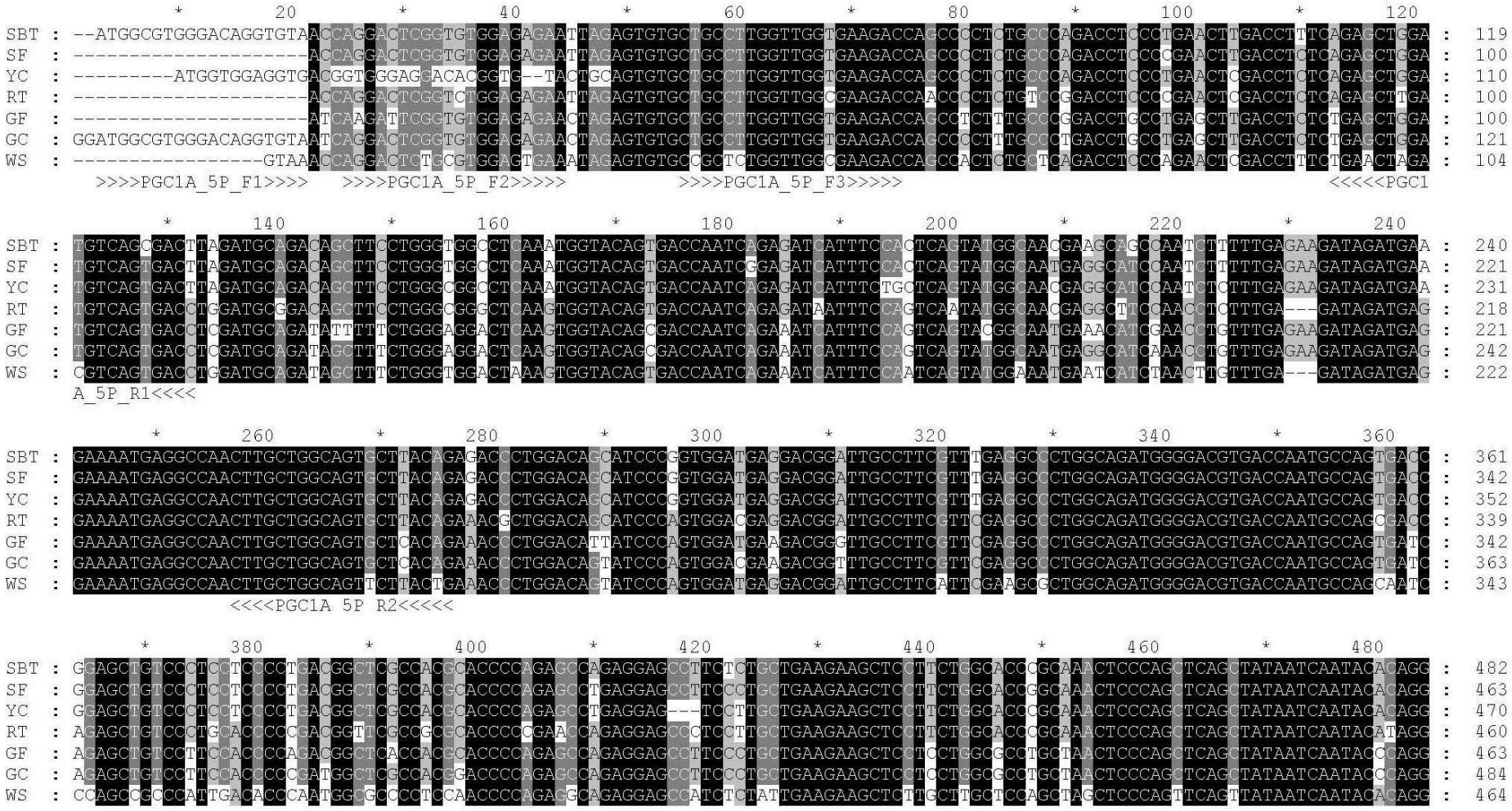


Figure 6.1: A representative construction of the 2,799 bp SBT PGC-1α cDNA sequence from multiple PCR products. Each box represents a different PCR product which was sequenced. The arrows at the top of the figure represent the representative primers that were used to clone and sequence the SBT PGC-1α cDNA. A minimum of three sequencing passes were used for each region of the PGC-1α cDNA to construct the full sequence.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SF : ----------ACCAGGACTCGGTGTGGAGAGAATTAGAGTGTGCTGCCTTGGTTGGTGAAGACCAGCCCCTCTGCCCAGACCTCCCCGAACTTGACCTCTCAGAGCTGGA : 100
YC : ---------ATGGTGGAGGTGACGGTGGGAGGACACGGTG--TACTGCAGTGTGCTGCCTTGGTTGGTGAAGACCAGCCCCTCTGCCCAGACCTCCCTGAACTCGACCTCTCAGAGCTGGA : 110
RT : ----------ACCAGGACTCGGTCTGGAGAGAATTAGAGTGTGCTGCCTTGGTTGGCGAAGACCAACCCCTCTGTCCGGACCTCCCCGAACTCGACCTCTCAGAGCTTGA : 100
GF : ----------ATCAAGATTCGGTGTGGAGAGAACTAGAGTGTGCTGCCTTGGTTGGTGAAGACCAGCCTCTTTGCCCGGACCTGCCTGAGCTTGACCTCTCTGAGCTGGA : 100
GC : GGATGGCGTGGGACAGGTGTAATCAGGACTCGGTGTGGAGAGAACTAGAGTGTGCTGCCTTGGTTGGTGAAGACCAGCCCCTTTGCCCTGACCTGCCTGAGCTTGACCTCTCTGAGCTGGA : 121
WS : ----------GTAAACCAGGACTCTGCGTGGAGTGAAATAGAGTGTGCCGCTCTGGTTGGCGAAGACCAGCCACTCTGCTCAGACCTCCCAGAACTCGACCTTTCTGAACTAGA : 104
>>>>PGC1A_5P_F1>>>> >>>>PGC1A_5P_F2>>>>> >>>>PGC1A_5P_F3>>>>>> <<<<<PGC1
SBT : TGTCAGCGACTTAGATGCAGACAGGTTCCTGGGTGGCCTCAAATGGTACAGTGACCAATCAGAGATCATTTCCAGTCAGTATGGCAACGAAGCAGCCAATCTTTTTGAGAAGATAGATGAA : 240
SF : TGTCAGTGACTTAGATGCAGACAGCTTCCTGGGTGGCCTCAAATGGTACAGTGACCAATCGGAGATCATTTCCAGTCAGTATGGCAATGAGGCATCCAATCTTTTTGAGAAGATAGATGAA : 221
YC : TGTCAGTGACTTAGATGCAGACAGCTTCCTGGGCGGCCTCAAATGGTACAGTGACCAATCAGAGATCATTTCTGGTCAGTATGGCAACGAGGCATCCAATCTCTTTGAGAAGATAGATGAA : 231
RT : TGTCAGTGACCTGGATGCGGACAGCTTCCTGGGCGGGCTCAAGTGGTACAGTGACCAATCAGAGATAATTTCCAGTCAATATGGCAACGAGGCTTCCAACCTCTTTGA---GATAGATGAG : 218
GF : TGTCAGTGACCTCGATGCAGATATTTTTCTGGGAGGACTCAAGTGGTACAGCGACCAATCAGAAATCATTTCCAGTCAGTACGGCAATGAAACATCGAACCTGTTTGAGAAGATAGATGAG : 221
GC : TGTCAGTGACCTCGATGCAGATAGCTTTCTGGGAGGACTCAAGTGGTACAGCGACCAATCAGAAATCATTTCCAGTCAGTATGGCAATGAGGCATCAAACCTGTTTGAGAAGATAGATGAG : 242
WS : CGTCAGTGACCTGGATGCAGATAGCTTTCTGGGTGGACTAAAGTGGTACAGTGACCAATCAGAAATCATTTCCAATCAGTATGGAAATGAATCATCTAACTTGTTTGA---GATAGATGAG : 222
A_5P_R1<<<<
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SF : GAAAATGAGGCCAACTTGCTGGCAGTGCTTACAGAGACCCTGGACAGCATCCCGGTGGATGAGGACGGATTGCCTTCGTTTGAGGCCCTGGCAGATGGGGACGTGACCAATGCCAGTGACC : 342
YC : GAAAATGAGGCCAACTTGCTGGCAGTGCTTACAGAGACCCTGGACAGCATCCCGGTGGATGAGGACGGATTGCCTTCGTTTGAGGCCCTGGCAGATGGGGACGTGACCAATGCCAGTGAGC : 352
RT : GAAAATGAGGCCAACTTGCTGGCAGTGCTTACAGAAACGCTGGACAGCATCCCAGTGGACGAGGACGGATTGCCTTCGTTCGAGGCCCTGGCAGATGGGGACGTGACCAATGCCAGCGAGC : 339
GF : GAAAATGAGGCCAACTTGCTGGCAGTGCTCACAGAAACCCTGGACATTATCCCAGTGGATGAAGACGGGTTGCCTTCGTTCGAGGCCCTGGCAGATGGGGACGTGACCAATGCCAGTGATC : 342
GC : GAAAATGAGGCCAACTTGCTGGCAGTGCTCACAGAAACCCTGGACAGTATCCCAGTGGACGAAGACGGTTTGCCTTCGTTCGAGGCCCTGGCAGATGGGGACGTGACCAATGCCAGTGATC : 363
WS : GAAAATGAGGCCAACTTGCTGGCAGTTCTTACTGAAACCCTGGACAGTATCCCAGTGGATGACGACGGATTGCCTTCATTCGAAGCGCTGGCAGATGGGGACGTGACCAATGCCAGCAATC : 343
<<<<PGC1A 5P R2<<<<<
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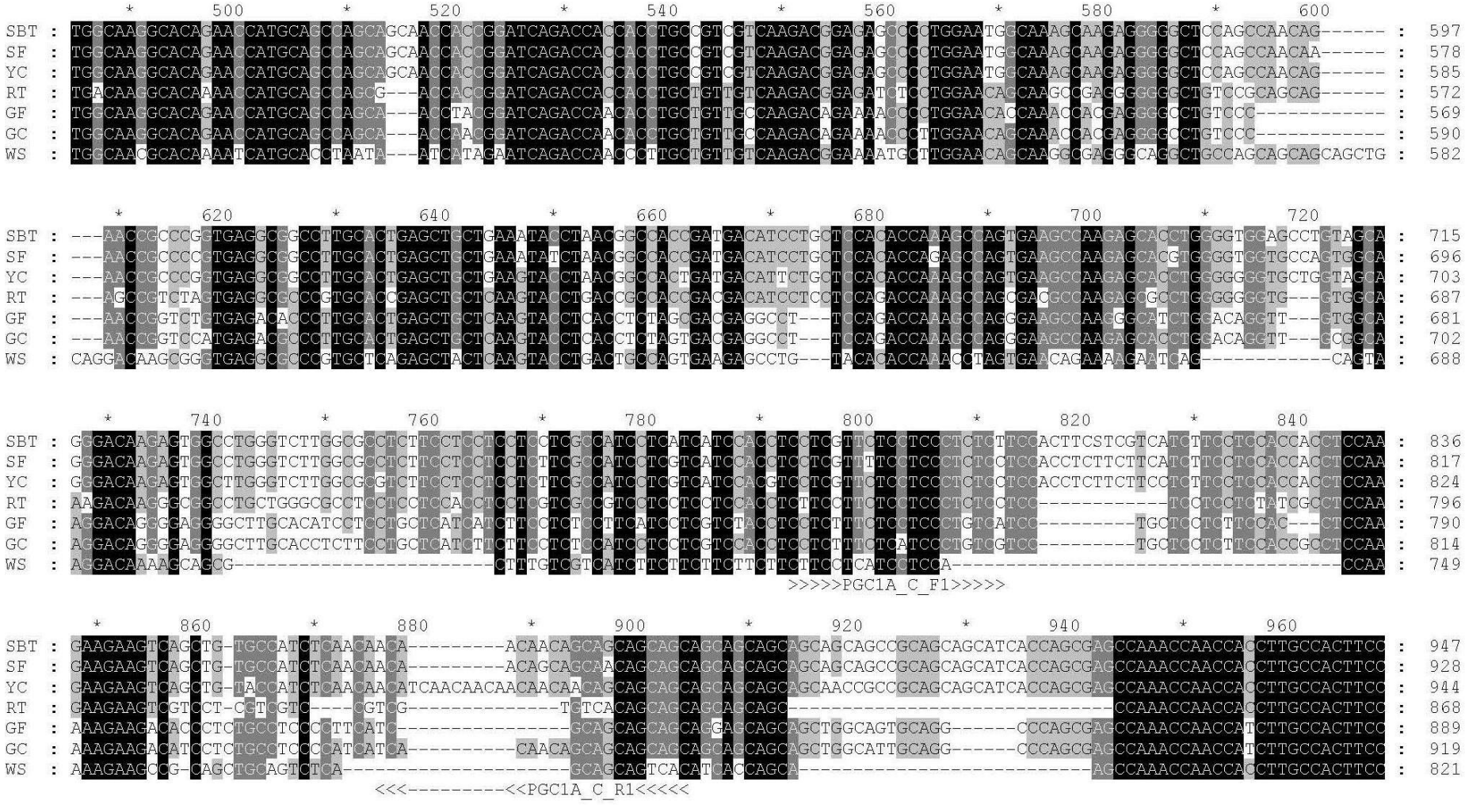
SF : GGAGCTGTCCCTCCTCCCCTGACGGCTCGCCACGCACCCCAGAGCCTGAGGAGCCTTCCCTGCTGAAGAAGCTCCTTCTGGCACCGGCAAACTCCCAGCTCAGCTATAATCAATACACAGG : 463
YC : GGAGCTGTCCCTCCTCCCCTGACGGCTCGCCACGCACCCCAGAGCCTGAGGAG---TCCTTGCTGAAGAAGCTCCTTCTGGCACCGGCAAACTCCCAGCTCAGCTATAATCAATACACAGG : 470
RT : AGAGCTGTCCCTGCACCCCGACGGTTCGCCGCGCACCCCCGAACCAGAGGAGCCGCTCCTTGCTGAAGAAGCTCCTTCTGGCACCGGCAAACTCCCAGCTCAGCTATAATCAATACATAGG : 460
GF : AGAGCTGTCCTTCCACCCCAGACGGCTCACCACGCACCCCAGAGCCAGAGGAGCCTTCCCTGCTGAAGAAGCTCCTCCTGGCGCCTGCTAACTCCCAGCTCAGCTATAATCAATAGCCAGG : 463
GC : AGAGCTGTCCTTCCACCCCCGATGGCTCGCCACGGACCCCAGAGCCAGAGGAGCCTTCCCTGCTGAAGAAGCTCCTGCTGGCGCCTGCTAACTCCCAGCTCAGCTATAATCAATAGCCAGG : 484
WS : CCAGCCGCCCATTGACACCCAATGGCGCCCCTCCAACCCCAGAGGCAGAGGAGCCATCTCTATTGAAGAAGCTCTTGCTTGCTCCAGCTAGCTCCCAGTTCAGTTATAATCAATACACAGG : 464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SF : TGGCAAGGCACAGAACCATGCAGCCAGCAGCAACCACCGGATCAGACCACCACCTGCCGTCGTCAAGACGGAGAAGCGCCTGGAATGGCAAAGCAAGAGGGGGCTCCAGCCAACAA------ : 578
YC : TGGCAAGGCACAGAACCATGCAGCCAGCAGCAACCACCGGATCAGACCACCACCTGCCGTCGTCAAGACGGAGAAGCGCCTGGAATGGCAAAGCAAGAGGGGGCTCCAGCCAACAG------ : 585
RT : TGACAAGGCACAAAACCATGCAGCCAGCG---ACCACCGGATCAGACCACCACCTGCTGTTGTCAAGACGGAGATCTCCTGGAACAGCAAGCCGAGGGGGGGCTGTCCGCAGCAG------ : 572
GF : TGGCAAGGCACAGAACCATGCAGCCAGCA---ACCTACGGATCAGACCAACACCTGCTGTTGCCAAGACAGAAAACCCCTGGAACAGCAAACCACGAGGGGCCTGTCCC---------- : 569
GC : TGGCAAGGCACAGAACCATGCAGCCAGCA---ACCAACGGATCAGACCAACACCTGCTGTTGCCAAGACAGAAAACCCTTGGAACAGCAAACCACGAGGGGCCTGTCCC---------- : 590
WS : TGGCAACGCACAAAATCATGCACCTAATA---ATCATAGAATCAGACCAACCCTTGCTGTTGTCAAGACGGAAAATGCTTGGAACAGCAAGGCGAGGGCAGGCTGCCAGCAGCAGCAGCTG : 582
SBT : ---AACCGCCCGGTGAGGCGGCCTTGCACTGAGCTGCTGAAATACCTAACGGCCACCGATGACATCCTGGTCCAGACCAAAGCCAGTGAAGCCAAGAGCACCTGGGGTGGAGCCTGTAGCA : 715
SF : ---AACCGCCCCGTGAGGCCGCCTTGCACTGAGCTGCTGAAATATCTAACGGCCACCGATGACATCCTGGTCCAGACCAGAGCCAGTGAAGCCAAGAGCACGTGGGGTGGTGCCACTGGCA : 696
YC : ---AACCGCCCGGTGAGGCGGCCTTGCACTGAGCTGCTGAAGTACCTAACGGCCACTGATGACATTCTGGTCCAGACCAAAGCCAGTGAAGCCAAGAGCACCTGGGGGGGTGCTGGTAGCA : 703
RT : ---AGCCGTCTAGTGAGGCGCCGTGCACCGAGCTGCTCAAGTACCTGACCGCCACCGACGACATCCTCCGTCCAGACCAAAGCCAGCGACGCCAAGAGGGCCTGGGGGGCTG---GTGGCA : 687
GF : ---AACCGGTCTGTGAGACGCCTTGCACTGAGCTGCTCAAGTACCTCACCTCTAGCGACGAGGCCT---TCCAGACCAAAGCCAGGGAAGCCAAGGGCATCTGGACAGGTT---GTGGCA : 681
GC : ---AACCGGTCCATGAGACGCCTTGCACTGAGCTGCTCAAGTACCTCACCTCTAGTGACGAGGCCT---TCCAGACCAAAGCCAGGGAAGCCAAGAGCACCTGGACAGGTT---GCGGCA : 702
WS : CAGGACAAGCGGGTGAGGCGCCGTGGTCAGAGCTACTCAAGTACCTGACTGCCAGTGAAGAGCCTG---TACAGACCAAACCTAGTGAACAGAAAAGAATCAG----------CAGTA : 688
SBT : GGGACAAGAGTGGCCTGGGTCTTGGCGCCTCTTCCTCCTCCTCCTCGCCATCCTCATCATCCACCTCCTCGTTGTCCTCCCTCTCTTCCACTTCSTCGTCATCTTCCTCCACCACCTCCAA : 836
SF : GGGACAAGAGTGGCCTGGGTCTTGGCGCCTCTTCCTCCTCCTCTTCGCCATCCTCGTCATCCACCTCCTCGTTTTCCTCCCTCTCCTCCACCTCTTCTTCATCTTCCTCCACCACCTCCAA : 817
YC : GGGACAAGAGTGGCTTGGGTCTTGGCGCGGTCTTCCTCCTCCTCTTCGCCATCCTCGTCATCCACGTCCTCGTTGTCCTCCCTCTCCTCCACCTCTTCTTCCTCTTCCTCCACCACCTCCAA : 824
RT : AAGACAAGGGCGGCCTGCTGGGGCGCCTCCTCCTCCACCTCCTCGTCGCCGTCCTCCTCCTCCACCTCTTCCTTCTCCTCCCTCTCCTCG------------TCCTCCTCTATCGCCTCCAA : 796
GF : AGGACAGCGGAGGGGCTTGCACATCCTCCTGCTCATCATCTTCCTCTCCTTCATCCTCGTCTACCTCCTCTTTCTCCTCCCTGTCATCG----------TGCTCCTCTTCCAC----CTCCAA : 790
GC : AGGACAGCGGAGGGGCTTGCACCTCTTCCTGCTCATCTTCTTCCTCTCCATCCTCCTCGTCCACCTCCTCTTTCTCATCCCTGTCGTCG----------TGCTCCTCTTCCACCGCCTCCAA : 814
WS : AGGACAAAAGCAGCG-----------------------CTTTGTCGTCATCTTCTTCTTCTTGTTCTTCCTCATCCTCCA----------------------------------CCAA : 749
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SF : GAAGAAGTCAGCTG-TGCCATCTCAACAACA---------ACAGCAGCAACAGCAGCAGCAGCAGCAGCAGCAGCCGCAGCAGCATCACCAGCGAGCCAAACCAACCAGCTTGCCACTTCC : 928
YC : GAAGAAGTCAGCTG-TACCATCTCAACAACATCAACAACAACAACAACAGCAGCAGCAGCAGCAGCAGCAACCGCCGCAGCAGCATCACCAGCGAGCCAAACCAACCAGCTTGCCACTTCC : 944
RT : GAAGAAGTCGTCCT-CGTGGTC----CGTCG--------------TGTCACAGCAGCAGCAGCAGC--------------------------------CCAAACCAACCAGCTTGCCACTTCC : 868
GF : AAAGAAGACACCTCTGCCTCCCGTTCATC----------------GCAGCAGCAGCAGGAGCAGCAGCTGGCAGTGCAGG------CCCAGCGAGCCAAACCAACCATCTTGCCACTTCC : 889
GC : AAAGAAGACATCCTCTGCCTCCCGATCATCA----------CAACAGCAGCAGCAGCAGCAGCAGCTGGCATTGCAGG------CCCAGCGAGCCAAACCAACCATCTTGCCACTTCC : 919
WS : AAAGAAGCCG-CAGCTGCAGTCTCA----------------------GCAGCAGTCACATCACCAGCA--------------------------AGCCAAACCAACCAGCTTGCCACTTCC : 821
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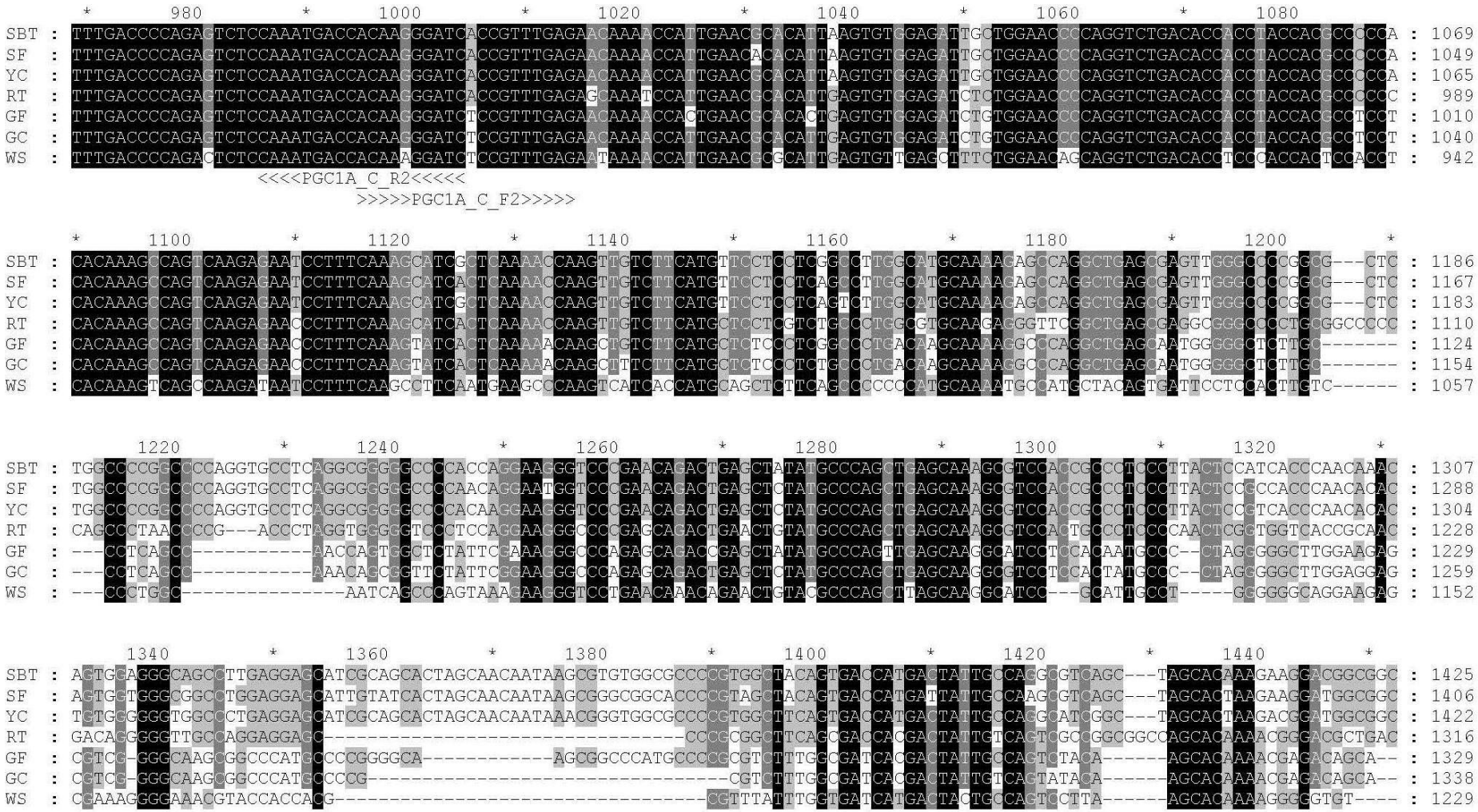
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1000
1020
1040
1060
1080
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SF : TTTGACCCCAGAGTCTCCAAATGACCACAAGGGATCACCGTTTGAGAACAAAACCATTGAACACACATTAAGTGTGGAGATTGCTGGAACCCCAGGTCTGACACCACCTACCACGCCGCCA : 1049
YC : TTTGACCCCAGAGTCTCCAAATGACCACAAGGGATCACCGTTTGAGAACAAAACCATTGAACGCACATTAAGTGTGGAGATTGCTGGAACCCCAGGTCTGACACCACCTACCACGCCGCCA : 1065
RT : TTTGACCCCAGAGTCTCCAAATGACCACAAGGGATCACCGTTTGAGAGCAAATCCATTGAACGCACATTGAGTGTGGAGATCTCTGGAACCCCAGGTCTGACACCACCTACCACGCCGCCC : 989
GF : TTTGACCCCAGAGTCTCCAAATGACCACAAGGGATCTCCGTTTGAGAACAAAACCACTGAACGCACACTGAGTGTGGAGATCTGTGGAACCCCAGGTCTGACACCACCTACCACGCCTCCT : 1010
GC : TTTGACCCCAGAGTCTCCAAATGACCACAAGGGATCTCCGTTTGAGAACAAAACCATTGAACGCACATTGAGTGTGGAGATCTGTGGAACCCCAGGTCTGACACCACCTACCACGCCTCCT : 1040
WS : TTTGACCCCAGACTCTCCAAATGACCACAAAGGATCTCCGTTTGAGAATAAAACCATTGAACGCGCATTGAGTGTTGAGCTTTCTGGAACAGCAGGTCTGACACCTCCCACCAGTCCACCT : 942
<<<<PGC1A_C_R2<<<<<
>>>>>PGC1A_C_F2>>>>>
1100
1120
1140
1160
1180
1200
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1220
1240
1260
1280
1300
1320
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1340
1360
1380
1400
1420
1440
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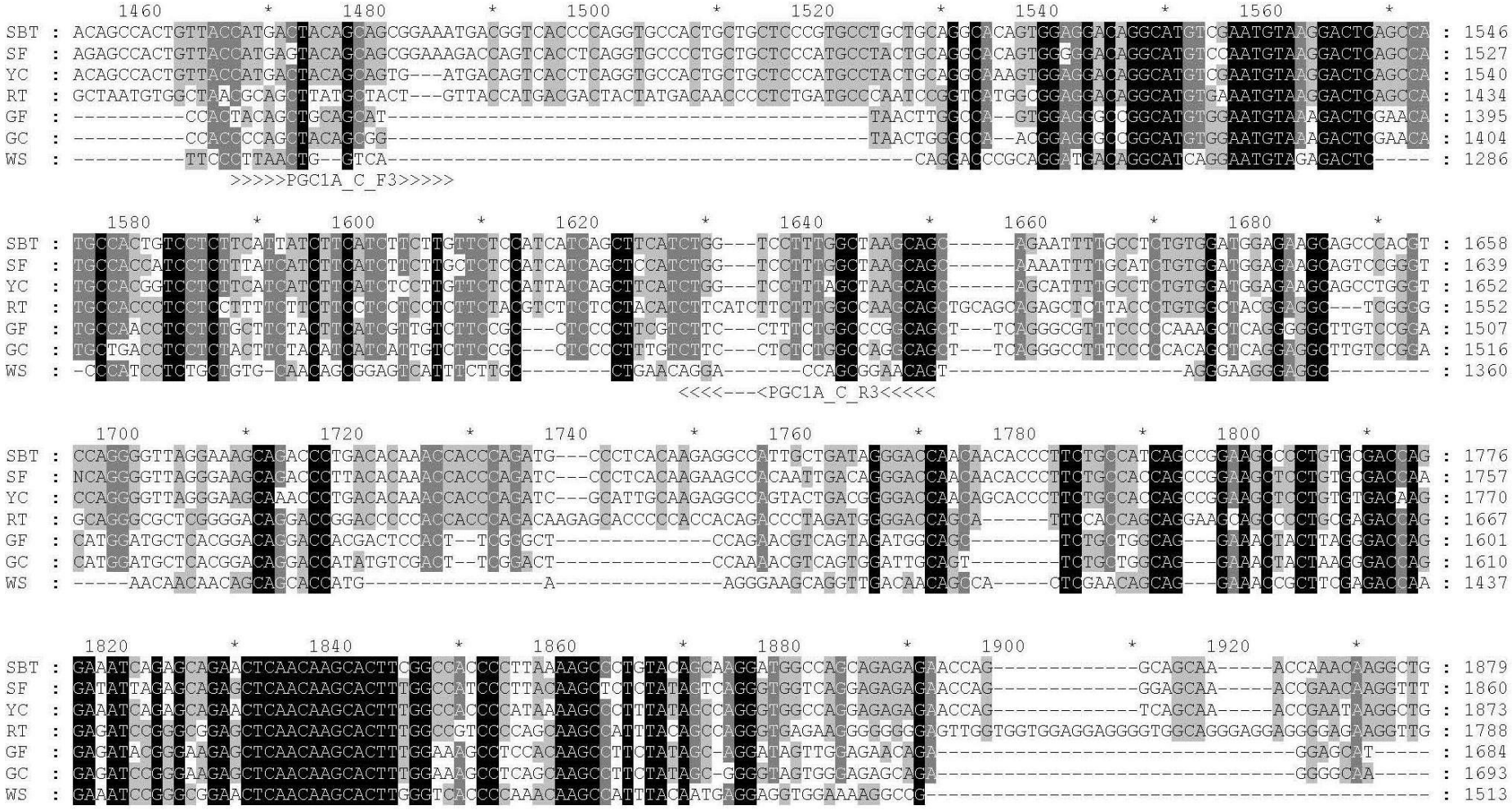
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RT : GCTAATGTGGCTAACGCAGCTTATGCTACT---GTTACCATGACGACTACTATGACAACCCCTCTGATGCCCAATCCGGTCATGGCGGAGGACAGGCATGTGAAATGTAAGGACTCAGCCA : 1434
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GC : ----------CCACCCCAGCTACAGCGG----------TAACTGGGCCA--ACGGAGGGCCGGCATGTGGAATGTAAAGACTCGAACA : 1404
WS : ----------TTCCCTTAACTG--GTCA----------CAGGACCCGCAGGATGACAGGCATCAGGAATGTAGAGACTC----- : 1286
>>>>>PGC1A_C_F3>>>>>
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SF : TGCCACCATCCTCTTTATCATCTTCATCTTCTTGCTCTCCATCATCAGCTCCATCTGG---TCCTTTGGGCTAAGCAGC------AAAATTTTGCATCTGTGGATGGAGAAGCAGTCCGGGT : 1639
YC : TGCCACGGTCCTCTTCATCATCTTCATCTCCTTGTTCTCCATTATCAGCTTCATCTGG---TCCTTTAGGCTAAGCAGC------AGCATTTTGCCTCTGTGGATGGAGAAGCAGCCTGGGT : 1652
RT : TGCCACCCTCCTCCTTTTCTTCTTCCTCCTCCTCTTCTACGTCTTCTCCTACATCTTCATCTTCTTTGGGCCAAGCAGCTGCAGCAGAGCTCTTACCCTGTGGCTACGGAGGC---TCGGCG : 1552
GF : TGCCAACCTCCTCTGCTTCTACTTCATCGTTGTCTTCCGC---CTCCCCTTCGTCTTC---CTTTCTGGCCCGGCAGGT---TCAGGGCGTTTCCCCCAAAGCTCAGGGGCTTGTCCGGA : 1507
GC : TGCTGACCTCCTCTACTTCTACATCATCATTGTCTTCCGC---CTCCCCTTTGTCTTC---CTCTCTGGGCCAGGCAGGT---TCAGGGCCTTTCCCCCACAGCTCAGGAGGCTTGTCCGGA : 1516
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<<<<---<PGC1A_C_R3<<<<<
1700 * 1720 * 1740 * 1760 * 1780 * 1800 *
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SF : NCAGGGGTTAGGGAAGCAGACCCTTACACAAACCACCCAGATC---CCCTCACAAGAAGCCACAATTGACAGGGAGCAACAACACCCTTCTGCCACCAGCCGGAAGCTCCTGTGCGACCAA : 1757
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GC : CATGGATGCTCACGGACAGGACCATATGTCGACT--TCGGACT--------------CCAAAACGTCAGTGGATTGCAGT--------TCTGCTGGCAG---GAAACTACTAAGGGACCAG : 1610
WS : -----AACAACAACAGCAGCACCATG---------------A--------------AGGGAAGCAGGTTGACAACAGCCA-----CTCGAACAGCAG---GAAACCGCTTCGAGACCAA : 1437
1820 * 1840 * 1860 * 1880 * 1900 * 1920 *
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RT : GAGATCCGGGCGGAGCTCAACAAGCACTTTGGCCGTCCCCAGCAAGCCATTTACAGCCAGGGTGAGAAGGGGGGGGAGTTGGTGGTGGAGGAGGGGTGGCAGGGAGGAGGGGAGAAGGTTG : 1788
GF : GAGATACGGGAAGAGCTCAACAAGCACTTTGGAAAGCCTCCACAAGCCTTCTATAGC-AGGATAGTTGGAGAACAGA--------------------------------GGAGCAT----- : 1684
GC : GAGATCCGGGAAGAGCTCAACAAGCACTTTGGAAAGCCTCAGCAAGCCTTCTATAGC-GGGGTAGTGGGAGAGCAGA--------------------------------GGGGCAA----- : 1693
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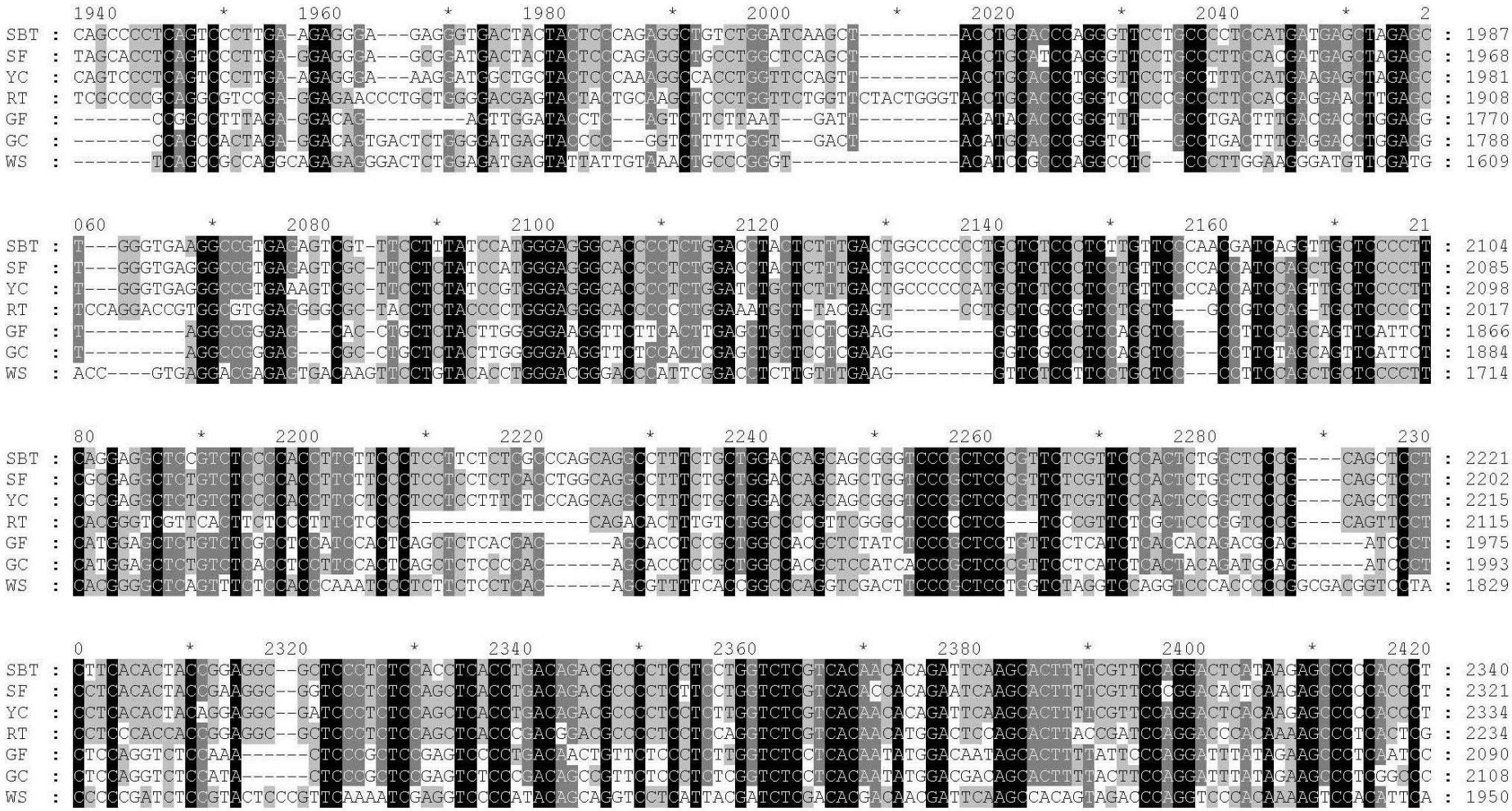

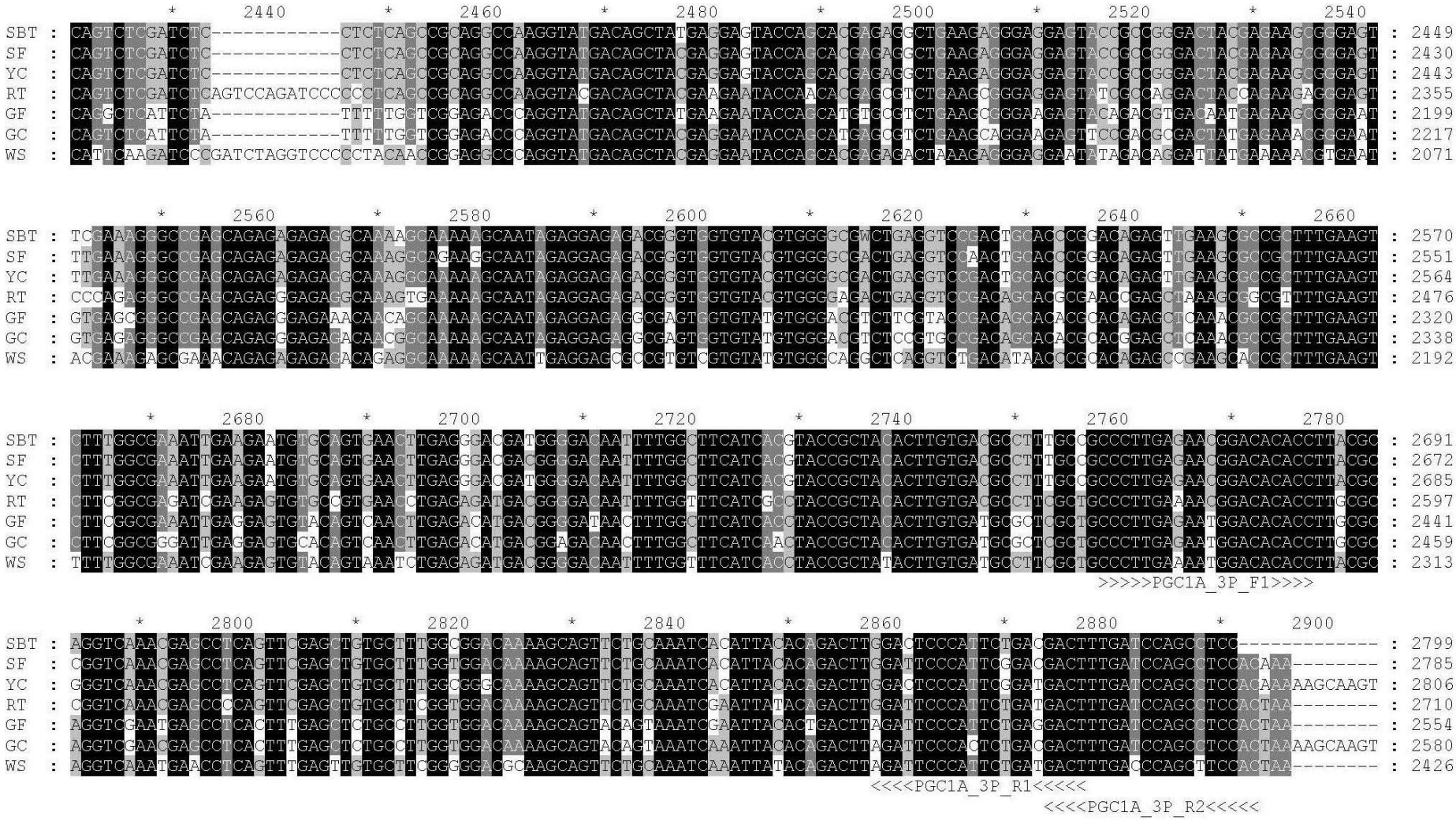
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2500
2520
2540
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YC : CAGTCTCGATCTC----------CTCTCAGCCGCAGGCCAAGGTATGACAGCTACGAGGAGTACCAGCACGGAGAGGCTGAAGAGGGAGGAGTACCGCCGGGACTACGAGAAGCGGGAGT : 2443
RT : CAGTCTCGATCTCAGTCCAGATCCCCCCCTCAGCCGCAGGCCAAGGTACGACAGCTACGAAGAATACCAACACGGAGCGTCTGAAGCGGGAGGAGTATCGCCAGGACTACGAGAAGAGGGAGT : 2355
GF : CAGGCTCATTCTA----------TTTTTGGTCGGAGACCCAGGTATGACAGCTATGAAGAATACCAGCATGTGCGTCTGAAGCGGGAAGAGTACAGACGTGAGAATGAGAAGCGGGAAT : 2199
GC : CAGTCTCATTCTA----------TTTTTGGTCGGAGACCCAGGTATGACAGCTACGAGGAATACCAGCATGAGCGTCTGAAGCAGGAAGAGTTCCGACGCGACTATGAGAAACGGGAAT : 2217
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GC : GTGAGAGGGCCGAGCAGAGGGAGAGACAACGGCAAAAAGCAATAGAGGAGAGGCGAGTGGTGTATGTGGGAGGTCTCCGTGCCGACAGCACACGCACGGAGCTCAAACGCCGCTTTGAAGT : 2338
WS : ACGAAAGAGCGAAACAGAGAGAGAGACAGAGGCAAAAAGCAATTGAGGAGCGCCGTGTCGTGTATGTGGGCAGGCTCAGGTCTGACATAACCCGCACAGAGCCGAAGCAGCGCTTTGAAGT : 2192
2680
2700
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2840
2860
2880
2900
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YC : GGGTCAAACGAGCCTCAGTTCGAGCTGTGCTTTGGCGGGCAAAAGCAGTTCTGCAAATCACATTACACAGACTTGGACTCCCATTCGGATGACTTTGATCCAGCCTCCACAAAAAGCAAGT : 2806
RT : CGGTCAAACGAGCCCCAGTTCGAGCTGTGCTTCGGTGGACAAAAGCAGTTCTGCAAATCGAATTATACAGACTTGGATTCCCATTCTGATGACTTTGATCCAGCCTCCACTAA-------- : 2710
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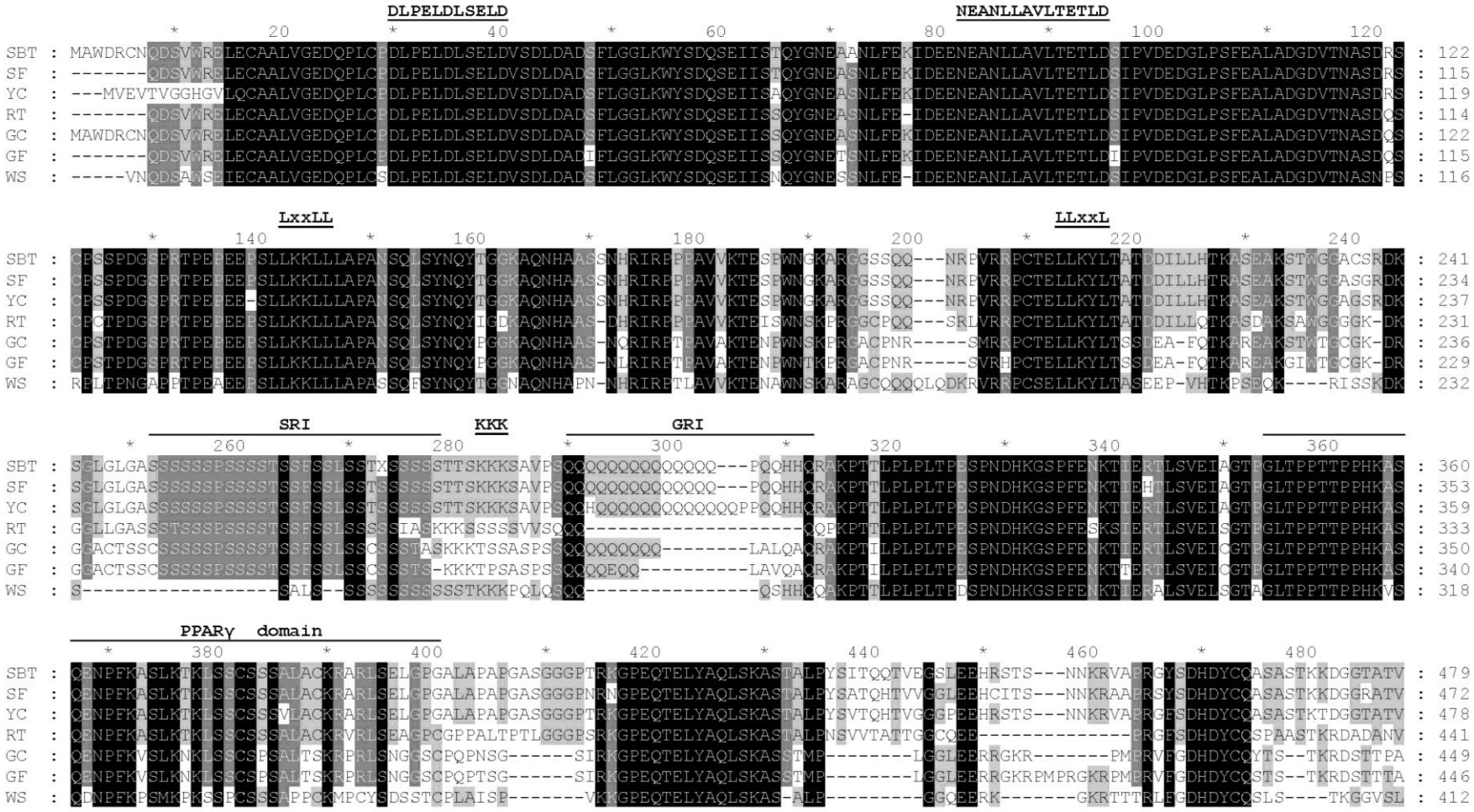
WS : AGGTCAAATGAACCTCAGTTTGAGTTGTGCTTCGGGGGACGCAAGCAGTTCTGCAAATCAAATTATACAGACTTAGATTCCCATTCTGATGACTTTGACCCAGCTTCCACTAA-------- : 2426
<<<<PGC1A_3P_R1<<<<<
<<<<PGC1A_3P_R2<<<<<

Figure 6.2: An alignment of the nucleotide sequences of PGC-1α cDNAs from various fish species. The sequence for the SBT PGC-1α cDNA was determined in the present study. The other sequences for swordfish (SF, *Xiphias gladius*, FJ710607.1), yellow croaker (YC, *Larimichthys crocea*, XM_010733812.1), rainbow trout (RT, *Oncorhynchus mykiss*, FJ710605.1), goldfish (GF, *Carassius auratus*, FJ710611.1), grass carp (GC, *Ctenopharyngodon idella*, JN195739.1) and white sturgeon (WS, *Acipenser transmontanus*, FJ710613.1) were downloaded from the NCBI Nucleotide database. The binding sites for each of the primers are shown below the corresponding sequence. Primer names flanked by >>> indicate forward primers and primer names flanked by <<< indicate reverse primers. Shading represents the degree of conservation across all aligned sequences where black = 100%, dark grey = 70%, light grey = 50% and no shading = <50%.

6.3.2 SBT PGC-1α amino acid sequence

The SBT PGC-1α predicted amino acid sequence compared to the sequences predicted from various fish species is shown in Fig. 6.3. The SBT PGC-1α amino acid sequence contained all of the characteristic motifs that have been previously reported for fish PGC-1α proteins. These included the two activation domains (AD1 and AD2) (Sadana and Park 2007), two leucine-rich motifs (L2 and L3) (Oberkofler *et al.* 2002, LeMoine *et al.* 2010b), the fish-specific serine and glutamine-rich insertions (SRI and GRI, respectively) (LeMoine *et al.* 2010b), a tri-lysine motif (Vercauteren *et al.* 2006, LeMoine *et al.* 2010b), the PPARγ binding site, and an RNA recognition motif (predicted from the ExPASy prosite calculator (prosite.expasy.org) and the annotated rat PGC-1α sequence (NM_031347)). The two activation domains (DLPELDLSELD and NEANLLAVLTETLD) were present at amino acids 30-40 and 82-95, the leucine-rich motifs (LxxLL and LLxxL) were present at amino acids 142-146 and 213-217, respectively and the tri-lysine motif (KKK) was present at amino acids 282-284. Each of these regions were 100% conserved across all of the aligned sequences. Furthermore, the SBT PGC-1α sequence showed a serine rich insertion (SRI) directly upstream of the tri-lysine motif and a glutamine rich insertion (GRI) directly downstream of the tri-lysine motif both of which were somewhat, though not completely, conserved across the different fish species. Interestingly, the PPARγ binding domain was well conserved at the N-terminal end but there were substantial variations between the different fish species at the C-terminal end of this domain. The final 145 amino acids at the N-terminus of the PGC-1α sequence showed substantial conservation between the different fish species and incorporated a well conserved RNA recognition motif. Comparison of the predicted SBT PGC-1α amino acid sequence with the predicted

sequences from other fish species in the alignment showed that the SBT sequence was most similar to swordfish with 91% identity (Table 6.2). The SBT sequence also showed substantial similarity to yellow croaker (identity = 88%) with the remaining species showing ≤ 71% identity.


DLPELDLSELD
NEANLLAVLTETLD
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LLxxL
SRI
KKK
GRI
PPARγ domain

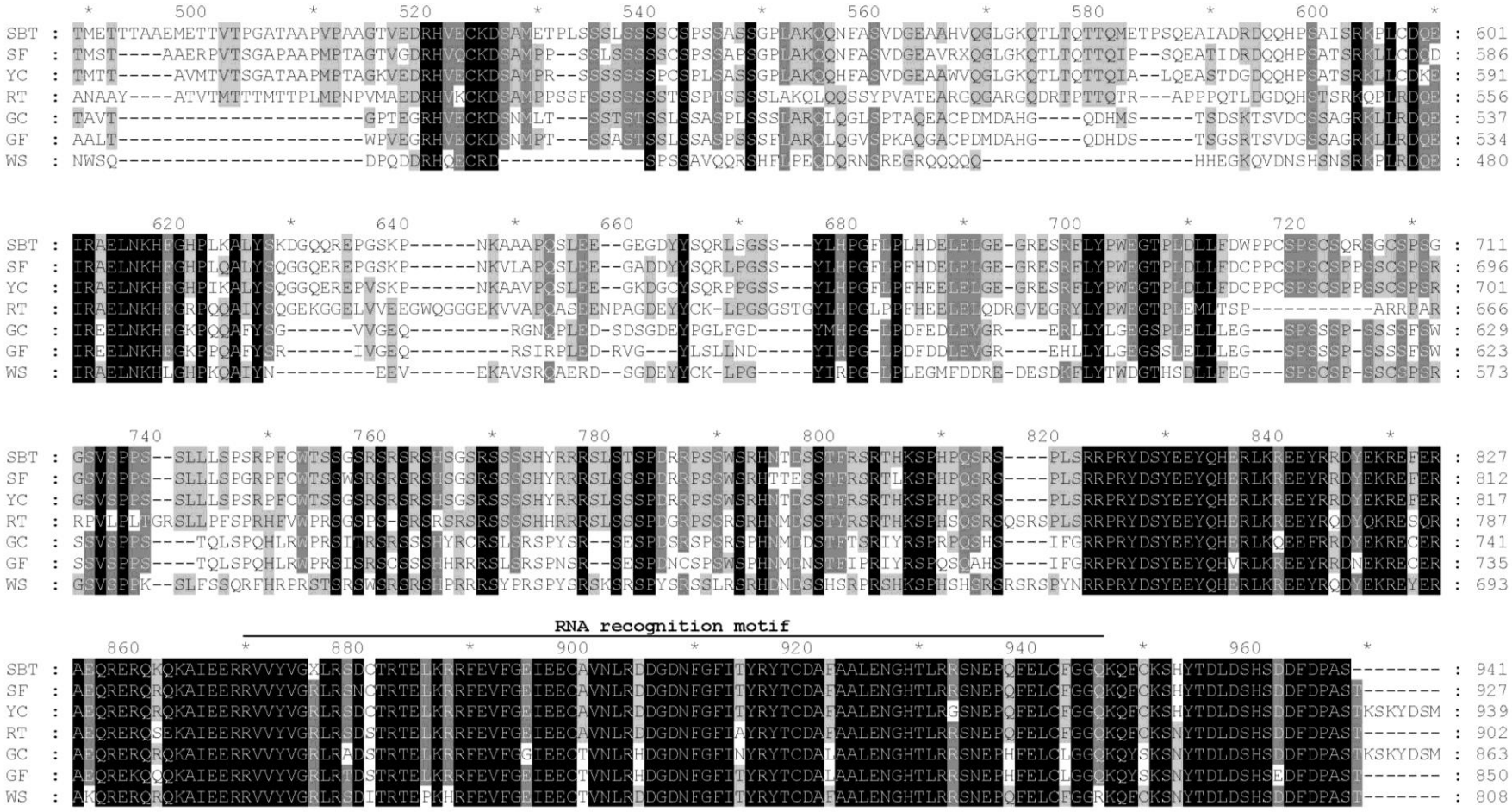
RNA recognition motif

Figure 6.3: An alignment of the predicted amino acid sequences of PGC-1α cDNAs from various fish species. The sequence for the SBT PGC-1α cDNA was determined in the present study. The sequences for the other fish species were translated from the NCBI nucleotide database mRNA sequences for swordfish (SF, *Xiphias gladius*, FJ710607.1), yellow croaker (YC, *Larimichthys crocea*, XM_010733812.1), rainbow trout (RT, *Oncorhynchus mykiss*, FJ710605.1), goldfish (GF, *Carassius auratus*, FJ710611.1), grass carp (GC, *Ctenopharyngodon idella*, JN195739.1) and white sturgeon (WS, *Acipenser transmontanus*, FJ710613.1). The shading represents the degree of conservation across all aligned sequences where black = 100%, dark grey = 70%, light grey = 50% and no shading = <50%. The annotated regions are the activation domain (1 - 186), the NRF-1 binding domain (187 - 430), the MEF2c binding domain (431 - 722) and the RNA binding domain (723 - end). Underlined sections indicate motifs that are characteristic of PGC-1α proteins and where relevant, the conserved sequence(s) are indicated, as inferred from existing literature and an annotated rat PGC-1α sequence (NM_031347). SRI = serine-rich insertion, GRI = glutamine-rich insertion.

Table 6.2: Percent identities of the different fish PGC-1α amino acid sequences compared to each other and the SBT PGC-1α sequence.

	Swordfish	Yellow croaker	Pufferfish	Rainbow trout	Grass carp	Goldfish	White sturgeon	Zebrafish
SBT	91	88	71	67	61	60	57	27
Swordfish		89	72	68	60	61	58	27
Yellow croaker			75	66	62	59	56	25
Pufferfish				56	54	51	49	19
Rainbow trout					59	60	56	28
Grass carp						87	56	35
Goldfish							58	33
White Sturgeon								25

6.3.3 Phylogenetic analysis of the SBT PGC-1α amino acid sequence

A phylogenetic tree comparing the SBT PGC-1α sequence with PGC-1α sequences from various fish, mammalian, and avian species and PGC-1β sequences from fish is shown in Fig. 6.4. The tree showed clear separation of the three different classes present: mammals (Mammalia), birds (Aves) and ray-finned fishes (Actinopterygii). Within the Actinopterygii clade, all the included sequences for PGC-1β were grouped in a single clade with the PGC-1α sequences forming a separate clade. Within the clade containing the PGC-1α sequences, perciform (yellow croaker and swordfish), tetradontiform (pufferfish) and scombrid (SBT) orders formed a single clade. A second clade was formed from acipenseriform (white sturgeon) and cypriniform (zebrafish) orders and a third clade contained the salmoniform (rainbow trout) and cypriniform (grass carp and goldfish) orders.

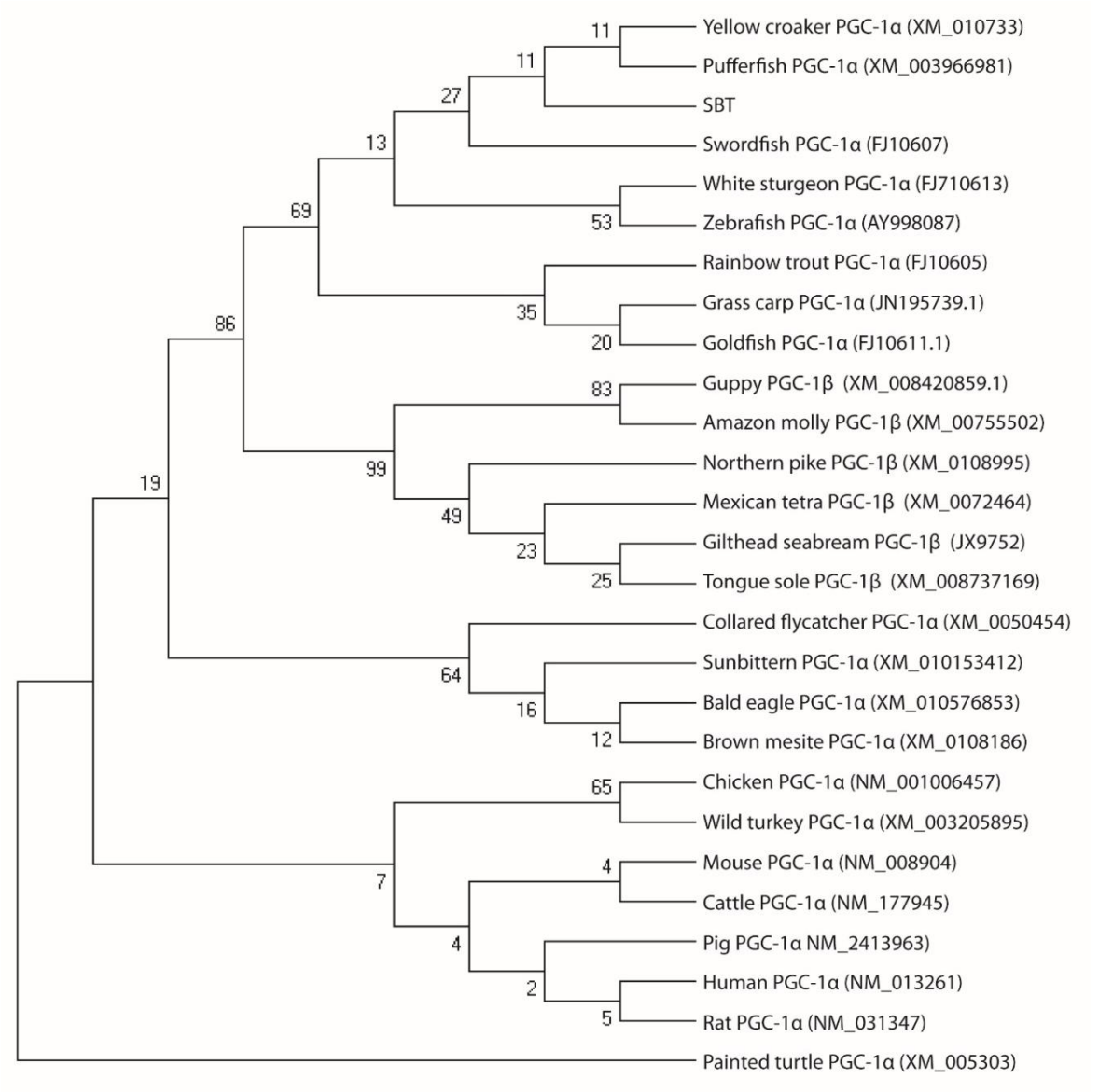

Figure 6.4: Maximum parsimony tree comparing the predicted SBT PGC-1α amino acid sequence with predicted PGC-1α amino acid sequences from fish, mammals and birds as well as predicted PGC-1β amino acid sequences from fish. The sequences were translated from the NCBI nucleotide database. They were for yellow croaker (*Larimichthys crocea*), pufferfish (*Takifugu rubripes*), swordfish (*Xiphias gladius*), white sturgeon (*Acipenser transmontanus*), zebrafish (*Danio rerio*), rainbow trout , grass carp (*Ctenopharyngodon idella*), goldfish (*Carassius auratus*), guppy (*Poecilia reticulata*), Amazon molly (*Poecilia formosa*), Northern pike (*Esox lucius*), Mexican tetra (*Astyanax mexicanus*), gilthead seabream (*Sparus aurata*), tongue sole (*Cynoglossus semilaevis*), collared flycatcher (*Ficedula albicollis*), sunbittern (*Eurypyga helias*), brown mesite (*Mesitornis unicolor*), bald eagle (*Haliaeetus leucocephalus*), chicken (*Gallus gallus*), wild turkey (*Meleagris gallopavo*), mouse (*Mus musculus*), cattle (*Bos taurus*), pig (*Sus scrofa*), human, rat (*Rattus norvegicus*) and painted turtle (*Chrysemys picta bellii*). The sequences were obtained from the NCBI Nucleotide database and the accession number for each sequence is shown on the tree and the numbers on the nodes represent the frequency that the tree topology was replicated after 1000 bootstrap iterations.

6.4 Discussion

6.4.1 The SBT PGC-1α nucleotide and amino acid sequences

The sequence of a PGC-1α cDNA from SBT was successfully determined through the sequencing of multiple overlapping fragments. Each region of the PGC-1α cDNA was sequenced a minimum of three times using either forward or reverse primers, or a combination of the two. The cDNA coded for a predicted amino acid sequence that contained the main characteristic features of a PGC-1α protein, as determined by comparison to fish and mammalian PGC-1α amino acid sequences. The majority of the ORF was successfully cloned and sequenced including the start codon. The deduced amino acid sequence contained all four domains that are present in a full-length PGC-1α protein; an activation domain (AD), a nuclear respiratory factor 1 (NRF-1) binding domain, a myocyte enhancer factor 2c (MEF2c) binding domain and an RNA binding domain, as inferred from an annotated rat PGC-1α sequence (Lin *et al.* 2005, LeMoine *et al.* 2010b). The two activation motifs in PGC-1α, DLPELDLSELD and NEANLLAVLTETLD are present with high levels of conservation in vertebrate PGC-1α proteins and are required for the transcriptional co-activation activity of PGC-1α (Sadana and Park 2007, LeMoine *et al.* 2010b). For example, Sandana and Park (2007) found that the deletion of these activation domains in mouse PGC-1α reduced the stimulation of transcription of carnitine palmitoyl transferase 1 α (CPT-1α) to 57% and CPT-1β to 70% of what was seen when the activation domains were intact. The proteins that CPT-1α and CPT-1α encode play a critical role in fatty acid β-oxidation by transporting fatty acids into the mitochondria. It was proposed that the activation domains present in the PGC-1α protein allow it to act as a bridge between nuclear receptors and transcription factors (Sadana and Park 2007). This allows the nuclear receptors to induce transcription of

genes such as CPT-1α and CPT-1β more effectively in the presence of PGC-1α (Sadana and Park 2007). The predicted amino acid sequences from the different fish species described here showed substantial conservation in the activation domain and both of the activation motifs were present near the N-terminal end of the SBT sequence. The first of two leucine-rich motifs, LxxLL, was also found within the activation domain. A second leucine-rich motif, LLxxL, was found at the N-terminal end of the NRF-1 binding domain. These leucine-rich motifs are involved in the interaction of PGC-1α with NRF-1 and estrogen-related receptor alpha (ERRα) and showed 100% conservation with the other fish species in the alignment (Corton and Brown-Borg 2005, Sadana and Park 2007).

A further feature within the NRF-1 binding domain was a conserved tri-lysine (KKK) motif. In PGC-1 related coactivator (PRC), a close relative of PGC-1α, this motif constitutes part of the minimal NRF-1 binding domain (Vercauteren *et al.* 2006). The KKK motif was flanked by a serine-rich insertion towards the N-terminal end and a glutamine-rich insertion towards the C-terminal end. These insertions are specific to fish PGC-1α proteins (LeMoine *et al.* 2010b) and were present at different lengths for the different fish species included in the alignment. The serine and glutamine-rich insertions appeared to be longer in the perciform (yellow croaker and swordfish), tetradontiform (pufferfish) and scombrid (SBT) fish than other fish species. The SBT PGC-1α contained the equal longest serine-rich insertion and the second-longest glutamine-rich insertion and these insertions were most similar in length to the swordfish PGC-1α. LeMoine *et al.* (2010b) proposed that the proximity of the insertions may place them within the putative NRF-binding domain and could

therefore affect the interaction of PGC-1α with NRF-1 in fish. However, this remains to be investigated.

Another important region is the PPARγ interaction domain which was partially conserved across the different fish species. The divergence within the serine and glutamine-rich insertions and in the C-terminal end of the PPARγ interaction domain contributed substantially to a lack of conservation between different fish species in N-terminal region of the alignment. The variability in the PPARγ interaction domain is intriguing as it indicates that there may be differences in the ability of PGC-1α to act as a co-activator to PPARγ. Alternatively, it may also indicate divergence in these sequences due to some selective pressure, for example, the environment that each species is adapted to. PPARγ plays an important role in adipogenesis in mammals (Schoonjans *et al.* 1996, Guan *et al.* 2005); therefore if there is a lower capacity for PGC-1α to act as a co-activator to PPARγ in SBT compared to mammalian species, it could indicate a low capacity for PGC-1α to induce adipogenesis in SBT.

PGC-1α may play an important role in muscle biogenesis through co-activation of MEF2c (Yu *et al.* 1992, Black and Olson 1998, Lin *et al.* 2005). Furthermore, MEF2c is involved in fatty acid β-oxidation in muscle tissues. For example, Baldán *et al.* (2004) transfected a monkey kidney cell line (CV-1), with MEF2c and/or PPARα. The cells transfected with MEF2c alone showed approximately 5-fold higher activation of the CPT-1β promoter and this activation was increased a further 3-fold in the cells transfected with both MEF2c and PPARα. The MEF2c domain was the most poorly conserved domain in the fish PGC-1α amino acid sequences and, similar to the sequence divergence in the PPARγ domain, this sequence

divergence could suggest a poor interaction of PGC-1α with MEF2c, or divergent evolution of these two genes in fish. The RNA binding domain was well conserved between the different fish species and the predicted SBT PGC-1α amino acid sequence contained a RNA recognition motif from amino acids 867-943. The high level of conservation seen in this region is consistent with previous reports of PGC-1α in fish and other vertebrates (LeMoine *et al.* 2010b)

6.4.3 Phylogenetic analysis of the SBT PGC-1α amino acid sequence

Phylogenetic analysis showed that the SBT PGC-1α amino acid sequence was most similar to the corresponding sequence from swordfish. SBT and swordfish are among a small number of marine fish which are regionally endothermic (Block *et al.* 1993). However, Little *et al.* (2010) found that swordfish and other billfishes were evolutionarily very distant from tuna and other scombrid fish and proposed that the physiological similarities are the result of convergent evolution due to similar selective pressures. It is important to note that there are distinct differences between the endothermy observed in swordfish and tunas. Endothermy in swordfish is made possible by a heater organ, which has evolved from ocular muscle tissue and raises the temperature of both the eyes and brain through the uncoupling of electron transfer from ATP synthesis (Block *et al.* 1993, Dickson and Graham 2004, Sepulveda *et al.* 2007). This heater organ exhibits a high rate of oxidative metabolism but lacks contractile machinery that it typical of other muscles (Block 1994, Block and Finnerty 1994). In contrast, tunas exhibit endothermy in their red muscle in addition to their eyes and brain. There is no evidence for uncoupling in the red muscle tissue and the capacity for red muscle endothermy is believed to be due to their constantly repeating muscle contraction. This muscle contraction is necessary as

it powers continuous swimming, which is necessary to ensure adequate oxygen intake (because tunas are ram ventilators) and to counter their natural negative buoyancy (Magnuson 1972). There are also several physiological and structural adaptations that facilitate red muscle endothermy, including the presence of vascular counter-current heat exchangers and the more interior localisation of the red muscle tissue, which conserves heat (Block *et al.* 1993, Dickson and Graham 2004, Graham and Dickson 2004). The common process of high metabolic rate, coupled with the similarity between SBT and swordfish PGC-1α and the convergent evolution of endothermy in these species is intriguing and warrants further investigation of the role of PGC-1α in the evolution of endothermy in tunas and swordfishes.

In conclusion, this chapter provides the first report of a PGC-1α cDNA sequence from any tuna species and since PGC-1α is important in the regulation of mitochondrial biogenesis and function in vertebrates, this study lays the foundation for further work to define the regulation of metabolic processes in tunas. The PGC-1α defined here corresponds to the canonical PGC-1α. However, some of the PCR reactions conducted for the experiments reported on in this chapter yielded multiple products and this may indicate that some of the other isoforms of PGC-1α may be present in SBT. This would be an interesting subject for further investigation.

Chapter 7 – Analysis of gene expression in SBT tissues

7.1 Aims and background

The aim of this chapter was to investigate the gene expression of PGC-1α, PPARγ, CS, COX1 and UCP2 in the tissues of farmed and wild-caught SBT.

PGC-1α exhibits a substantial level of control on metabolism through its ability to co-activate a broad range of transcription factors. These include NRF-1 and NRF-2 (mitochondrial biogenesis), PPARs (fatty acid β-oxidation, adipocyte differentiation), ERRα (mitochondrial biogenesis) and TRβ (mitochondrial biogenesis, synthesis and degradation of fatty acids, glucose homeostasis) (further details can be found in Section 1.6.2 and Fig. 1.2). The PPARγ gene is typically found expressed to high levels in preadipocytes and adipocytes and is most commonly recognised for its role in adipogenesis (Schoonjans *et al.* 1996, Guan *et al.* 2005). CS has been previously used as an indicator of mitochondrial abundance (Moyes *et al.* 1992, Dalziel *et al.* 2005). The COX1 gene encodes a protein which is located in the inner mitochondrial membrane and therefore is an indicator of the abundance of the electron transfer chain components within that membrane (Khalimonchuk and Rödel 2005). Thus, it could be expected that tissues with high levels of aerobic metabolism (e.g. red muscle) will have a greater requirement for mitochondria and thus will express CS and COX1 to a greater extent than tissues such as white muscle, which rely more heavily on anaerobic, glycolytic metabolism. UCP2 gene expression and protein abundance have both been shown to increase during cold acclimation in temperate eelpout (*Zoarces viviparous*) and warm acclimation in Antarctic eelpout (*Pachycara brachycephalum*) (Mark *et al.* 2006). It was proposed that these results could indicate a role in thermal adaptation and/or form part of a response to thermal stress (Mark *et al.* 2006). Thus, it is an interesting

target to investigate, especially in tuna red muscle, due to the unusual capacity for endothermy in this tissue (Dickson and Graham 2004).

It was hypothesised that:

1. The gene expression of PGC-1α would be higher in tissues with a large number of mitochondria (e.g. red muscle) than those with a lower number of mitochondria (e.g. white muscle) due to its role in mitochondrial biogenesis
2. The gene expression of CS and COX1 would be higher in tissues with a large number of mitochondria compared to those with a lower number of mitochondria due to their role of the enzymes they encode in mitochondrial respiration.
3. PGC-1α gene expression would correlate with PPARγ and UCP2 gene expression, indicating an interaction between the proteins that these gene encode

7.2 Materials and methods

7.2.1 Harvest of tissues

7.2.1.1 Harvest of tissues from farmed SBT

Tissue samples from farmed SBT were collected as described in Section 2.6.2.1.

7.2.1.2 Harvest of tissues from wild-caught SBT

Tissue samples from wild-caught SBT were collected as described in Section 2.6.2.2

7.2.2 RNA extraction

RNA was extracted from the tissues according to the protocol described in Section 2.6.4

7.2.3 cDNA synthesis

A total of 1 µg of RNA was used to synthesise cDNA according to the protocol described in Section 2.6.5.

7.2.4 Design, synthesis and validation of primers

Design, synthesis and validation of primers were conducted according to the protocol described in Section 2.5. The primers used to assess gene expression by qRT-PCR are shown in Table 7.1

Table 7.3: A summary of the primer sets used to assess gene expression in the SBT tissues. The efficiency values were obtained by qRT-PCR using a 10-fold dilution series of the template spanning ≥ 5 orders of magnitude (Section 2.5.9.

Target gene	**Forward primer sequence (5´ → 3´)**	**Reverse primer sequence (5´ → 3´)**	**Efficiency**
β-actin	ACCCACACAGTGCCCATCTA	TCACGCACGATTTCCCTCT	0.91
ELF-1α	CCCCTGGACACAGAGACTTC	GCCGTTCTTGGAGATACCAG	0.94
CS	CTGGACTGGTCCCACAACTT	GGACAGGTAGGGGTCAGACA	0.96
COX1	GGAGCTGTATTCGCCATTGT	AGGAAGTGCTGTGGGAAGAA	0.91
PGC-1α	AACAACACCCTTCTGCCATC	GTTTGCTGCCTGGTTCTCTC	0.92
PPARγ	TCTTCAGACGCACAATCAGG	GTGACATGCCAACATTGAGG	0.95
UCP2	AGACCATTGCCAGGGATGAA	CACGGCATGTTGTCTGTCAT	0.96

7.2.5 Analysis of gene expression by qRT-PCR

The qRT-PCR reactions were conducted using 50 ng of cDNA template according to the protocol described in Section 2.6.6. The fluorescence curves produced for each sample were used to calculate the threshold cycle (Ct) value. The relative expression of each of the different genes was calculated in six different ways in order to determine the effects of different normalisation methods on the data.

1) Gene expression per μg RNA was calculated using the formula 2^{-Ct} x 20, as each reaction contained 50 ng of cDNA as template (i.e. 50 ng x 20 = 1 μg).
2) Gene expression per mg tissue was calculated by multiplying the results from (1) by the RNA yield in μg RNA/mg tissue.
7. β-actin-normalised gene expression per μg RNA was calculated by the equation $2^{-\Delta Ct}$, where ΔCt = Ct (gene of interest) – Ct (β-actin) (Livak and Schmittgen 2001).
3) β-actin-normalised gene expression per mg tissue was calculated by multiplying the result from (3) by the RNA yield in μg RNA/mg tissue.
4) ELF-1α-normalised gene expression per μg RNA was calculated by the equation $2^{-\Delta Ct}$, where ΔCt = Ct (gene of interest) – Ct (ELF-1α) (Livak and Schmittgen 2001).
5) ELF-1α-normalised gene expression per μg RNA was calculated by multiplying the result from (5) by the RNA yield in μg RNA/mg tissue.

7.2.6 Statistical analyses

The data were analysed according to the protocol described in Section 2.8. Specifically, significant differences in the data were determined using one-way ANOVA followed by Tukey's *post-hoc* comparisons. These analyses were chosen to

correct for multiple comparison bias and to reduce the incidence of type 1 statistical errors. Data were considered statistically different from one another when $p < 0.05$.

7.3 Results

7.3.1 RNA yield

The RNA yield from each of the tissues from the farmed SBT is shown in Fig. 7.1. Liver tissue yielded the most RNA, with a value of 1.76 μg RNA/mg tissue and this was significantly higher than for all of the other tissues. The tail-cut muscle tissue (presumed to be mostly white muscle) yielded the least RNA with a value of 0.59 μg RNA/mg tissue and this was significantly lower than both the liver and gill tissue, but not significantly different to heart. Similar results were seen for the tissues from wild-caught SBT (Fig. 7.2). The yields of RNA were in the order of liver > red muscle > white muscle however the only significant difference was between liver and white muscle.

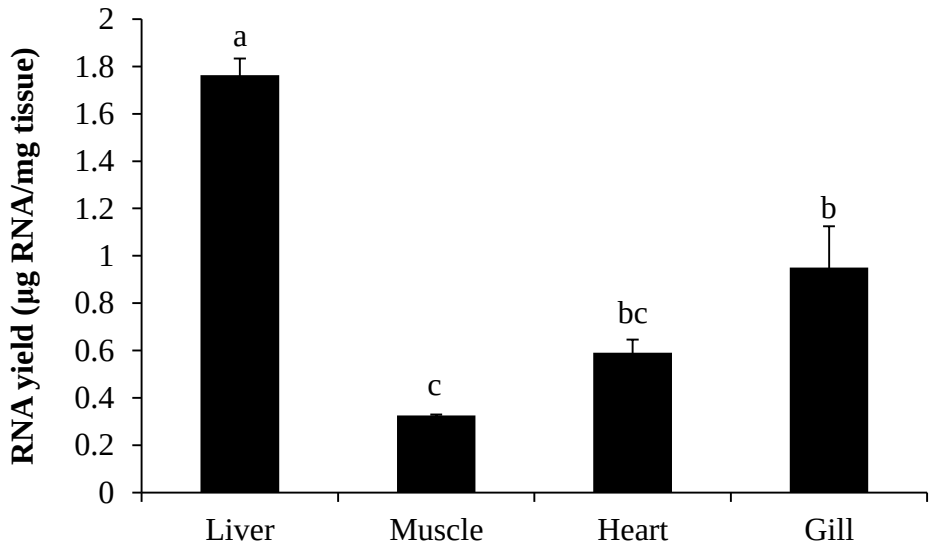

Figure 7.1: RNA yield from different tissues of farmed SBT. The muscle tissue was from the tail-cut and is presumed to be mostly white muscle. The RNA was extracted from three different fish and the data are the mean ± the standard error of the mean (n = 3). Different letters indicate significant differences between the different tissues ($p < 0.05$).

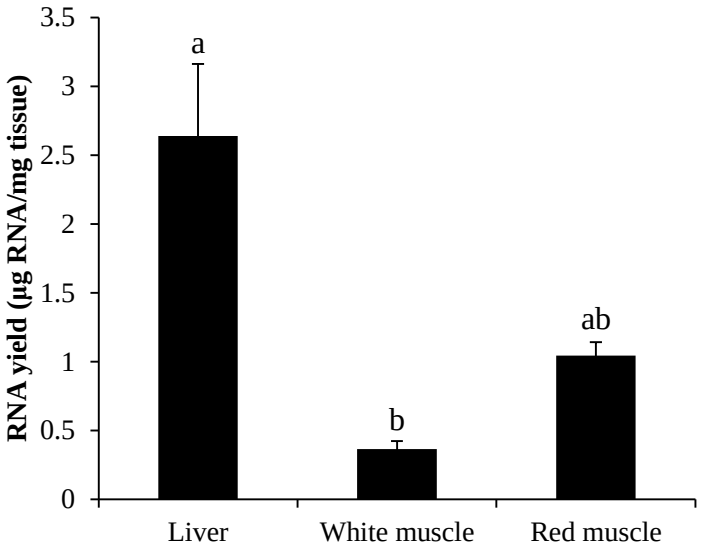

Figure 7.2: RNA yield from different tissues of wild caught SBT. The RNA was extracted from three different fish and the data are the mean ± the standard error of the mean (n = 3). Different letters indicate significant differences between the different tissues ($p < 0.05$).

7.3.2 Expression of the β-actin and ELF-1α housekeeping genes

The expression of the housekeeping genes β-actin and ELF-1α under different normalisation conditions in farmed SBT is shown in Fig. 7.3. The expression of β-actin was significantly higher in gill than any other tissues regardless of whether expression was presented per μg RNA or per mg tissue weight (Fig. 7.3A and 7.3B, respectively). The expression of ELF-1α did not differ significantly between the different tissues regardless of whether expression was presented per μg RNA or per mg tissue weight (Fig. 7.3C and 7.3D, respectively).

The expression of β-actin and ELF-1α in the tissues of wild-caught SBT is shown in Fig. 7.4. When the data were presented per μg RNA, the greatest level of expression was seen in the red muscle but this was not significantly different from either the white muscle or the liver (Fig. 7.4A). A very similar expression pattern was observed when the data was presented per mg tissue and there were no significant differences between the different tissues (Fig. 7.4B). The expression of ELF-1α in the different tissues was qualitatively more similar than that of β-actin, especially between red and white muscle (Fig. 7.4C, 7.4D). There were no significant differences in ELF-1α expression between any of the tissues regardless of whether the data were presented per μg RNA or per mg tissue (Fig. 7.4C, 7.4D, respectively).

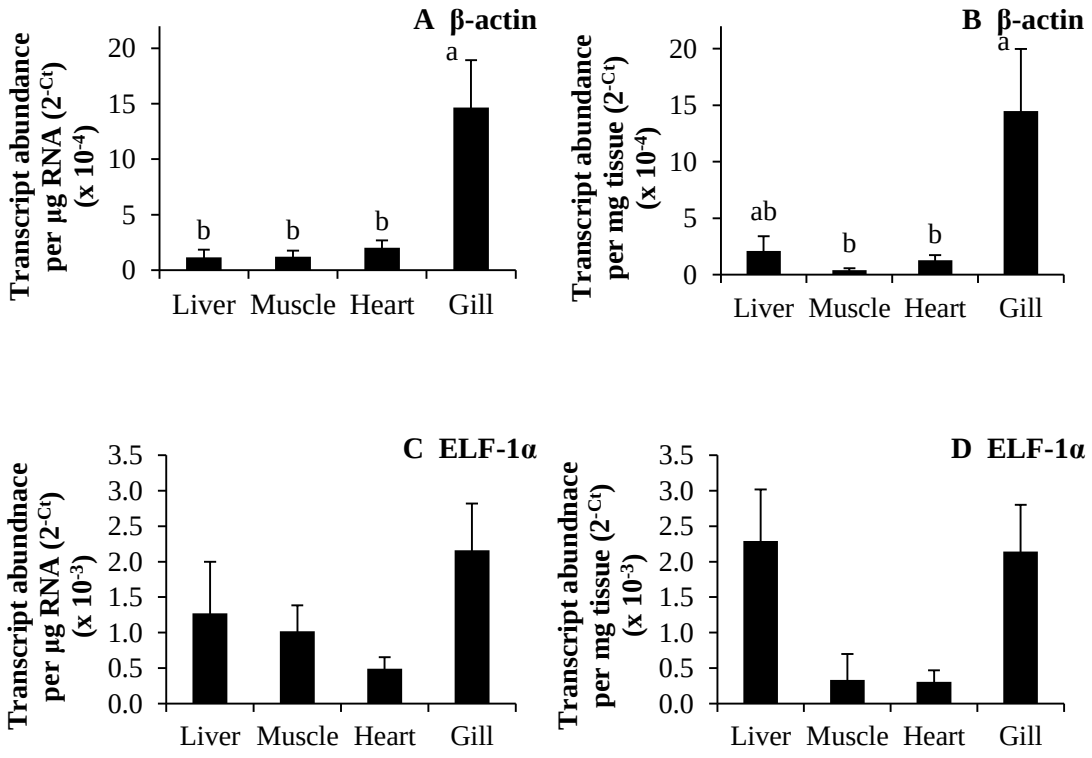

Figure 7.3: Expression of housekeeping genes in the tissues of farmed SBT. The data are presented as transcript abundance for β-actin per µg RNA (A), β-actin per mg tissue (B), ELF-1α per µg RNA (C) and ELF-1α per mg tissue (D). The RNA was extracted from three different fish and the data are the mean ± the standard error of the mean (n = 3). Within each panel, significant differences are indicated by different letters ($p < 0.05$). The transcript abundances were calculated using 2^{-Ct} where Ct is the threshold cycle number.

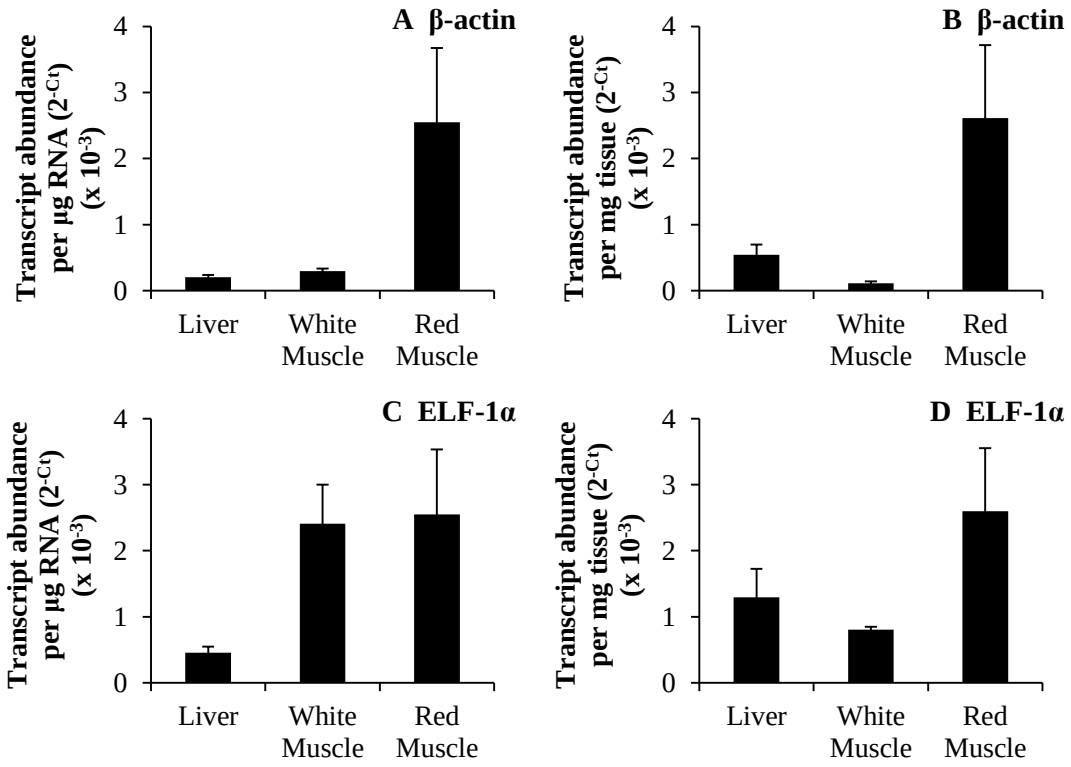

Figure 7.4: Expression of housekeeping genes in the tissues of wild-caught SBT. Data are presented as gene expression of β-actin per µg RNA (A), gene expression of β-actin per mg tissue (B), gene expression of ELF-1α per µg RNA (C) and gene expression of ELF-1α per mg tissue (D). The RNA was extracted from three different fish and the data are the mean ± the standard error of the mean (n = 3). Within each panel, significant differences are indicated by different letters ($p < 0.05$). The transcript abundances were calculated using the formula 2^{-Ct} where Ct is the threshold cycle number.

7.3.3 PGC-1α gene expression

The gene expression of PGC-1α in the tissues of farmed SBT is shown in Fig. 7.5. Prior to normalisation with the housekeeping genes, the expression of PGC-1α in gill was significantly greater than in either liver or muscle, but not heart when the data were presented per μg RNA (Fig. 7.5A). Similarly, when the data were presented per mg tissue, the expression in gill was significantly greater than in all other tissues (Fig. 7.5B). The different normalisation methods changed the relative abundances of the different transcripts but with each of the different normalisation methods, there were no significant differences in transcript abundance between the different tissues (Fig. 7.5C, 7.5D, 7.5E, 7.5F).

The gene expression of PGC-1α in the different tissues of wild-caught SBT is shown in Fig. 7.6. With the exception of the non-normalised transcript levels expressed per μg RNA, the levels of expression were all in the order of liver > red muscle > white muscle. However, there were no statistically significant differences in the data, regardless of the housekeeping gene used for normalisation or whether the data were presented per μg RNA or per mg tissue.

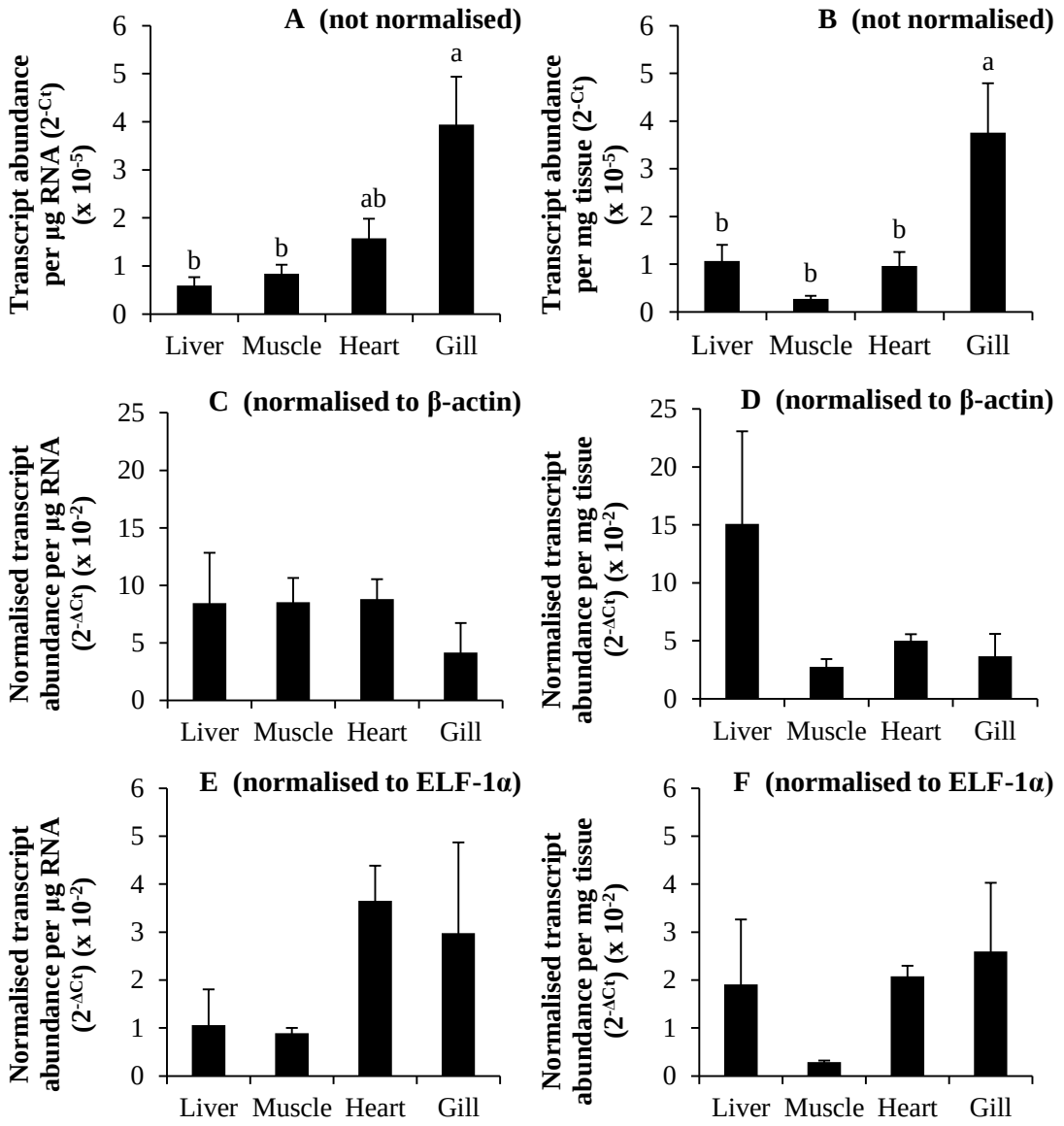

Figure 7.5: Relative abundances of PGC-1α transcripts in the different tissues of farmed SBT. Data are presented as gene expression per µg RNA (A), gene expression per mg tissue (B), gene expression per µg RNA normalised to the expression of β-actin (C) gene expression per mg tissue normalised to the expression of β-actin (D) gene expression per µg RNA normalised to the expression of ELF-1α (E) and gene expression per mg tissue normalised to the expression of ELF-1α (F). The RNA was extracted from three different fish and the data are the mean ± the standard error of the mean (n = 3). Within each panel, significant differences are indicated by different letters ($p < 0.05$). The transcript abundances were calculated using the formula 2^{-Ct} (Panels A and B) or $2^{-\Delta Ct}$ (Panels C through F) where Ct is the threshold cycle number.

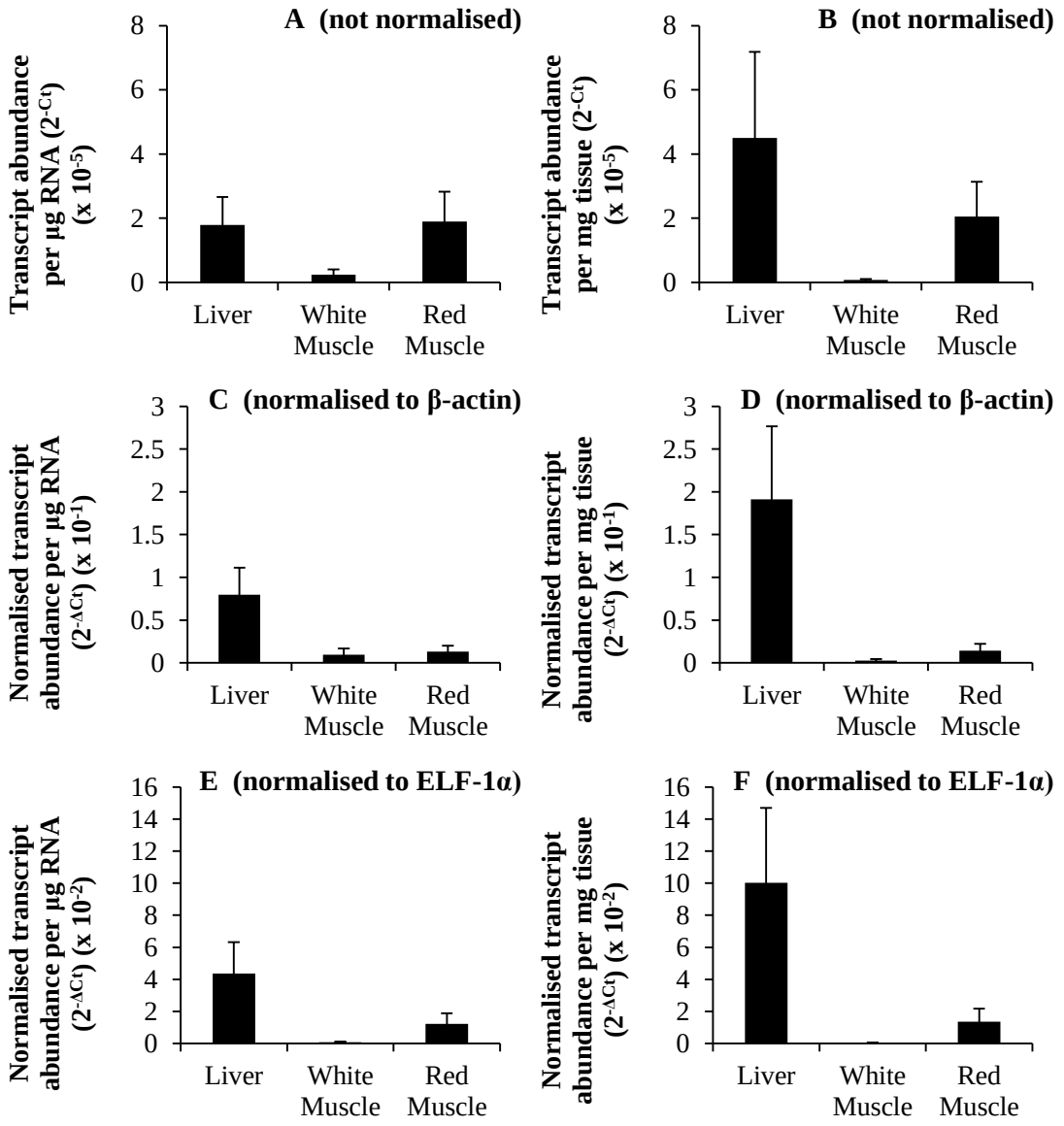

Figure 7.6: Relative abundances of PGC-1α transcripts in the different tissues of wild caught SBT. Data are presented as gene expression per µg RNA (A), gene expression per mg tissue (B), gene expression per µg RNA normalised to the expression of β-actin (C) gene expression per mg tissue normalised to the expression of β-actin (D) gene expression per µg RNA normalised to the expression of ELF-1α (E) and gene expression per mg tissue normalised to the expression of ELF-1α (F). The RNA was extracted from three different fish and the data are the mean ± the standard error of the mean (n = 3). The transcript abundances were calculated using the formula 2^{-Ct} (Panels A and B) or $2^{-\Delta Ct}$ (Panels C through F) where Ct is the threshold cycle number.

7.3.4 PPARγ gene expression

There was substantial similarity in the pattern of gene expression between PPARγ and PGC-1α in the tissues of the farmed SBT (Fig. 7.7). Prior to normalisation, PPARγ expression was significantly higher in gill tissue than other tissues, regardless of whether the data was expressed per μg RNA or per mg tissue (Fig. 7.7A, 7.7B, respectively). The only exception was in the data expressed per mg tissue. In this case, there was not a significant difference in the expression between gill and liver. When the data were normalised to the expression of β-actin and presented per μg RNA, there were no significant differences in expression between the different tissues (Fig. 7.7C). However, when the data was presented per mg tissue, there was significantly higher expression in the liver compared to all of the other tissues (Fig. 7.7D). When the expression of PPARγ was normalised to the expression of ELF-1α, there were no significant differences regardless of whether the data was presented per μg RNA or per mg tissue (Fig. 7.7E, 7.7F, respectively).

The expression of PPARγ in wild-caught SBT showed a similar trend to what was observed for PGC-1α (Fig. 7.8). The levels of expression were all in the order of liver > red muscle > white muscle, with the exception of the non-normalised transcript levels expressed per μg RNA which showed expression levels in the order of red muscle > liver > white muscle. Similar to the data for PGC-1α gene expression, these trends in gene transcript levels were not significantly different between the different tissues in any of the cases.

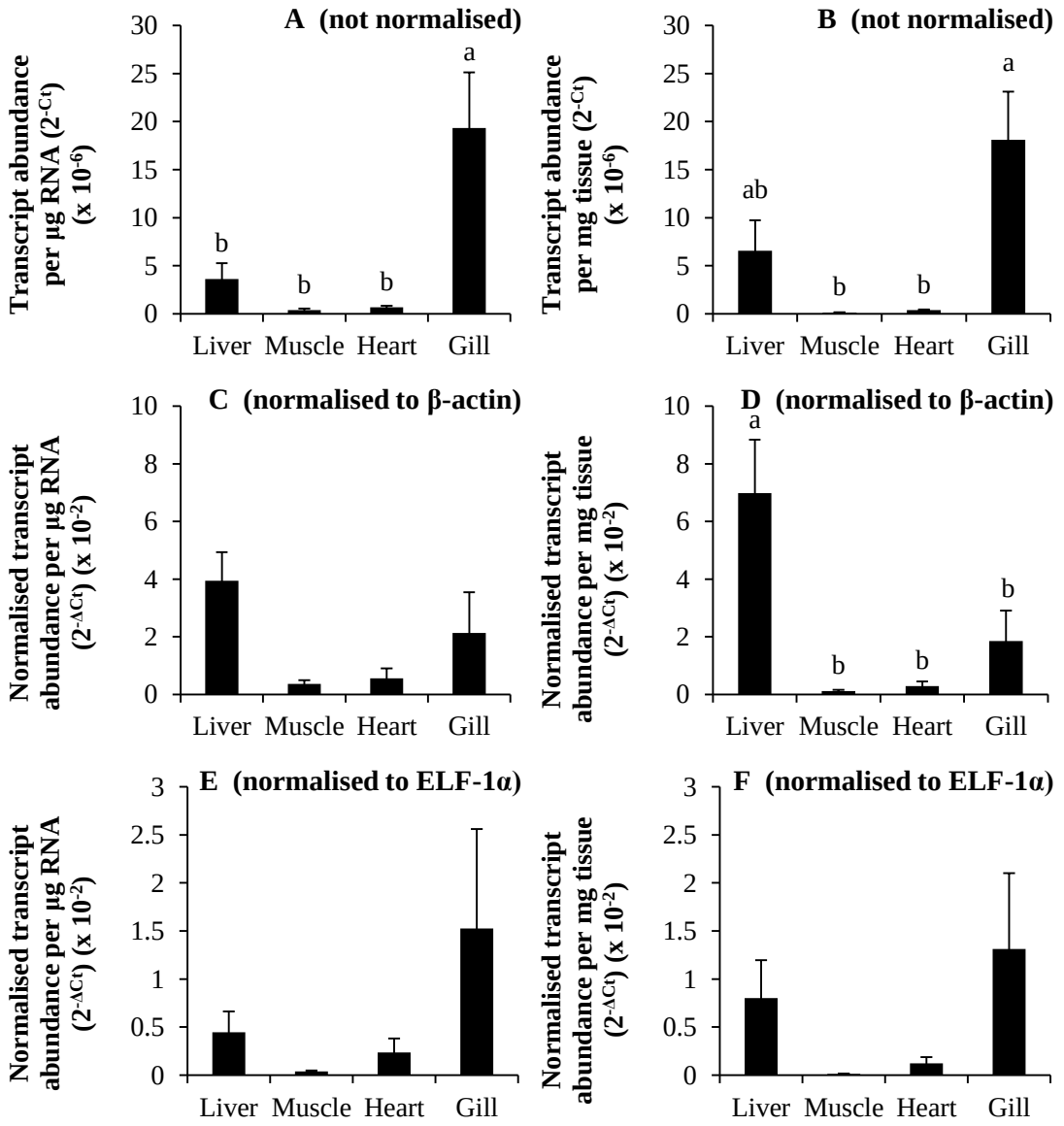

Figure 7.7: Relative abundances of PPARγ transcripts in the different tissues of farmed SBT. Data are presented as gene expression per µg RNA (A), gene expression per mg tissue (B), gene expression per µg RNA normalised to the expression of β-actin (C) gene expression per mg tissue normalised to the expression of β-actin (D) gene expression per µg RNA normalised to the expression of ELF-1α (E) and gene expression per mg tissue normalised to the expression of ELF-1α (F). The RNA was extracted from three different fish and the data are the mean ± the standard error of the mean (n = 3). Within each panel, significant differences are indicated by different letters ($p < 0.05$). The transcript abundances were calculated using the formula 2^{-Ct} (Panels A and B) or $2^{-\Delta Ct}$ (Panels C through F) where Ct is the threshold cycle number.

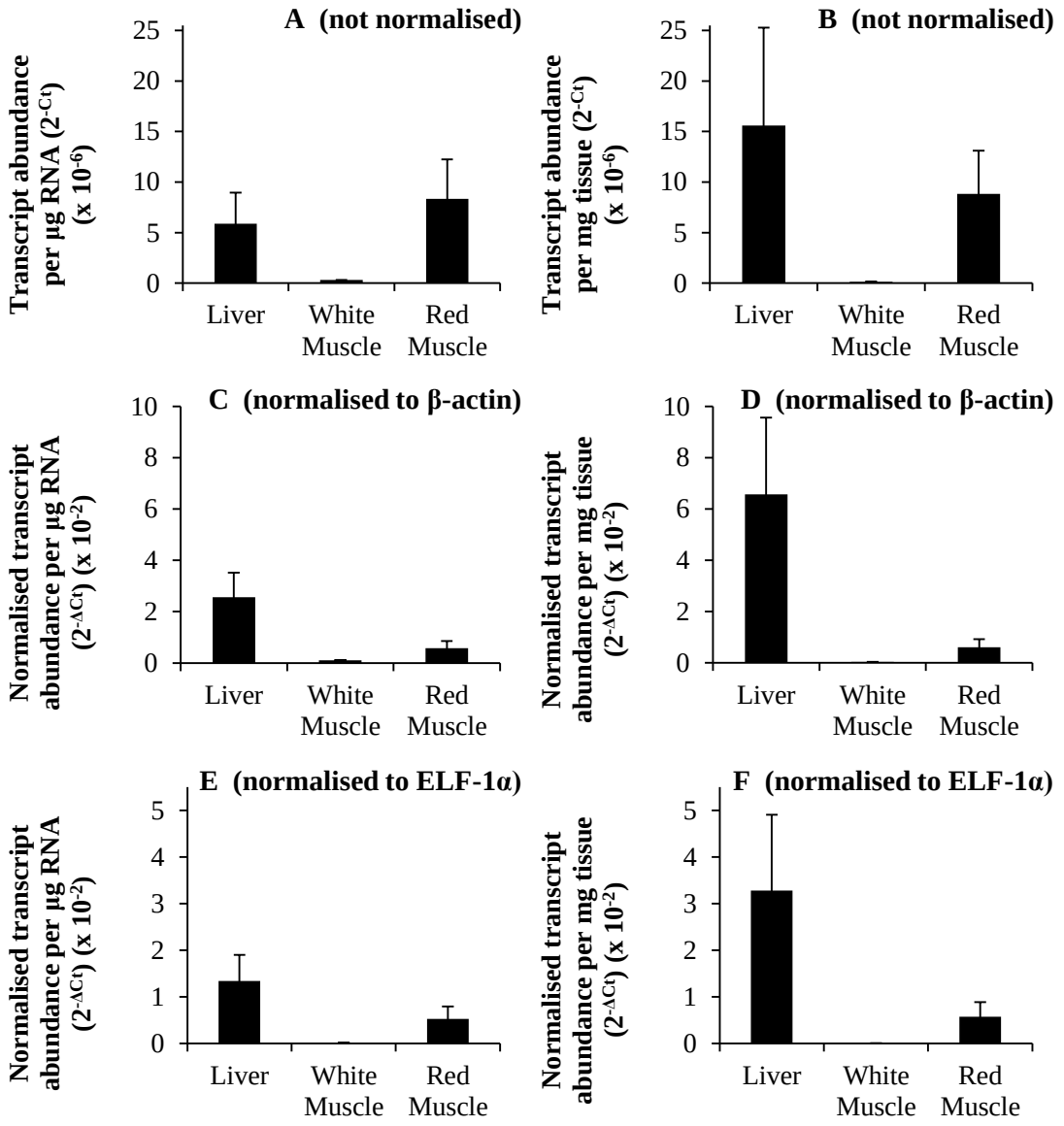

Figure 7.8: Relative abundances of PPARγ transcripts in the different tissues of wild caught SBT. Data are presented as gene expression per µg RNA (A), gene expression per mg tissue (B), gene expression per µg RNA normalised to the expression of β-actin (C) gene expression per mg tissue normalised to the expression of β-actin (D) gene expression per µg RNA normalised to the expression of ELF-1α (E) and gene expression per mg tissue normalised to the expression of ELF-1α (F). The RNA was extracted from three different fish and the data are the mean ± the standard error of the mean (n = 3). The transcript abundances were calculated using the formula 2^{-Ct} (Panels A and B) or $2^{-\Delta Ct}$ (Panels C through F) where Ct is the threshold cycle number.

7.3.5 CS gene expression

The expression of the CS gene in the tissues of the farmed SBT is shown in Fig. 7.9. When the data were not normalised to any housekeeping gene, the gene expression per μg RNA showed the greatest number of transcripts in the muscle tissue but this was not significantly different from the other tissues (Fig. 7.9A). When the gene expression was expressed per mg tissue, the expression was similar between the different tissues and there were no significant differences between the different tissues (Fig. 7.9B). After normalisation to β-actin, the expression of CS was significantly higher in muscle than all other tissues and this result was seen regardless of whether the data were presented per μg RNA or per mg tissue (Fig. 7.9C and 7.9D, respectively). Similarly, when the data were normalised to the expression of ELF-1α and expressed per μg RNA, the muscle tissue showed significantly higher levels of CS expression than all other tissues (Fig. 7.9E). Furthermore, the expression of CS was significantly higher in heart than in liver. Interestingly, when the CS gene expression was normalised to ELF-1α and expressed per mg tissue, there were no significant differences between the different tissues (Fig. 7.9F).

A similar trend was seen in the tissues of wild-caught SBT (Fig. 7.10). When CS gene expression was not normalised to any housekeeping gene and presented per μg RNA, the expression was significantly higher in white muscle than red muscle or liver (Fig. 7.10A). When the data were expressed per mg tissue, a similar trend was observed with the exception that there was no significant difference between red and white muscle (Fig. 7.10B). When the expression of CS was normalised to β-actin and expressed per μg RNA, the highest expression was seen in white muscle but there

were no significant differences between the different tissues (Fig. 7.10C). Presentation of the data per mg tissue decreased the magnitude of the difference between the different tissues but also decreased the variation between the different replicates and thus the expression per mg tissue was significantly higher in white muscle than both liver and red muscle (Fig. 7.10D). When CS gene expression was normalised to ELF-1α and expressed per μg RNA, the expression was significantly higher in white muscle than liver and red muscle (Fig. 7.10E). There were no significant differences between the different tissues when the expression was normalised to ELF-1α expression and presented per mg tissue (Fig. 7.10F).

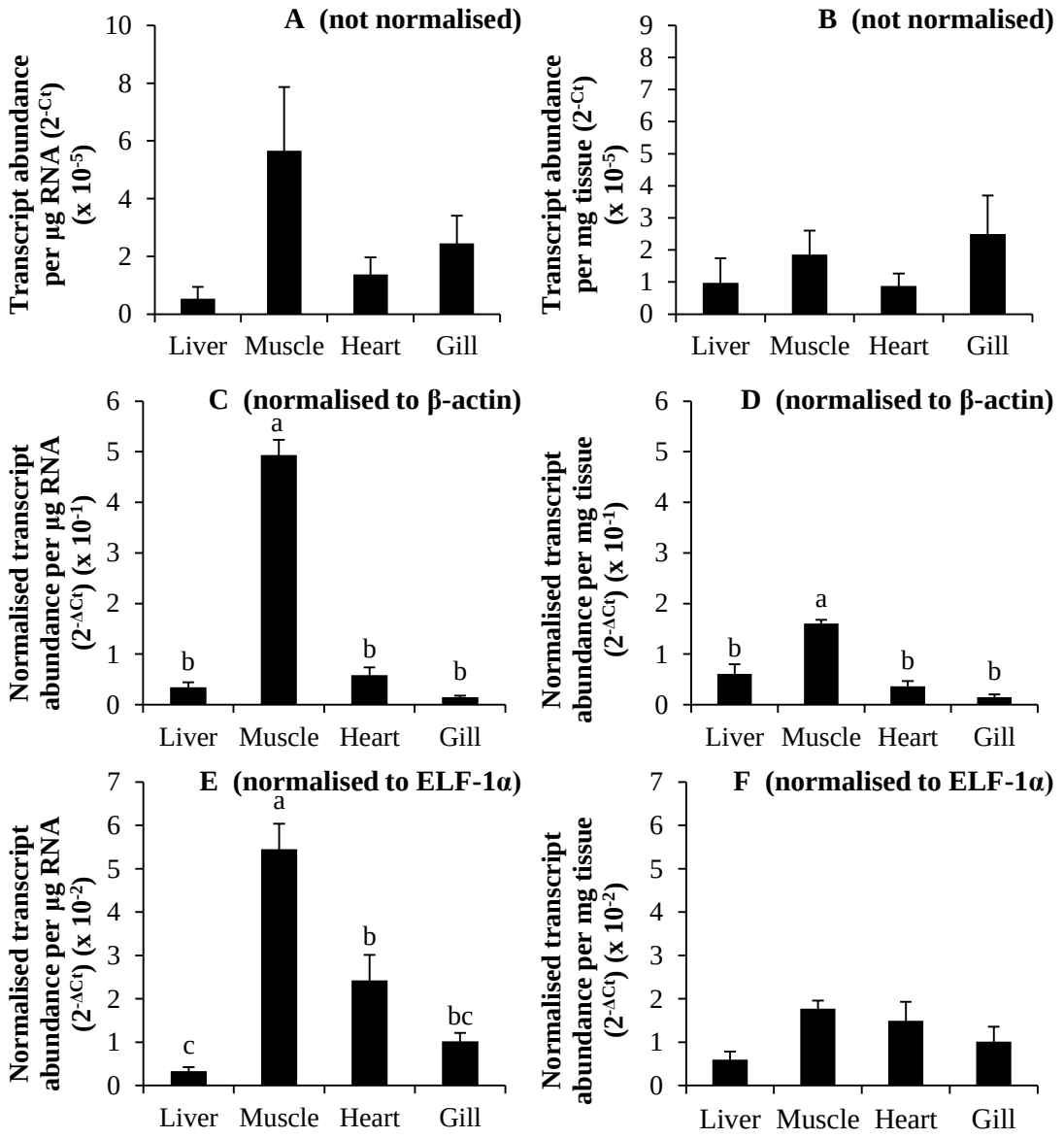

Figure 7.9: Relative abundances of CS transcripts in the different tissues of farmed SBT. Data are presented as gene expression per µg RNA (A), gene expression per mg tissue (B), gene expression per µg RNA normalised to the expression of β-actin (C) gene expression per mg tissue normalised to the expression of β-actin (D) gene expression per µg RNA normalised to the expression of ELF-1α (E) and gene expression per mg tissue normalised to the expression of ELF-1α (F). The RNA was extracted from three different fish and the data are the mean ± the standard error of the mean (n = 3). Within each panel, significant differences are indicated by different letters ($p < 0.05$). The transcript abundances were calculated using the formula 2^{-Ct} (Panels A and B) or $2^{-\Delta Ct}$ (Panels C through F) where Ct is the threshold cycle number.

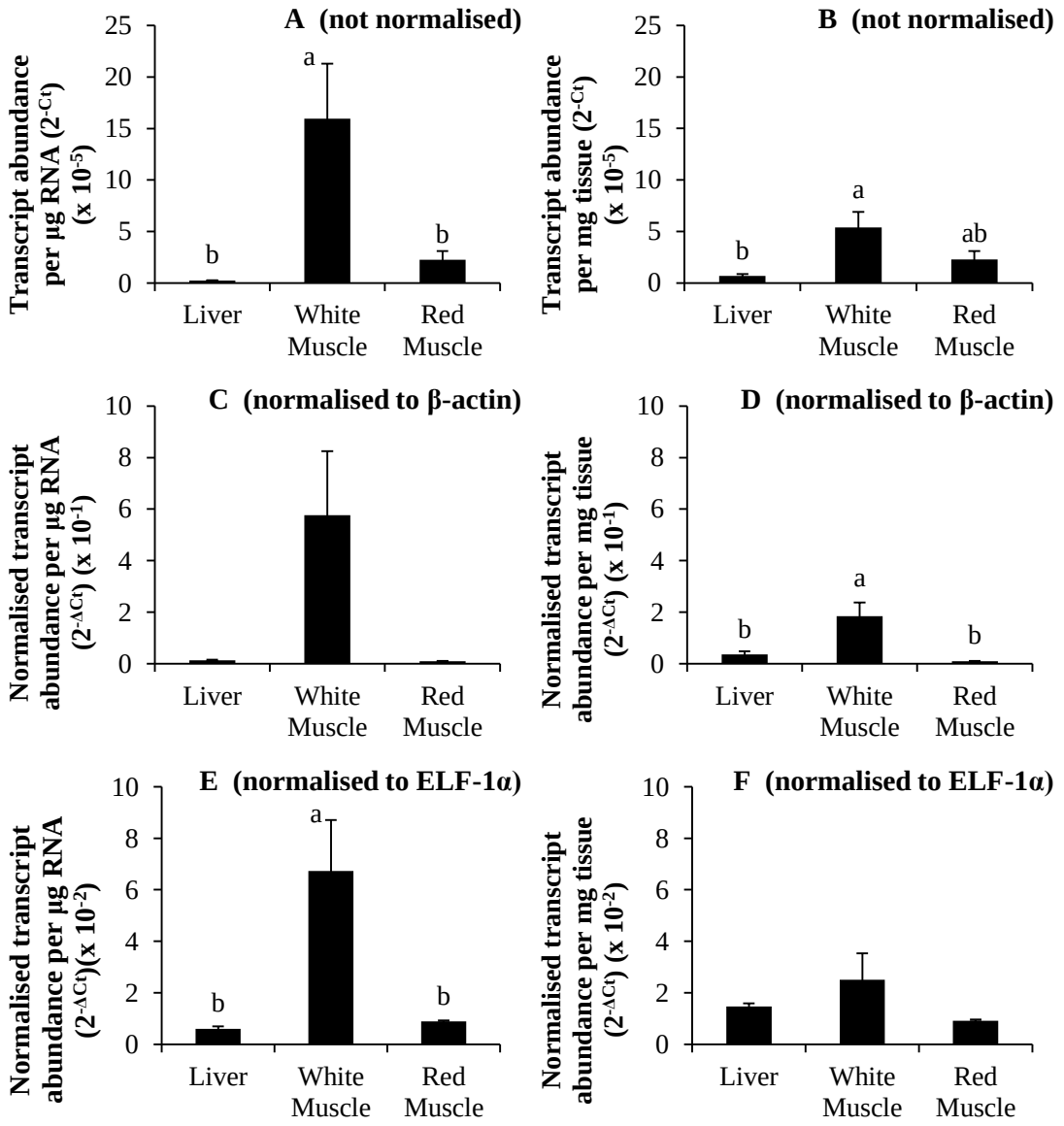

Figure 7.10: Relative abundances of CS transcripts in the different tissues of wild caught SBT. Data are presented as gene expression per µg RNA (A), gene expression per mg tissue (B), gene expression per µg RNA normalised to the expression of β-actin (C) gene expression per mg tissue normalised to the expression of β-actin (D) gene expression per µg RNA normalised to the expression of ELF-1α (E) and gene expression per mg tissue normalised to the expression of ELF-1α (F). The RNA was extracted from three different fish and the data are the mean ± the standard error of the mean (n = 3). Within each panel, significant differences are indicated by different letters ($p < 0.05$). The transcript abundances were calculated using the formula 2^{-Ct} (Panels A and B) or $2^{-\Delta Ct}$ (Panels C through F) where Ct is the threshold cycle number.

7.3.6 COX1 gene expression

The expression of the COX1 gene in the tissues of the farmed SBT is shown in Fig. 7.11. When the data were expressed per μg of RNA, there was a significantly higher abundance of COX1 transcripts in the heart when compared to liver and gill, but not compared to muscle (Fig. 7.11A). The presentation of the data as gene expression per mg tissue inflated the relative abundance of transcripts in the liver and deflated the relative abundance of transcripts in muscle (Fig. 7.11B). The heart tissue showed the highest COX1 gene expression and this was significantly higher than in both muscle and gill. The normalisation of COX1 gene expression to the gene expression of the different housekeeping genes changed the relative abundances of the different transcripts and resulted in no significant differences in transcript abundance between the different tissues, regardless of whether the data were expressed per μg RNA or per mg tissue (Figs. 7.11C through F).

The expression of the COX1 gene in the different tissues of wild-caught SBT is shown in Fig. 7.12. Prior to normalisation with the housekeeping genes and when presented per μg RNA, COX1 transcript abundance was significantly greater in white muscle than liver or red muscle (Fig. 7.12A). However, when COX1 expression was calculated per mg tissue, the relative abundance of the transcript in the liver was substantially inflated and the relative abundance in white muscle was substantially deflated. Thus, when the data were presented per mg tissue, liver had the highest level of COX1 expression and this was significantly higher than expression in red muscle but not significantly different to white muscle (Fig. 7.12B). Red muscle and white muscle expression did not differ significantly from one another. When normalised to β-actin, and calculated per μg RNA, COX1 gene expression in white

muscle was significantly higher than in red muscle but was not significantly different to the expression in liver (Fig. 7.12C). However, red and white muscle expression levels were not significantly different from one another when the data were presented per mg tissue (Fig. 7.12D). There were no significant differences in gene expression between any tissues when COX1 gene expression was normalised to the expression of ELF-1α and the data were expressed per μg RNA (Fig. 7.12E). However, when the data were normalised to ELF-1α expression and expressed per mg tissue, the level of expression was significantly higher in liver when compared to both red and white muscle (Fig. 7.12F). Red and white muscle expression levels did not differ significantly from one another.

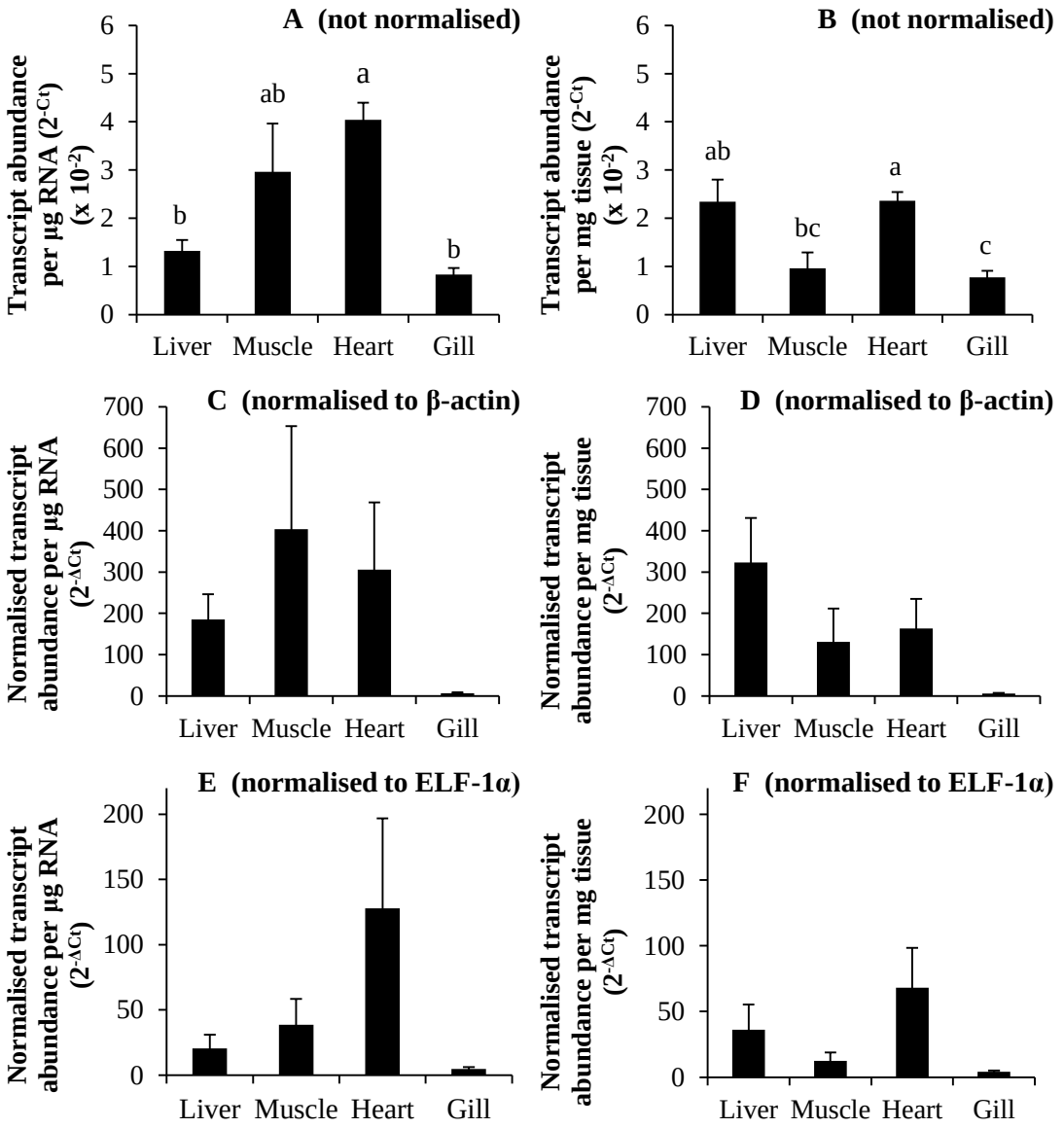

Figure 7.11: Relative abundances of COX1 transcripts in the different tissues of farmed SBT. Data are presented as gene expression per µg RNA (A), gene expression per mg tissue (B), gene expression per µg RNA normalised to the expression of β-actin (C) gene expression per mg tissue normalised to the expression of β-actin (D) gene expression per µg RNA normalised to the expression of ELF-1α (E) and gene expression per mg tissue normalised to the expression of ELF-1α (F). The RNA was extracted from three different fish and the data are the mean ± the standard error of the mean (n = 3). Within each panel, significant differences are indicated by different letters ($p < 0.05$). The transcript abundances were calculated using the formula 2^{-Ct} (Panels A and B) or $2^{-\Delta Ct}$ (Panels C through F) where Ct is the threshold cycle number.

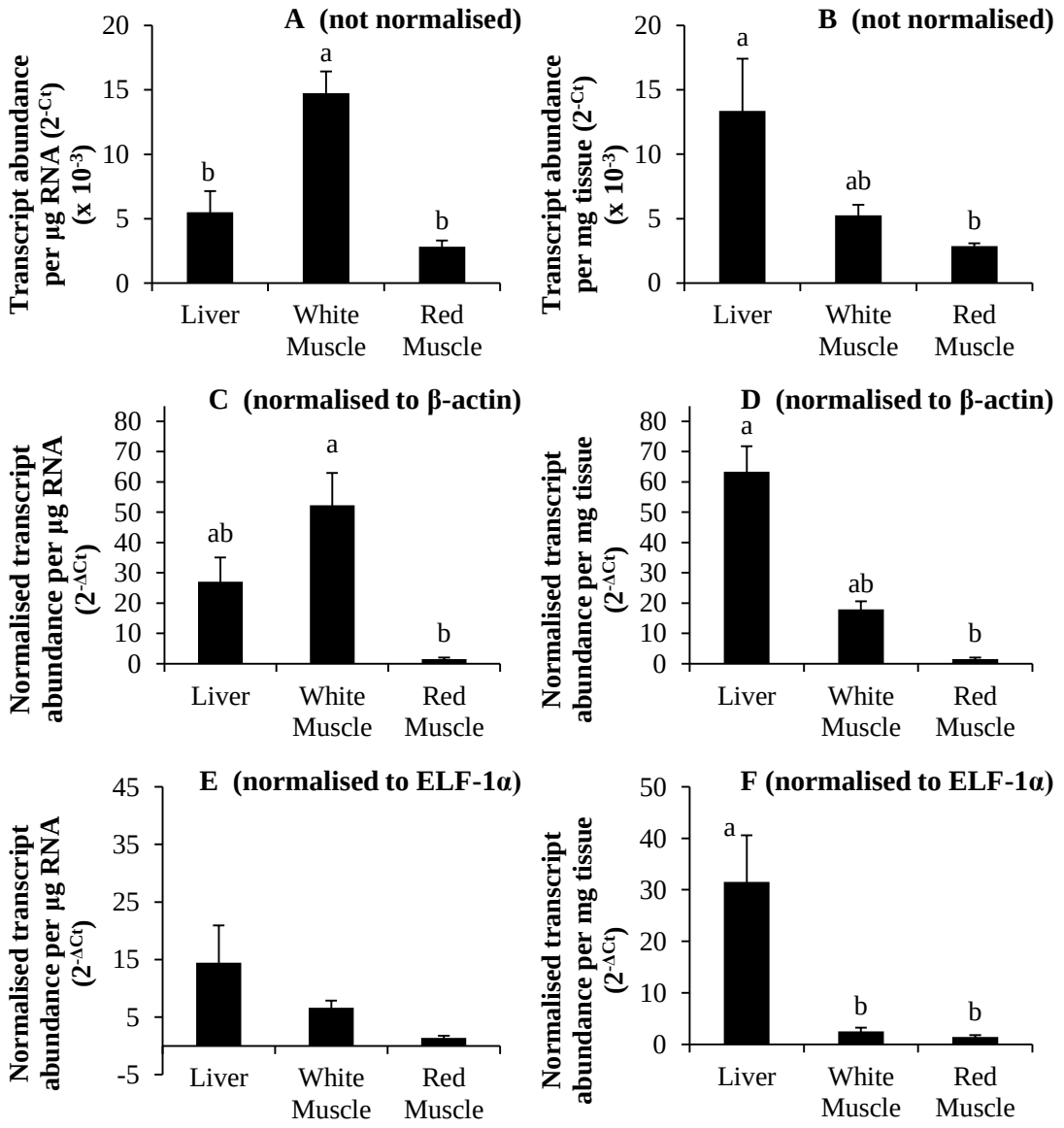

Figure 7.12: Relative abundances of COX1 transcripts in the different tissues of wild caught SBT. Data are presented as gene expression per µg RNA (A), gene expression per mg tissue (B), gene expression per µg RNA normalised to the expression of β-actin (C) gene expression per mg tissue normalised to the expression of β-actin (D) gene expression per µg RNA normalised to the expression of ELF-1α (E) and gene expression per mg tissue normalised to the expression of ELF-1α (F). The RNA was extracted from three different fish and the data are the mean ± the standard error of the mean (n = 3). Within each panel, significant differences are indicated by different letters ($p < 0.05$). The transcript abundances were calculated using the formula 2^{-Ct} (Panels A and B) or $2^{-\Delta Ct}$ (Panels C through F) where Ct is the threshold cycle number.

7.3.7 UCP2 gene expression

The expression of the UCP2 gene in the tissues from farmed SBT is shown in Fig. 7.13. Prior to normalisation with the housekeeping genes, there were no significant differences in transcript abundance between the different tissues regardless of whether the data were presented per μg RNA or per mg tissue (Fig. 7.13A, 7.13B, respectively). When normalised to the expression of β-actin and presented per μg RNA, there was significantly greater expression of the UCP2 gene in muscle than all other tissues (Fig. 7.13C). The same result was seen when the data were presented per mg tissue but the magnitude of the difference was reduced (Fig. 7.13D). When UCP2 gene expression was normalised to ELF-1α gene expression and presented per μg RNA, the muscle expression was significantly higher than the liver expression, but not significantly different to heart and gill expression (Fig. 7.13E). However, when the data were normalised to ELF-1α expression and presented per mg tissue, there were no significant differences in gene expression between the different tissues (Fig. 7.13F).

The gene expression of UCP2 in the tissues of wild-caught SBT is shown in Fig. 7.14. When UCP2 expression was not normalised to any housekeeping genes and was expressed per μg RNA, there were significantly higher levels of transcript in white muscle compared to red muscle and liver (Fig. 7.14A). When the data were expressed per mg tissue, there were no significant differences between the different tissues (Fig. 7.14B). When the expression data were normalised to β-actin expression and expressed per μg RNA, the UCP2 transcript was significantly more abundant in white muscle than either red muscle or liver (Fig. 7.14C). A similar trend was seen when the data were expressed per mg tissue but the only significant difference was

that expression in white muscle was significantly higher than in red muscle (Fig. 7.14D). The UCP2 gene expression normalised to ELF-1α and presented as expression per μg RNA showed a similar trend with the highest level of transcripts in the white muscle, but this was not statistically different from the level of transcript in liver or red muscle (Fig. 7.14E). There were no significant differences between the different tissue types when the data were normalised to ELF-1α and presented per mg tissue (Fig. 7.14F).

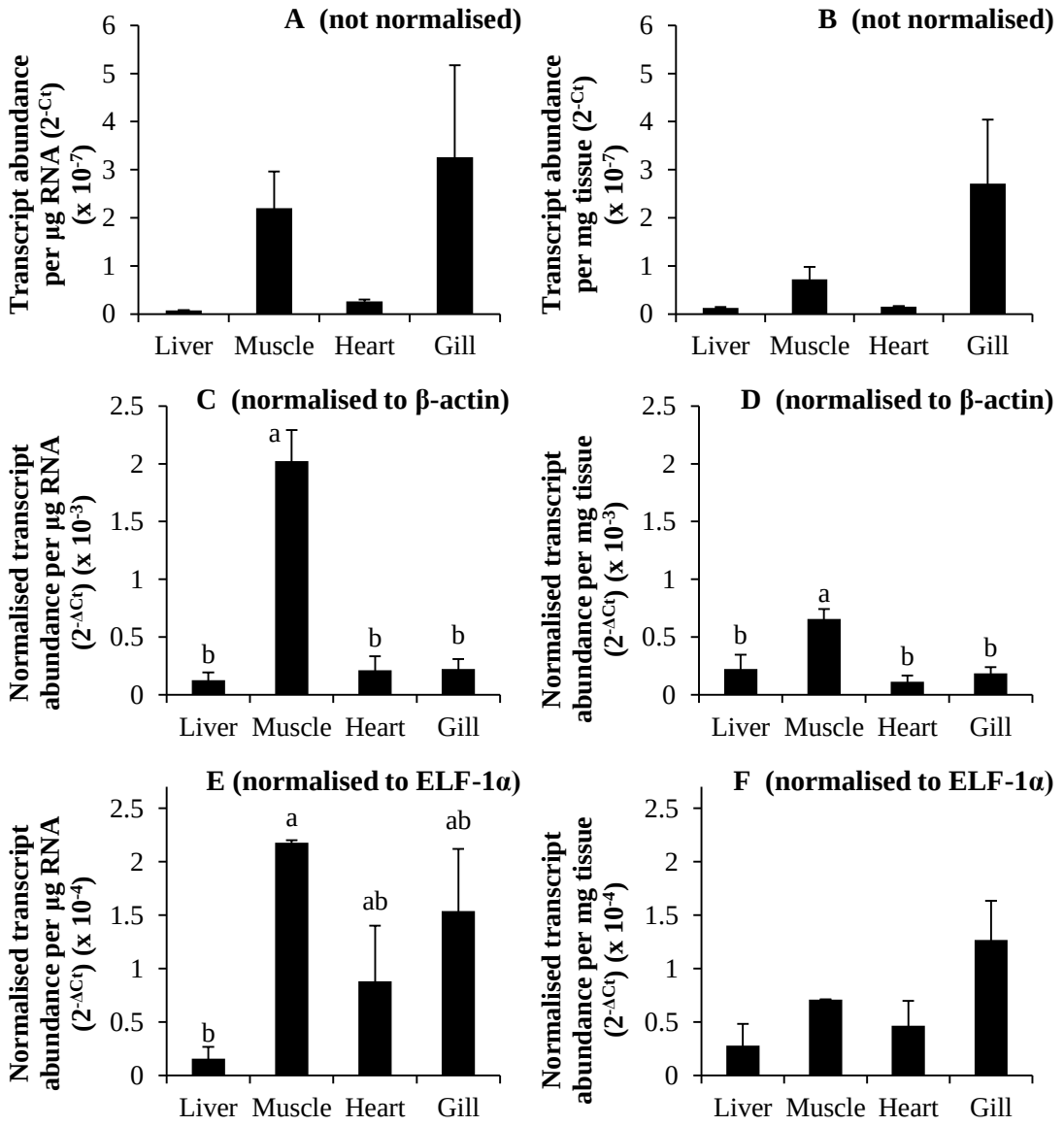

Figure 7.13: Relative abundances of UCP2 transcripts in the different tissues of farmed SBT. Data are presented as gene expression per µg RNA (A), gene expression per mg tissue (B), gene expression per µg RNA normalised to the expression of β-actin (C) gene expression per mg tissue normalised to the expression of β-actin (D) gene expression per µg RNA normalised to the expression of ELF-1α (E) and gene expression per mg tissue normalised to the expression of ELF-1α (F). The RNA was extracted from three different fish and the data are the mean ± the standard error of the mean (n = 3). Within each panel, significant differences are indicated by different letters ($p < 0.05$). The transcript abundances were calculated using the formula 2^{-Ct} (Panels A and B) or $2^{-\Delta Ct}$ (Panels C through F) where Ct is the threshold cycle number.

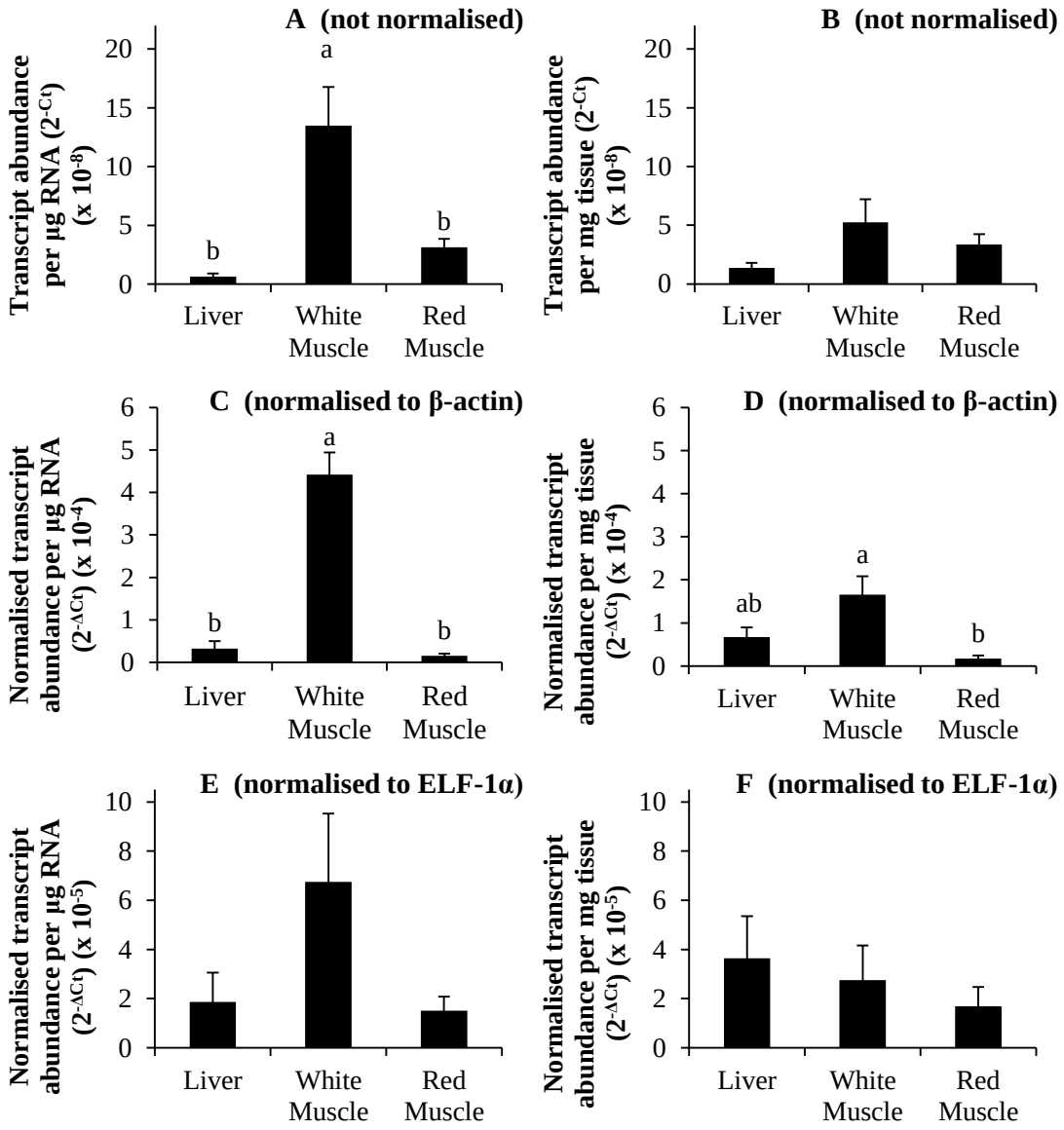

Figure 7.14: Relative abundances of UCP2 transcripts in the different tissues of wild caught SBT. Data are presented as gene expression per µg RNA (A), gene expression per mg tissue (B), gene expression per µg RNA normalised to the expression of β-actin (C) gene expression per mg tissue normalised to the expression of β-actin (D) gene expression per µg RNA normalised to the expression of ELF-1α (E) and gene expression per mg tissue normalised to the expression of ELF-1α (F). The RNA was extracted from three different fish and the data are the mean ± the standard error of the mean (n = 3). Within each panel, significant differences are indicated by different letters ($p < 0.05$). The transcript abundances were calculated using the formula 2^{-Ct} (Panels A and B) or $2^{-\Delta Ct}$ (Panels C though F) where Ct is the threshold cycle number.

7.4 Discussion

7.4.1 RNA yield

There are often substantial differences in the amount of RNA obtained from different tissue types (Dalziel *et al.* 2005). For example, liver tissue typically yields high amounts of RNA due to the abundance of RNA in this tissue and the ease of cellular homogenisation and RNA extraction (Chomczynski and Mackey 1995, Gayral *et al.* 2011). In contrast, muscle tissue has more fibrous cells, which are lower in RNA concentration and can be more difficult to homogenise and therefore typically yields lower amounts of RNA (Chomczynski and Mackey 1995, Gayral *et al.* 2011). The RNA content from the different tissues from farmed SBT followed expected trends with tissue types such as liver, yielding significantly more RNA than white muscle.

7.4.2 Expression of the β-actin and ELF-1α housekeeping genes

Radonić *et al.* (2004) proposed that many of the common genes used for normalisation of qRT-PCR data were not adequate when comparing gene expression in different tissue types. It was proposed that the RNA polymerase II (RPII) gene was the most suitable candidate for comparing between tissues, or, if this gene was not able to be used, that multiple reference genes should be used (Radonić *et al.* 2004). It was not possible to utilise RPII due to a lack of sequence data for fish species, so as an alternative, both β-actin and ELF-1α were used as normalisation genes. The results presented here show that expression of β-actin was as much as 15-fold higher in gill tissue than other tissues and thus all normalisation of results to β-actin expression should be interpreted in consideration of this imbalance in expression. The expression of ELF-1α was much more consistent between the different tissues and is

therefore a more useful candidate for normalising the expression data for the various target genes presented here.

7.4.3 PGC-1α gene expression

The results presented here show that there were high levels of PGC-1α transcript detected in the gill compared to other tissues when the data were not normalised to housekeeping genes. The normalisation of the data to either β-actin or ELF-1α eliminated these differences. If PGC-1α gene expression is, indeed high in the gill tissue, then it could be indicative of high numbers of mitochondria in the gills of SBT. An alternative explanation may be that there are changes in PGC-1α gene expression in the gills because this tissue is exposed to high levels of oxygen. Tissues exposed to high levels of oxygen can be susceptible to oxidative attack by reactive oxygen species (ROS) (Gutteridge and Halliwell 1990, Gutteridge 1995). Nearly all higher organisms have evolved antioxidant enzymes as a mechanism to detoxify ROS and reduce their impact on normal tissue function (Gutteridge 1995). Over-expression of PGC-1α in C2C12 mouse myoblast cells, which had been differentiated to form myotubes, was shown to increase the endogenous gene expression of the antioxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase 1 (GPx1) (St-Pierre *et al.* 2003). As a result, it has been proposed that PGC-1α can modulate ROS metabolism (Lin *et al.* 2005). Therefore, it may be expected that fish tissues such gill, which facilitate oxygen transfer between water and the blood stream, would show high expression of the PGC-1α gene in order to counter the deleterious effects of ROS. Further investigation is needed to define if there is an important role for PGC-1α in SBT gill tissue.

During the course of this project, a review was published that demonstrated that at least seven different isoforms of PGC-1α are present in mammals and that they have distinct roles in

regulating gene expression in muscle (Fig. 7.15, Chan and Arany 2014). Ruas *et al.* (2012) described a truncated form of PGC-1α, termed PGC-1α4 and transgenic mice expressing PGC-1α4 showed increased insulin-like growth factor 1 gene expression and hypertrophy in white muscle and this was accompanied by an increase in muscular strength and endurance. However, the most recognised role of PGC-1α in mammals is stimulating mitochondrial biogenesis (Moyes 2003, Liu and Lin 2011, Chan and Arany 2014). The primers used in this study amplify a region in the SBT PGC-1α gene that is present in all isoforms identified by Chan and Arany (2014) with the exception of PGC-1α4 and a similar small isoform, termed NT-PGC-1α (Zhang *et al.* 2009). Thus it is expected that PGC-1α gene expression would be high in tissues that have a high abundance of mitochondria, such as red muscle. The expression of PGC-1α in heart has been reported to be high in both mammals and fish (Puigserver *et al.* 1998, LeMoine *et al.* 2008), which is consistent with the fact that the heart muscle requires a large number of mitochondria. This is because the heart is constantly β-oxidising fatty acids to fuel repeated beating (Moyes *et al.* 1992, Graham and Dickson 2004). The results presented here are consistent with the previous literature showing that when gene expression data were normalised to ELF-1α, the heart showed substantial expression of the PGC-1α transcript. The expression of PGC-1α in the red muscle of wild-caught SBT was not significantly different than what was seen in white muscle or liver. This was unexpected based on previous findings that in other closely related tuna species the activity of CS and COX1 as indicators of mitochondrial abundance were 6-10 fold-higher in red muscle than white muscle (Dalziel *et al.* 2005). However, there appeared to be a trend towards higher expression in red muscle compared to white muscle. The inclusion of a greater number of fish in future analyses may increase the statistical power to a sufficient level to detect differences between PGC-1α expression in red and white muscle.

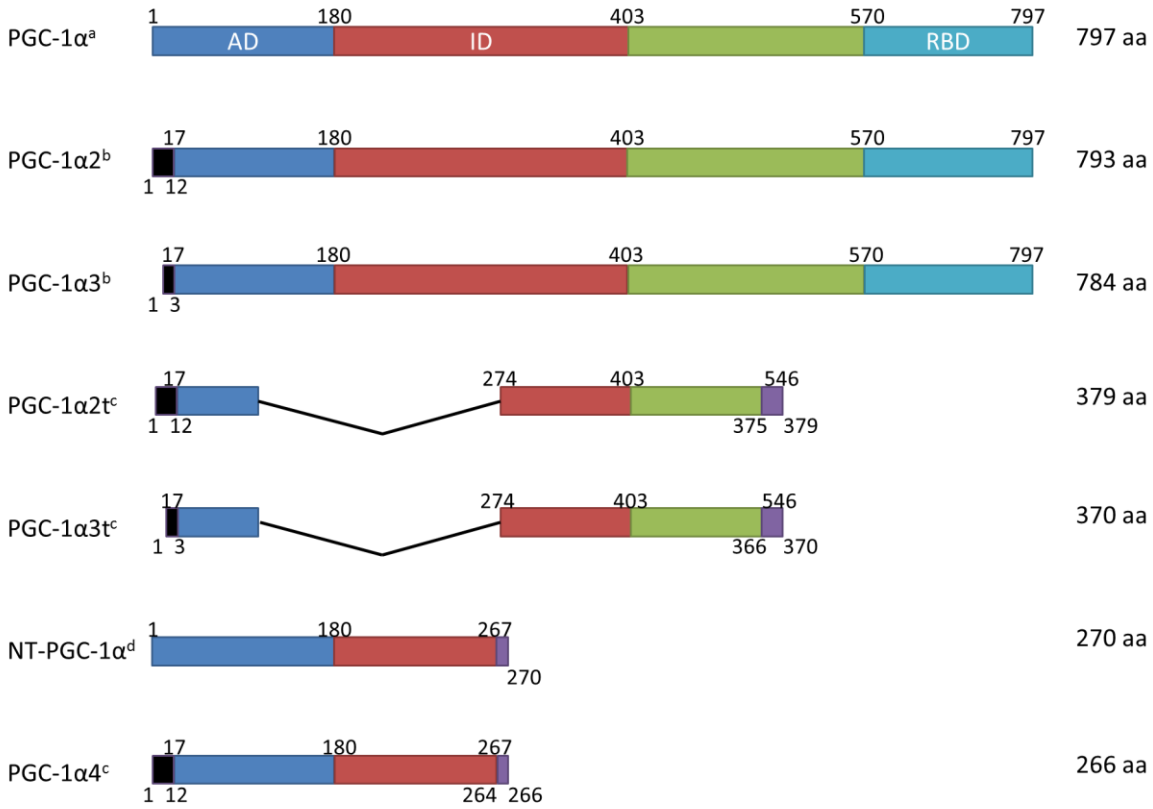

Figure 7.15: The various isoforms of PGC-1α that have been defined in mammals. AD: Activation domain. ID: Inhibitory domain. RBD: RNA binding domain. The sections in black at the N-terminal end are the result of the use of an upstream transcription start site and the use of an alternative first exon. [a]Originally described by Puigserver *et al.* (1998) and accepted as the canonical PGC-1α. [b]Described by Miura *et al.* (2008) and Chinsomboon *et al.* (2009). [c]First described by Ruas *et al.* (2012). [d]First described by Zhang *et al*. (2009). Figure adapted from Fig. 2 in Chan and Arany (2014).

7.4.4 PPARγ gene expression

One of the transcription factors that is co-activated by PGC-1α is PPARγ and the results presented here show a strong correlation in the gene expression of PPARγ and PGC-1α in SBT. PPARγ is a transcription factor that promotes adipogenesis and stimulates the transport of free fatty acids to adipocytes to form triglycerides (Puigserver *et al.* 1998, Guan *et al.* 2005, Agawa *et al.* 2012). Studies in goldfish and zebrafish have found that the gene expression of PGC-1α did not always correlate well with the gene expression of the PGC-1α targets that have been described in mammals, including members of the PPAR family (LeMoine *et al.* 2008, LeMoine *et al.* 2010a). For example, after 4 weeks of exercise training in zebrafish, PGC-1α gene expression was significantly increased in both red and white muscle, but the expression of the medium chain acyl-CoA dehydrogenase (MCAD) gene, which is a PPARα target involved in fatty acid β-oxidation, was unchanged (LeMoine *et al.* 2010a). In contrast, LeMoine *et al.* (2008) found that in goldfish MCAD gene expression correlated well with PGC-1α gene expression in white muscle, but not in red muscle, heart or liver in response to warm or cold acclimation. PGC-1α gene expression was found to correlate poorly with PPARα, CS and COX1 gene expression in white muscle, heart and liver (LeMoine *et al.* 2008). PGC-1α gene expression correlated with PPARα, but not CS or COX1 gene expression in red muscle (LeMoine *et al.* 2008). The results from these studies suggested that PGC-1α may have a different function in fish than in mammals. The results presented here showed a strong correlation between PPARγ and PGC-1α gene expression. Since PPARγ promotes adipogenesis and triacylglyceride synthesis (Schoonjans *et al.* 1996, Guan *et al.* 2005), this result may suggest that PGC-1α is involved in fatty acid anabolism in SBT more so than fatty acid catabolism, which is its dominant effect in mammals. Other members of the PPAR family that are involved in catabolic pathways, such as PPARα, which is involved in fatty acid β-oxidation (Baldán *et al.* 2004), may be investigated in the future to

define if PGC-1α also stimulates fatty acid catabolism. This research is important as fatty acid and lipid catabolism fuels the high respiratory output in SBT red muscle tissue. This, in turn may contribute to the capacity for endothermy in SBT.

7.4.5 CS and COX1 gene expression

Previous work has shown that in skipjack tuna (*Katsuwonus pelamis*), bigeye tuna (*Thunnus obesus*) and yellowfin tuna (*Thunnus albacares*), the enzyme activities of CS and COX were 6-10 fold higher in red muscle than white muscle (Dalziel *et al.* 2005). This is likely to be because the CS and COX enzymes are located in the mitochondria and one of the defining differences between the red muscle and white muscle is that red muscle contain an abundance of mitochondria (Wiegand and Remington 1986, Moyes *et al.* 1992, Khalimonchuk and Rödel 2005). This in turn results in substantially higher respiratory capacity in red muscle compared to white muscle (Moyes *et al.* 1992, Dickson and Graham 2004). To assess the abundance and activity of mitochondria, the nuclear-encoded CS gene and the mitochondrially-encoded COX1 gene were chosen. As indicated above, many studies which have used CS and COX1 as indicators of mitochondrial abundance and capacity, respectively, have measured enzyme activity instead of gene expression (e.g. Moyes *et al.* 1992, Dickson and Graham 2004, Dalziel *et al.* 2005). Gene expression analysis was chosen in this study to investigate (a) if gene expression for CS and COX1 correlated with the previously reported CS and COX enzyme activity for three other closely related tuna species (Dalziel *et al.* 2005), (b) to investigate if there was any evidence for greater numbers of mitochondria in red muscle compared with white muscle, as indicated by CS and COX1 gene expression and (c) to assess whether the level of expression of PGC-1α and/or PPARγ correlated with the abundance of CS, COX1 and UCP2 gene transcripts. The results presented here did not show the expected 6-10 fold higher gene expression of CS and COX1 in the red muscle compared

to white, as was expected from the previously reported enzyme activity data (Dalziel *et al.* 2005). In fact, the results presented here indicate that both CS and COX1 gene expression were actually greater in white muscle than red muscle. Previous studies have suggested, however, that in fish, gene expression of CS and COX1 may not correlate with enzyme activity. For example, LeMoine *et al.* (2008) found that CS mRNA abundance increased 3-fold in red muscle and 6-fold in liver but CS activity decreased by 50% in red muscle and was unchanged in liver in goldfish fed a high fat diet. This lack of correlation between gene expression and enzyme activity may explain the unexpected results found in our study. The lack of correlation between the gene expression data described here and the enzyme activity data found in previous studies (Dalziel *et al.* 2005, LeMoine *et al.* 2008), suggests that there may be strong post-transcriptional regulation of both CS and COX1 in SBT. Indeed, post-transcriptional limitation of CS expression has been previously shown during cold acclimation in eelpout (*Zoarces viviparous*), using ribosomal RNA as an indicator of translational capacity (Hardewig *et al.* 1999). The unexpected finding that the CS and COX1 genes were expressed to a greater level in white muscle than red muscle necessitates further research to define if the gene expression correlates to enzyme activity in SBT.

7.4.6 UCP2 gene expression

The overall abundance of the UCP2 transcript was extremely low compared to all other genes investigated here and was close to the limits of detection. Low levels of UCP2 gene expression have also been reported in the liver, red muscle, white muscle and heart of Atlantic Salmon (Torstensen *et al.* 2009). These low levels of expression and the fact that expression was extremely low in the endothermic red muscle suggest that UCP2 is unlikely to play a role in thermogenesis and the development of endothermy in SBT. Low expression of UCP2 has been associated with hypoxia and this could explain the low levels of UCP2 seen

here, especially in the farmed fish (Bermejo-Nogales *et al.* 2014). Tuna have an extremely high requirement for oxygen due to their impressive metabolic rate and have evolved adaptations in order to take up as much oxygen as possible from the water (Brill 1996, Brill and Bushnell 2001). These include ram ventilation and high gill filament surface area to facilitate increased gas exchange (Brill 1996, Brill and Bushnell 2001). Thus it is likely that tuna respond to changes in oxygen availability more readily than other fish species. Intensive farming is known to create a hypoxic environment due to a large number of fish in a relatively small area and this is further exacerbated by the fact that sampling for the tissues was conducted during a commercial SBT harvest. This is because the process of collecting SBT for harvest involves 'crowding' of the fish into a small area of the cage for capture, which further reduces available oxygen (Poli *et al.* 2005, Oppedal *et al.* 2011). The presence of a hypoxic environment in culture does not, however, explain the low levels of UCP2 transcript in the wild-caught SBT. An alternative explanation is that UCP2 is chronically expressed at low levels in these tissues as a physiological adaptation of SBT to cope with hypoxic environments that are regularly encountered during normal swimming and foraging. Tunas are known to dive as far as 1000 m in search of prey (Graham and Dickson 2004, Patterson *et al.* 2008) and the water at these depths contains extremely low oxygen levels compared to the oxygen-rich surface water (Stramma *et al.* 2008, Stramma *et al.* 2010). Reduced uncoupling lowers the futile cycling of the respiratory electron transfer chain (Bermejo-Nogales *et al.* 2014). Therefore, under hypoxic conditions, this ensures that the limited oxygen is utilised to support ATP production. This hypothesis is supported by the tendency for UCP2 expression to be lower in tissues that exhibit higher aerobic respiration (e.g. heart, red muscle) than tissues that are involved in predominantly anaerobic respiration (e.g. white muscle). PPARα has been shown to increase UCP2 gene expression in rats fed a diet enriched in LC-PUFA (Murase *et al.* 2001). Since PGC-1α functions as a coactivator of

PPARα, it was expected that UCP2 gene expression would correlate with PGC-1α gene expression. The results presented here show that UCP2 gene expression was not correlated with PGC-1α gene expression suggesting that PGC-1α may not exhibit transcriptional control over UCP2. However, further investigation of other members of the UCP family may provide a link to PGC-1α in SBT.

The results shown here were intentionally presented in such a way that showed that the choice of housekeeping gene and the method of normalisation can have a significant impact on the gene expression results. For example, the expression of UCP2 was significantly different between red and white muscle when normalised to the expression of β-actin but not when normalised to the expression of ELF-1α. Furthermore, the expression of COX1 was significantly different between red and white muscle when the data were presented per μg RNA. However, there were no significant differences when the data were presented per mg tissue, which is arguably more biologically relevant. Thus, it is clear that the choice of normalisation method can have significant impacts. The data are presented here to show the effects of the different normalisation methods and we propose that gene expression data should be published in this format to provide information on whether the normalisation method is driving changes in the data. Presentation of data in this manner will increase the transparency of the results and allow for a more robust interpretation of gene expression data.

In conclusion, the gene expression of PGC-1α correlated well with the gene expression of its co-activation target, PPARγ, suggesting that some co-activation ability is present in SBT. However, the gene expression of PGC-1α did not seem to correlate with the abundance of mitochondria, as shown by the gene expression of mitochondrial markers CS and COX1. This discrepancy could potentially be explained by post-transcriptional regulation of CS and

COX1, but it appears unlikely that PGC-1α influences mitochondrial abundance in SBT in the same way that occurs in mammals. Furthermore, it appears unlikely that PGC-1α controls UCP2 gene expression. However, the role of UCP2 in SBT is still largely undefined and thus warrants further investigation. Further work may also include the analysis of CS and COX1 enzyme activity in SBT as well as an investigation of the expression of CS, COX1, PGC-1α and PPARγ during the development of juvenile SBT. This work could assist in defining whether these genes play a role in the changes in metabolic physiology and the onset of endothermy that occur during the ontogeny of young SBT.

Chapter 8 – Conclusions and future directions

This thesis has reported on an investigation of fatty acid metabolism in the SBT-E1 cell line, the sequencing of PGC-1α, an important transcriptional regulator, from SBT liver, and the expression of PGC-1α and several of its key targets in the SBT-E1 cell line and in various tissues from wild-caught and farmed SBT.

In Chapters 3 and 4, the effects of different fatty acid supplements on cell proliferation, fatty acid profiles, and fatty acid metabolism were investigated in the SBT-E1 cell line. The cells were found to be susceptible to fatty acid supplementation-induced oxidative stress and this sensitivity was substantially higher for cells supplemented with highly unsaturated fatty acids. The negative effects of fatty acid supplementation on cell proliferation were also more severe when the cells were supplied with fatty acids that had been stored for approximately 1 month, and thus may have been partially oxidised. A 125 μM concentration of vitamin E was found to be optimal to counteract the oxidative effect of the highly unsaturated fatty acid supplements. This concentration of vitamin E did not have any effect on the fatty acid profile of the SBT-E1 cells. The supplementation of the cells with different fatty acids, however, did substantially alter the cellular fatty acid profiles. There was some evidence that when 22:6n-3 was supplied, it was selectively retained by the cells and this is presumably due to the fact that a combination of peroxisomes and mitochondria are required for oxidation of this fatty acid. Substantial elongation of 20:5n-3 but very little evidence of desaturation of any fatty acids was found in assays using [1-^{14}C] labelled fatty acids. Substantial levels of β-oxidation of both 18:3n-3 and 20:5n-3 were found. The selective retention of 22:6n-3 combined with the high levels of elongation and β-oxidation of 20:5n-3 led to a high 22:6n-3/20:5n-3 ratio, which has been described previously in tuna tissues. The minimal progress of fatty acids through the desaturation/elongation pathway was consistent with the presumption that large

predatory marine fish have a plethora of LC-PUFA in their diet and thus have limited capacity for endogenous synthesis of these fatty acids. Interestingly, the expression of Δ6 Fads and Elovl5 genes showed the opposite of what was expected based on the [1-^{14}C] desaturation/elongation assays with high levels of Δ6 Fads and low levels of Elovl5 gene expression but high level of Elovl5 enzyme activity and low levels of Δ6 Fads enzyme activity. This could suggest the presence of an undescribed Elovl2 in tunas which may be responsible for the substantial elongation of 20:5n-3 to 22:5n-3. This may be a subject for future investigation.

In Chapter 5, the focus shifted from investigating the fates of individual fatty acids to the broader subject of metabolic regulation. The target of this investigation was the PGC-1α transcriptional co-activator. This enzyme has been shown to be a key regulator of mitochondrial biogenesis in mammals, but only limited studies have been done in fish. The gene expression of PGC-1α, PPARγ (a co-activation target of PGC-1α), COX1 and CS (indicators of mitochondrial abundance) and UCP2 (a representative downstream target of PGC-1α) were investigated in the SBT-E1 cells. PGC-1α gene expression was up-regulated in cells cultured in medium with the FBS concentration reduced to 2% (v/v) for 24 h. This may indicate that fatty acid depravation stimulates mitochondrial biogenesis through increased expression of PGC-1α. Interestingly, the cells supplied with the reduced serum concentration together with 20:5n-3 did not exhibit significantly increased PGC-1α gene expression, indicating 20:5n-3 was able to overcome some of the effects of reduced serum concentration. There were minimal changes in the gene expression of CS and COX1 and UCP2 in the SBT-E1 cells.

In Chapter 6, the sequencing of the PGC-1α cDNA from SBT liver tissue was described. This is the first PGC-1α cDNA that has been sequenced from any tuna species. The cDNA sequence bore all of the defining characteristics of a functional PGC-1α cDNA and phylogenetic analysis showed that it was most closely related to the PGC-1α cDNA from swordfish. This finding was interesting as PGC-1α was discovered in mammals due to its influence on thermogenesis and both SBT and swordfish are endothermic, albeit via different physiological processes.

In Chapter 7, the gene expression of PGC-1α, PPARγ, CS, COX1 and UCP2 were investigated in different tissues of both wild-caught and farmed SBT. The gene expression patterns for PGC-1α and PPARγ followed similar trends and interestingly, were higher in red muscle, which has endothermic capability, than white muscle, which is ectothermic. However, the level of PGC-1α did not appear to correlate with the expression of CS, COX1 or UCP2. Post-transcriptional regulation of CS and COX1 could explain some of the unexpected results. However, further work is needed to determine the role of PGC-1α in SBT.

In conclusion, this thesis has used a variety of methods to describe and understand fatty acid metabolism and the transcriptional control of processes associated with fatty acid metabolism in a SBT cell line and SBT tissues. The results of this thesis have advanced the current understanding of SBT fatty acid metabolism. Furthermore, the work with PGC-1α has provided a foundation to further investigate its role in the transcriptional regulation of fatty acid catabolism and anabolism to define if this gene influences the development of endothermy in SBT.

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Appendix A – Published manuscript 1

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Cell proliferation and long chain polyunsaturated fatty acid metabolism in a cell line from southern bluefin tuna (*Thunnus maccoyii*)

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Abbreviations

ALA	Alpha-linolenic acid (18:3 n-3)
ARA	Arachidonic acid (20:4 n-6)
DHA	Docosahexaenoic acid (22:6 n-3)
Elovl	Elongase of very long chain fatty acids
EPA	Eicosapentaenoic acid (20:5 n-3)
Fads	Fatty acyl desaturase
FAME	Fatty acid methyl esters
FBS	Fetal bovine serum
FO	Fish oil
GC	Gas chromatography
LNA	Linoleic acid (18:2 n-6)
LC-PUFA	Long-chain polyunsaturated fatty acids
NRU	Neutral red uptake
n-3	Omega-3
n-6	Omega-6
PBS	Phosphate buffered saline
PUFA	Polyunsaturated fatty acids
SBT	Southern bluefin tuna
SBT-E1	Southern bluefin tuna epithelial-like cell line 1
Vitamin E	(+)-α-Tocopherol
VO	Vegetable oil

Abstract

Southern bluefin tuna (SBT, *Thunnus maccoyii*) aquaculture is a highly valuable industry but research on these fish is hampered by strict catch quotas and the limited success of captive breeding. To address these limitations, we have developed a SBT cell line (SBT-E1) and here we report on fatty acid metabolism in this cell line. The SBT-E1 cells proliferated well in standard Leibovitz's L-15 cell culture medium containing fetal bovine serum (FBS) as the source of fatty acids. Decreasing the FBS concentration decreased the cell proliferation. Addition of the C_{18} polyunsaturated fatty acids (PUFA) α-linolenic acid (ALA, 18:3n-3) or linoleic acid (LNA, 18:2n-6) to the cell culture medium had little effect on the proliferation of the cells whereas addition of the long-chain PUFA (LC-PUFA) arachidonic acid (ARA, 20:4n-6), eicosapentaenoic acid (EPA, 20:5n-3) or docosahexaenoic acid (DHA, 22:6n-3) significantly reduced the proliferation of the cells, especially at higher concentrations and especially for DHA. Addition of vitamin E to the culture medium overcame this effect suggesting that it was due to oxidative stress. The fatty acid profiles of the total lipid from the cells reflected those of the respective culture media with little evidence for desaturation or elongation of any of the fatty acids. The only exceptions were EPA and ARA which showed substantial elongation to 22:5n-3 and 22:4n-6, respectively, and DHA which was significantly enriched in the cells compared with the culture medium. The results are discussed in light of the dietary PUFA requirements of SBT in the wild and in aquaculture.

Key words

Elongases, Desaturases, Fatty acid metabolism, Fish nutrition, n-3 Fatty acids, Vitamin E, Polyunsaturated fatty acids (PUFA)

Introduction

Scombrids, finned fishes that include tunas and mackerels, are commonly exploited for human consumption. Possibly the most valuable scombrid in the southern hemisphere is the southern bluefin tuna (SBT, *Thunnus maccoyii*). Australia holds approximately 42% of the global fishing quota for this species (1). These fish are mostly captured as 2-4 year old juveniles in the Great Australian Bight and then 'ranched' prior to export to Japanese sashimi markets (2). As a result, knowledge regarding the nutritional requirements of these fish is critical to the viability of these commercial ventures. Development of a formulated feed was only realised in 2009 (3) and much of the industry still relies on capture fisheries to provide sardines (*Sardiops sagax*) or redbait (*Emmelichthys nitidus*) as tuna feed (4). Sardine fisheries have exhibited large variations in catches in the past and several experts have questioned their sustainability, and by extension, the sustainability of SBT ranching (5, 6). Furthermore, formulated feeds for SBT contain 100% fish oil as their source of dietary lipid (Dr R. Smullen, Ridley Aquafeed, personal communication) and global supplies of fish oil are static (7).

Fishing pressure on wild stocks of sardines and tunas can be alleviated through appropriate quota management, but the lack of understanding surrounding tuna nutrition needs to be addressed in order to secure a sustainable future for the SBT ranching industry. In their natural habitat, the flesh of top predator fish such as tunas is rich in omega-3 (n-3) long chain polyunsaturated fatty acids (LC-PUFA, defined as PUFA with ≥ 20 carbons and ≥ 3 double bonds (8)), particularly eicosapentaenoic acid (EPA; 20:5 n-3) and docosahexaenoic acid (DHA; 22:6 n-3) (9, 10).These fatty acids are synthesised almost exclusively by marine primary producers such as

phytoplankton and they accumulate with increasing trophic level (10-12). The result is that predatory fish such as tunas contain very high concentrations of DHA in their cellular lipids, primarily due to their high dietary intake of DHA (10, 13).

There are two main points to consider when discussing research on n-3 LC-PUFA in fish. Firstly, n-3 LC-PUFA have well documented benefits for human and fish health (14, 15). For example, DHA is important in human brain and eye development and is present at especially high concentrations in these organs (16). EPA and DHA are important modulators of the inflammatory response in vertebrates (17). The omega-6 (n-6) counterpart of EPA, arachidonic acid (20:4 n-6; ARA), is a precursor for pro-inflammatory eicosanoids, whereas EPA is a precursor of anti-inflammatory eicosanoids (17, 18). Therefore, the balance between EPA and ARA is critical in regulating the vertebrate inflammatory response. The second point of significance of EPA and DHA is that they are only produced in the oceans and the harvest of fish and fish oil (FO) from capture fisheries is considered to already be at capacity (19, 20). As a result, the industrial harvest of fish and fish oil is potentially unsustainable (21-24). This has forced the aquaculture industry to consider alternatives to fish protein and FO from wild-caught fish to feed farmed fish (19, 20, 24). For example, vegetable oil (VO) inclusion is common in aquafeeds in the Atlantic salmon industry (25, 26). For example, 100% fish oil replacement with rapeseed oil has no detrimental effects on the growth rate or final weight of Atlantic salmon (27). The inclusion of VO in feeds can translate to higher profits due to the ready availability and lower cost of VO compared to FO. Furthermore, replacing FO with VO can result in improved public perceptions of the industry, as VO is considered a more sustainable resource than FO. However, the problem with replacing FO with VO is that the fatty acid composition of fish lipids reflects that of their diet and therefore

fish fed VO-based diets contain significantly reduced concentrations of EPA/DHA in their flesh. Therefore, the physiological roles that these fatty acids support in the farmed fish are impaired and there are reduced human health benefits from consuming such fish (10, 28). Interestingly, tunas not only contain high levels of DHA in their lipids, but importantly they also contain high levels of DHA relative to EPA (12). This ratio is important as it suggests that tunas preferentially retain the DHA they consume rather than oxidising it (12). If tunas do, in fact, have this capability, it is possible that dietary FO may be partially substituted with VO without excessively negative impacts on the fatty acid composition of the flesh. However, this has not yet been investigated.

EPA and DHA can be synthesised *de novo* from their precursor α-linolenic acid (ALA, 18:3 n-3), which is abundant in certain VO, through a series of desaturation and elongation steps (10, 29; Fig. 1). Fish, like all vertebrates, do not have the Δ12 or Δ15 desaturase enzymes which convert oleic acid (18:1 n-9) to linoleic acid (LNA, 18:2 n-6) and ALA, respectively (10, 30). Therefore ALA and LNA are dietary essential fatty acids (EFA). ALA serves as the precursor for the synthesis of n-3 LC-PUFA and LNA as the precursor for the synthesis of n-6 LC-PUFA (10, 31). However, this process is inefficient in vertebrates and especially in marine fish, presumably due to an abundance of dietary EPA and DHA in their natural diet (10). The majority of work in the area of LC-PUFA biosynthesis in fish has been conducted with Atlantic salmon (*Salmo salar*) but also with species such as turbot (*Psetta maxima*) (30, 32), gilthead sea bream (*Sparus aurata*) (32-34) and Atlantic cod (*Gadus morhua*) (35, 36). To our knowledge, no studies have been conducted directly addressing LC-PUFA biosynthesis in tunas except for the recombinant expression and functional characterisation of some of the enzymes involved (13, 29).

Previously we cloned a Δ6 fatty acyl desaturase (Δ6 Fads) cDNA (M. Gregory and K. Schuller, unpublished data) and an elongase of very long chain fatty acids (Elovl) 5 cDNA from SBT (29). Functional characterization in yeast showed that the SBT Elovl5 gene product catalysed efficient C_{18} to C_{20} and C_{20} to C_{22} conversion of both n-3 and n-6 fatty acids but the SBT Δ6 Fads cDNA did not code for a functional protein (29). In contrast, Morais et al. (13) successfully cloned and functionally characterised a Δ6 Fads cDNA from Atlantic bluefin tuna (*Thunnus thynnus*) and showed that the gene product desaturated approximately 32% of the supplied 18:3 n-3 to 18:4 n-3 (13). Thus it appears that tunas do have a functional Δ6 Fads. In mammals, the Δ6 Fads is thought to catalyse the rate limiting step in LC-PUFA biosynthesis (37). However, this is apparently not always the case for fish. For example, Tocher et al. (38) found that conversion of ALA to EPA was limited by Elovl5 rather than Δ5 or Δ6 Fads in a turbot cell line. These results were confirmed by Ghioni et al. (30) who found that a cell line from turbot showed limited Elovl5 activity, whereas a cell line from Atlantic salmon showed limited Δ5 and possibly Δ6 Fads activity. The inter-species variability in this pathway illustrates the requirement for species-specific investigations of *de novo* LC-PUFA biosynthesis.

Strict fisheries quotas and the high commercial value of SBT make it prohibitively expensive to perform statistically robust feeding trials with this species and although larvae and juveniles can now be produced in captivity, they do not survive in large numbers or for long periods of time (39). As an alternative, we recently developed a SBT cell line (SBT-E1, 40). This is the first continuous cell line for any tuna species. Here we have used this cell line to investigate PUFA metabolism. Specifically, we have investigated the effects of PUFA supply on cell proliferation and the incorporation of PUFA into cellular lipids as well as the apparent conversion of ALA

and LNA to LC-PUFA. This is the first time that PUFA metabolism has been investigated *in vitro* in tuna as this is the first tuna cell line to have been established. The availability of this cell line provides the opportunity to further investigate PUFA metabolism in this commercially important fish species such as by quantifying the effects of different PUFA supplements on the expression of genes involved in LC-PUFA biosynthesis.

Materials and Methods

Cell line and stock cultures

All reagents were obtained from Life Technologies™ unless otherwise stated. The cells were from the recently established SBT-E1 cell line (40). Stock cultures were maintained as previously described (39). Briefly, the cells were cultured in a 25^{o}C incubator in 75 cm^{2} cell culture flasks (Corning) in 15 ml of Leibovitz's L-15 medium supplemented with 15 mM HEPES (pH 7.4) and 10% (v/v) fetal bovine serum (FBS). Subsequently, this is referred to as the standard culture medium. The cells were subcultured at a split ratio of 1:3 when the flasks had reached 80-90% confluence. To do this, the medium was removed, the cell surface was rinsed with phosphate buffered saline (PBS) and a 0.05% (w/v) solution of trypsin/EDTA in PBS was added to detach the cells from the flask. The detached cells were resuspended in the standard culture medium and seeded into fresh culture flasks. Cell numbers were estimated as required using the Trypan blue dye exclusion method (41).

PUFA supplementation

Purified ALA (18:3 n-3), LNA (18:2 n-6), EPA (20:5 n-3), ARA (20:4 n-6) and DHA (22:6 n-3) were obtained from Cayman Chemical Company in solution in

ethanol. Cell culture tested Vitamin E ((+)-α-Tocopherol) was obtained from Sigma-Aldrich in an oil solution. The solutions were diluted in 200-proof molecular biology grade ethanol to a concentration of 10 mg/mL for the PUFA or 0.2 M for the vitamin E on the day that the media were prepared. The diluted PUFA solutions were discarded after one use to avoid supplying the cells with PUFA that had become oxidised. To prepare the PUFA-supplemented media, the molar concentrations of the 10 mg/mL PUFA stock solutions were calculated using the molecular weights of the fatty acids and then the appropriate volume of each solution was mixed with 10 mM bovine serum albumin (BSA, Sigma) at a molar ratio of 2:1 (PUFA:BSA) before mixing with the culture medium. Vitamin E supplemented media were prepared by adding the diluted vitamin E solution to the PUFA supplemented media to a final concentration of 0 to 2 mM. The PUFA and/or vitamin E supplemented media were prepared on the day that each experiment commenced.

Cell proliferation experiments – testing the effects of different concentrations of FBS

Cells from stock culture flasks were seeded into 96-well plates at a concentration of 8,000 cells per well (25,000 cells cm^{-2}) in 100 µl of the standard culture medium (see above) but with the FBS concentration reduced to 2% (v/v). After the cells had adhered for 4 hours the medium was removed and replaced with 100 µl per well of the standard cell culture medium but with the FBS concentration set at 1, 2, 5 or 10% (v/v). There were 10 plates with 3 replicate wells per plate for each treatment. One plate was sacrificed each day for 10 days to estimate cell proliferation using the Neutral Red Uptake (NRU) assay (42) with some modifications. Briefly, the culture medium was removed and replaced with the NRU assay medium (standard culture medium but with the FBS concentration reduced to 5% (v/v) and 10 µg/mL of

Neutral Red (Sigma-Aldrich) added). The cells were then incubated for two hours at 25°C before being washed twice with 100 µl of PBS. Finally, the incorporated stain was solubilised with 100 µl of de-stain solution (50% (v/v) ethanol, 5% (v/v) acetic acid) with shaking for 5 min and the absorbance was measured at 540 nm using a plate reader. Absorbance at 540 nm was shown to have a linear correlation with cell number ($y = 8.04x + 0.015$ where x is million cells and y is the absorbance at 540 nm; $r^2 = 0.99$, data not shown).

Cell proliferation experiments – testing the effects of different PUFA

Cells from stock culture flasks were seeded into 96-well plates and left to adhere as described above. Once the cells had adhered, the medium was removed and replaced with 100 µl per well of the standard culture medium but with the FBS concentration reduced to 2% and 0-20 µM ALA, LNA, EPA, ARA or DHA added. There were 10 plates with 3 replicate wells per plate for each treatment. One plate was sacrificed each day for 10 days to estimate cell proliferation using the NRU assay as described above.

Vitamin E titration experiments

To determine the optimum concentration of vitamin E to prevent the apparent oxidative stress caused by DHA (see Results and Discussion), a range of vitamin E concentrations from 0-2 mM was tested, in treatments with and without DHA. To do this, cells from stock culture flasks were seeded into 24-well plates (Nunc™) at a concentration of 4.75×10^4 cells per well (25,000 cells cm^{-2}) in the standard culture medium but with the FBS concentration reduced to 2% (v/v). Once the cells had adhered (after 4 hours), the medium was removed and replaced with 600 µl of the standard culture medium but with the FBS concentration reduced to 2% (v/v) and

various concentrations of vitamin E added with or without 20 μM DHA. There were 3 replicate wells for each treatment. The volume of the culture medium (600 μl) was chosen because it was proportional to the volume used in the cell proliferation experiments conducted in 96-well plates. The cells were then cultured for 4 days before they were harvested and counted using the Trypan blue dye exclusion method (41).

Fatty acid profiling

Cells from stock culture flasks were seeded into 6-well plates at a concentration of 2.375×10^5 cells per well (25,000 cells cm^{-2}) in the standard culture medium but with the FBS concentration reduced to 2% (v/v). Once the cells had adhered (after 4 hours), the medium was removed and replaced with 3 mL of the standard culture medium but with the following modifications. Vitamin E (125 μM) was added to all treatments and for the PUFA treatments, the FBS concentration was reduced to 2%. The PUFA (ALA, LNA, EPA, ARA or DHA) were added at a concentration of 10 μM. The volume of the culture medium (3 mL) was chosen because it was proportional to the volume used in the cell proliferation experiments conducted in 96-well plates. The cells were allowed to proliferate for 4 days before they were harvested as described above. For each treatment, 3 wells were combined to provide sufficient cells for lipid extraction. This resulted in 3 replicates for each treatment. A 20 μl subsample of each cell suspension was taken to estimate viable cell number as before and the remainder was used for fatty acid analysis. Total lipid was extracted and fatty acid methyl esters (FAME) were produced using an isopropanol/methanol lipid extraction and a sulphuric acid-mediated fatty acid methylation as previously described (29). The FAME were analysed as previously described (29) using a Hewlett-Packard 6890 gas chromatograph (GC) with a 50 m capillary column. The

injector and detector temperatures were set to 250^{o}C and 300^{o}C, respectively. The initial oven temperature was 140^{o}C and rose to 220^{o}C at a rate of 5^{o}C per minute before a 3 minute hold at 220^{o}C. Individual FAME were identified by comparison to a reference mixture of known standards (GLC-463, NuCheck Prep Inc.). The peroxidation index was calculated from the fatty acid data using the formula of Hulbert et al. (43) as described in the footnote to Table 1.

Statistical analysis

Plotting of data and statistical analysis were conducted using the statistical package PASW Statistics 18 (SPSS Inc., Chicago). Significant differences in the data were determined using one-way analysis of variance (ANOVA) followed by p*ost-hoc* multiple comparisons using the Bonferroni method to correct for multiple-comparison bias (44). Specific details of the statistical analyses are included in the legends to the tables and figures. Significant differences are reported when $p < 0.05$.

Results

Effects of fetal bovine serum concentration on cell proliferation

The SBT-E1 cells cultured at FBS concentrations ≥ 2% (v/v) showed higher cell numbers at the end of the experiment compared with the beginning ($F_{9,20} > 32.7$, $p < 0.01$; Fig. 2). The 2% (v/v) FBS treatment showed significantly reduced cell proliferation compared to the 5% (v/v) and 10% (v/v) FBS treatments at day 9 ($F_{3,8} = 26.3$, $p < 0.01$). Therefore, 2% (v/v) FBS was chosen as a suitable intermediate concentration for the subsequent PUFA supplementation experiments so that both positive and negative effects of the PUFA on cell proliferation could be seen.

Effects of PUFA supplementation on cell proliferation

The SBT-E1 cells proliferated well at all fatty acid concentrations for the 18:3 n-3 and 18:2 n-6 treatments (Fig. 3A, B). The higher concentrations of both 18:3 n-3 (20 μM) and 18:2 n-6 (≥10 μM) showed lower proliferation compared to the control during the early stages of the experiment ($F_{4,10} = 9.7$, $p < 0.05$ and $F_{4,10} = 11.92$, $p < 0.05$, respectively). However this effect was not retained and there were no significant differences to the control at the conclusion of the experiment (Fig. 3A, B). The lower concentrations (≤ 10 μM) of the 20:5 n-3 and 20:4 n-3 treatments also showed good proliferation throughout the course of the experiment (Fig. 3C, D). In contrast, the 20 μM treatment for both 20:5 n-3 and 20:4 n-3 showed good proliferation for the first 4 days but exhibited significantly lower proliferation than the control from day 6 onwards for 20:5 n-3 ($F_{4,10} = 16.0$, $p < 0.01$) and day 5 onwards for 20:4 n-6 ($F_{4,10} = 10.3$, $p < 0.05$). The 22:6 n-3 treatments showed a negative dose-dependent response with the highest rate of cell proliferation observed

in the 2.5 μM treatment and complete inhibition of cell proliferation observed in both the 20 μM and 10 μM treatments by days 1 and 6, respectively (Fig. 3E).

Effects of vitamin E concentration on cell proliferation

Since the LC-PUFA treatments, especially the DHA treatment, showed lower cell proliferation than the other PUFA treatments, it was hypothesised that this could be due to oxidative stress. This was based on the fact that DHA is very highly unsaturated with 22 carbon atoms and 6 double bonds. To test this hypothesis, we cultured the cells with various concentrations of the antioxidant Vitamin E in the presence and absence of 20 μM DHA. In the presence of DHA, vitamin E supplementation positively affected cell proliferation at all vitamin E concentrations tested (Fig. 4, $F_{6,14} = 70.92$, $p < 0.01$). The highest number of cells was observed in the 250 μM vitamin E treatment but this was not significantly different from any of the other vitamin E treatments except for the 0 and 2000 μM treatments which both gave significantly lower cell numbers ($p < 0.01$). In contrast, in the absence of DHA, concentrations of vitamin E above 125 μM had significant negative effects on cell number ($p \leq 0.001$). Despite the changes in cell proliferation observed with the addition of vitamin E, there were no significant effects of vitamin E on cellular fatty acid composition in cells fed either 2% (v/v) FBS or 2% (v/v) FBS plus DHA (data not shown). Based on these results, a vitamin E concentration of 125 μM was chosen for the investigation of the effects of PUFA supplementation on the fatty acid profiles of the cells.

Effects of PUFA supplementation on cellular fatty acids

In all cases, the cells contained different proportions of fatty acids to those observed in the culture medium which was the standard culture medium (see Materials and

Methods) but with the FBS concentration reduced to 5% (v/v) (Table 1). Notably, there were significantly lower levels of saturated fatty acids in the cells (≤ 36.7%) compared with the medium (54.0%, $F_{7,16}$ = 11.0, p < 0.001) and greater proportions of PUFA in the cells (≥ 27.9%) compared with the medium (10.4%, $F_{7,16}$ = 46.5, p < 0.001). Interestingly, DHA showed a significantly higher level in the cells than in the medium when the cells were supplied with either 2% (v/v) or 10% (v/v) FBS. Specifically, there was an increase from 1.7% DHA in the medium to 6.6% DHA in the cells supplied with 2% (v/v) FBS and 8.2% DHA in the cells supplied with 10% (v/v) FBS ($F_{7,16}$ = 221.3, p <0.001). Supplementation with either n-3 or n-6 PUFA significantly increased the levels of the respective PUFA in the cells in all cases ($F_{7,16} \geq 22.6$, p < 0.001). These increases, however, did not necessarily translate into higher levels of products further along the LC-PUFA biosynthesis pathway. For example, addition of ALA led to increases in cellular ALA from trace amounts to 23% of the total fatty acids ($F_{7,16}$ = 22.6, p < 0.001). The GC trace did not contain a standard for 18:4 n-3 but there were no detectable changes in the region where 18:4 n-3 was expected to elute from the column (data not shown). Therefore, there was no detectable desaturation of ALA. In contrast, the cells fed ALA showed apparent C_{18} – C_{20} elongation with an increase in 20:3 n-3 from undetectable levels to 1.7% of the total fatty acids (Table 1). There was no detectable desaturation of 18:2n-6 to 18:3 n-6 (Table 1), and despite an apparent increase in the elongation product, 20:2 n-6, from 0.4% to 1.1% of total fatty acids, this change was not statistically significant ($F_{7,16}$ = 6.6, p = 0.109) (Table 1). A substantial amount of C_{20} – C_{22} elongation was seen in cells supplied EPA with significant increases in both EPA and 22:5 n-3 (the elongation product of EPA) ($F_{7,16}$ = 156.7, p <0.001; Table 1). Separate elution of 22:3 n-3 and 22:4 n-6 was not possible but the combined GC trace for these fatty acids supported substantial elongation of 20:4 n-6 with 22:3n-3 + 22:4 n-6 increasing

from 1.1% of the total fatty acids in the control cells to 11.0% in cells supplied with ARA ($F_{7,16}$ = 916.8 p <0.01; Table 1). Therefore the cells exhibited high rates of C_{20} – C_{22} elongation but only low rates of C_{18} – C_{20} elongation. In contrast, there were no significant increases in DHA concentration when the cells were supplied with either ALA or EPA. Therefore, the enzymatic steps involved in the synthesis of DHA from EPA seem to have limited activity.

The addition of vitamin E did not have any effect on the fatty acid composition of the cells (p > 0.99, data not shown). Likewise, the different concentrations of FBS, despite altering the proliferation of the cells (Fig. 2), did not have any significant effects on the proportions of most fatty acids in the cells (Table 1). The only exceptions were 14:0, which increased from 0.6% in the 10% (v/v) FBS treatment to 1.1% in the 2% (v/v) FBS treatment, and 20:3 n-3 which decreased from 0.2% in the 10% (v/v) FBS treatment to undetectable in the 2% (v/v) FBS treatment ($F_{7,16}$ = 994.3, p <0.05, Table 1).

A high peroxidation index value of 292.5 was seen in the cells supplied 10 μM DHA (Table 1). This value was significantly higher than the values for the other PUFA treatments and also for the 2% (v/v) and 10% (v/v) FBS controls ($F_{7,16}$ = 179.8, p <0.001; Table 1). In fact, the peroxidation index value for the cells treated with DHA was approximately twice that for either the control cells, or the cells treated with either ALA or LNA.

Discussion

The SBT-E1 cells showed a similar pattern of rapid proliferation during the early stages, before a plateau at day 6 for all concentrations of FBS (Fig. 1). Mostly likely, this was due either to an exhaustion of the available nutrients in the case of the low FBS concentrations or to a filling of the available growth area at the high FBS concentrations. The plateau at the low FBS concentrations was consistent with what has been reported for turbot (*Scophthalmus maximus*) and Atlantic salmon (*Salmo salar*) cells in culture (45). However the turbot and Atlantic salmon cells, despite being seeded at a similar density, appeared to show slower and more consistent proliferation over a longer period of time with cells continuing to proliferate after 10 days, compared to a plateau at 6 days for the SBT-E1 cells (Fig. 1; 45). Therefore, the higher initial rate of proliferation may have caused the SBT-E1 cells to fill the available growth area more rapidly. Furthermore, at high cell densities the SBT-E1 cells appeared to show alterations in morphology. For example, the cells formed more isolated colonies and cells within particular colonies packed closer together and became more uniform in shape causing an increase in the cell density within individual colonies. Evidence of contact inhibition was also apparent between adjacent colonies. These morphological changes could suggest cell differentiation. Differentiation of the cells could also explain the slower proliferation observed after several days without subculture.

The SBT-E1 cells showed good proliferation over the course of 7 days when supplemented with either ALA or LNA but the numbers for these cells were only moderately higher than those for the control cells at any time (Fig. 3A, 3B). The cell numbers for the 2.5 μM ALA and 10 μM LNA treatments were significantly higher

than those for the control at day 6 (p = 0.012 and 0.024, respectively), but this difference was not observed by day 7. This result was similar to what was seen in cultured turbot and Atlantic salmon cells which showed modest increases in proliferation (relative to the control) when supplied 5-25 μM ALA or LNA (45). The proliferation of the SBT-E1 cells was inhibited at high concentrations of EPA or ARA and this effect was even more pronounced in the cells supplied with DHA. This sensitivity to high concentrations of LC-PUFA, especially DHA, appeared to be much greater in the SBT-E1 cells than in cell lines from either turbot or Atlantic salmon (45). The differences could be due to the SBT-E1 cells having less effective antioxidant protection than the Atlantic salmon or turbot cell lines.

We hypothesized that the inhibition of cell proliferation by DHA may have been due to oxidative stress as the large number of double bonds in DHA makes it especially susceptible to oxidative attack and the peroxidation index for the cells supplied DHA was approximately double that for the cells supplied either ALA or LNA (46, 47). Therefore we tested the effects of various concentrations of vitamin E in the presence and absence of DHA. Our results showed that vitamin E did indeed reverse the inhibitory effects of DHA on cell proliferation (Fig. 4). Tocher and Dick (45) found that in turbot cells fed 25 μM EPA, cell proliferation could be improved with the addition of 1.8 mM vitamin E but the effects of vitamin E on cells supplied with DHA were not investigated in that study. Our results showed that in the SBT-E1 cells, a 63 μM concentration of vitamin E was sufficient to reverse the negative effects of 20 μM DHA on cell proliferation and the greatest benefit was seen in the presence of 250 μM vitamin E. Therefore, despite the SBT-E1 cells showing high sensitivity to apparent LC-PUFA-induced oxidative stress, this effect could readily be reversed by the addition of vitamin E.

Vitamin E improved the proliferation of the SBT-E1 cells both in the absence and in the presence of DHA but the optimal vitamin E concentration was different; lower in the absence of DHA than in the presence of DHA. This suggests, either that the basal cell culture medium was deficient in vitamin E or that the amount of vitamin E required by the cells varied according to the PUFA supplement. The high potential for DHA to cause oxidative stress, as evidenced by the high peroxidation index value for the cells supplied DHA (Table 1), may explain the higher optimum vitamin E concentration required in the presence of DHA compared with in the absence of DHA. In other words the positive effects of oxidative protection outweighed the negative effects of additional vitamin E when the cells were cultured in the presence of DHA. The biochemical basis for this is not known but previous studies have shown that high concentrations of vitamin E ($\geq$ 115 μM) inhibited cell proliferation in human mesangial and glomerular microvascular endothelial cells (48). Furthermore, Jóźwik et al. (49) found that when vitamin E was supplied to mice in high doses, there was destabilisation of the cellular membranes, leading to membrane leak and ultimately, apoptosis.

Tuna lipids have unusually high DHA:EPA ratios but the molecular basis for this is not known (9, 13). The SBT-E1 cells cultured in the presence of 2% (v/v) FBS had fatty acid profiles containing 1.1% EPA and 6.6% DHA giving a DHA:EPA ratio of 6.0. Similarly, when the cells were cultured in the presence of 10% (v/v) FBS, their fatty acid profiles contained 1.1% EPA and 8.2% DHA giving a DHA:EPA ratio of 7.5. The high DHA:EPA ratios observed in the SBT-E1 cells have also been observed in the muscle lipids of SBT consuming its natural diet in the wild but not in other bluefin tuna species. For example, SBT muscle lipids contained 4.8% EPA and

44.1% DHA giving a DHA:EPA ratio of 9.2 (50). For Atlantic Bluefin tuna (*Thunnus thynnus*), the values were 4.9% EPA and 12.1% DHA giving a DHA:EPA ratio of 2.5 (51). For Pacific Bluefin tuna (*Thunnus orientalis*), the values were 6.9% EPA and 23.3% DHA giving a DHA:EPA of 3.4 for dorsal skeletal muscle as well as 9.8% EPA and 20.2% DHA giving a DHA:EPA ratio of 2.1 for ventral skeletal muscle (52). Thus, the DHA:EPA ratio in the SBT-E1 cells approached that in the wild-caught SBT and exceeded that in the wild-caught Atlantic or Pacific Bluefin tuna.

The cell culture medium contained only 1.7% of the total fatty acids as DHA whereas the cells cultured with either 2% or 10% (v/v) FBS contained 6.6% and 8.2% of the total fatty acids as DHA. Thus there was apparent selective retention of DHA in the cellular lipids. This was not seen with EPA with the proportions of EPA being similar in the medium and in the cells. DHA is difficult to oxidise because the Δ4 bond must be removed by peroxisomal β-oxidation (10) and this could explain the selective retention of DHA reported here. Other fish species oxidise DHA by the same mechanism and therefore also show selective retention of DHA. However, the levels of DHA reported here in the SBT-E1 cells cultured with 10% (v/v) FBS were greater than those reported previously for other fish cells in culture (38). Here we found 8.2% DHA in the SBT-E1 cells cultured with 10% (v/v) FBS whereas previous studies have found only 1.3% DHA in rainbow trout gonad cells and only 3.9% DHA in turbot fin cells when these cells were cultured with 10% (v/v) FBS (38). The greater apparent selective retention of DHA in the SBT-E1 cells may explain the high DHA:EPA ratios observed in tuna tissues (9, 13).

Another explanation for the high DHA:EPA ratio in the SBT-E1 cells could be the apparently high level of conversion of EPA to 22:5n-3. In particular, the cells supplied EPA contained 14.1% of their total fatty acids as 22:5 n-3, a significant increase from the 4.6% found in the control cells (Table 1). This indicated substantial $C_{20} - C_{22}$ elongation. In a previous study, the proportion of 22:5 n-3 in SBT liver was reported as 5.33% of total fatty acids (29), which is similar to that observed for the cells supplemented with 2% (v/v) FBS in the present study. However there are currently no data on the activity of any Elovl enzymes in any SBT tissues and therefore further study to determine the capability of tuna to elongate EPA to 22:5 n-3 is needed. The apparently high level of elongation in the SBT-E1 cells could serve to remove EPA from the cells, lowering the proportion of EPA relative to DHA and increasing the DHA:EPA ratio.

ARA is the n-6 equivalent of EPA and occurs at the same point in the LC-PUFA biosynthesis pathway (Fig.1). In this study, the cells appeared to incorporate ARA more readily than EPA, with cells supplemented with ARA containing 22.3% of their fatty acids as ARA (Table 1). There also seemed to be a similarly high level of elongation of ARA in cells fed ARA as was seen in cells supplied EPA. In particular, the combined GC trace for 22:3 n-3 and 22:4 n-6 indicated an increase from 1.1% in cells supplied 2% FBS to 11.0% in cells supplied 2% FBS and 10 μM ARA (Table 1). The apparently substantial elongation of EPA and ARA to 22:5 n-3 and 22:4 n-6, respectively, is indicative of a high level of Elovl5 activity in the SBT-E1 cells. The data presented here show far less Elovl5 activity towards ALA and LNA compared with EPA and ARA suggesting a preference of the Elovl5 enzyme for C_{20} substrates over C_{18} substrates. Alternatively, there may be an Elovl2 enzyme that is active

towards C_{20} and C_{22} substrates but not C_{18} substrates in the SBT cells and this may catalyse the substantial elongation of EPA and ARA observed in this study.

One possible explanation for the apparently high rates of C_{20} PUFA elongation in the SBT-E1 cells could be the roles that EPA and ARA play in mediating the inflammatory response in vertebrates through the production of eicosanoids such as leukotrines and prostanoids (53). The majority of the eicosanoids that promote the inflammatory response are derived from ARA whereas the down-regulation of this pathway and the return of the tissue to homeostasis is due to metabolites of EPA (17, 18). Therefore, the balance between ARA and EPA concentrations needs to be tightly regulated as an abundance of ARA could lead to an exacerbated inflammatory response whereas an excess of EPA could lead to the silencing of what may otherwise be a healthy inflammatory response (17). As a result, whilst the use of the LC-PUFA biosynthesis pathway in the production of DHA may be redundant in predatory marine fish such as tunas, some of the enzymes involved, especially Elovl5, may still be utilised as regulators of these organisms' inflammatory response by elongating (and thereby removing) excess ARA or EPA.

The low levels of ALA and LNA elongation may be because the Elovl5 enzyme, in its normal cellular environment, utilises 18:4 n-3 and 18:3 n-6 as its C_{18} substrates. Previous research with a cell line from Atlantic Salmon showed 5.6% elongation of ALA, compared with 23.6% elongation of 18:4 n-3 (30). The corresponding figures for a cell line from turbot were 0.9% for ALA and 4.4% for 18:4 n-3 (30). Therefore, it is likely that there is competition between different C_{18} substrates for Elovl5-catalyzed elongation and the high concentrations of ALA and LNA used in this study provided a favourable environment for their elongation. Furthermore, and perhaps

more importantly, it is likely that there is also competition between C_{18} and C_{20} substrates for Elovl5-catalyzed elongation. The functional characterisation of SBT Elovl5 recombinantly expressed in yeast showed that there was a strong preference for 18:4 n-3 and 18:3 n-6 over C_{20} substrates (29). To investigate this further, the SBT-E1 cells could be supplied a combination of ALA and LNA or 18:4 n-3 and 18:3 n-6 along with EPA and ARA in a similar way as described by Gregory et al. (29).

In contrast to the apparent elongation that was observed for ALA, LNA and especially EPA and ARA, there was very little evidence of desaturation in any of the treatments. These data are consistent with the lack of desaturase activity observed in attempts to functionally characterise a SBT Δ6 Fads cDNA by expression in yeast (M.K. Gregory and K.A. Schuller, unpublished).

The data presented here were obtained with a cell line and one must be cautious in extrapolating cell line data to what may be observed in whole fish. A number of mammalian cell lines such as Chinese hamster ovary, mouse fibroblast and human liver cells have shown a reduction or complete loss of Δ6 Fads activity over the course of extensive culture (54). Furthermore, because fish sera of cell culture quality are not readily available, fish cell lines are usually cultured with mammalian sera (usually FBS). Mammalian sera contain substantially less EPA and DHA than is usually found in fish tissues. This means that cultured fish cells contain less EPA and DHA in their fatty acids than the fish tissues from which they originate (55). This could drive changes in the availability of substrates for Fads and Elovl enzymes and in turn change gene and enzyme expression patterns.

In summary, this study has characterised the effects of various PUFA on cell proliferation, PUFA incorporation into cellular lipids and PUFA metabolism in the SBT-E1 cell line. Studies on PUFA metabolism in SBT and other tuna species have been limited to date by the high commercial value of these species. This is the first *in vitro* study of fatty acid metabolism in any tuna species and the results show that the SBT-E1 cell line is a useful model. Future investigations with the cell line could include analyses of the effects of fatty acid supplementation on the expression of the Fads and Elovl genes involved in the *de novo* synthesis of EPA and DHA.

References

1. Timmiss, T (2011) Letter to SBT concession holders regarding SBT TAC's for 2011/12 and 2012/13. Australian Fisheries Management Authority.
2. Kirchhoff, NT, Leef, MJ, Ellis, D, Purser, J, Nowak, BF (2011) Effects of the first two months of ranching on the health of southern bluefin tuna *Thunnus maccoyii*. Aquaculture 315: 207-212.
3. Ridley corporation limited (2009) Ridley reports world's first formulated feed success for southern blue fin tuna. *Media release from Ridley corporation limited (RIC) on the Australian securities exchange 23/09/2009* [Online].
4. Musgrove, RJ, Carragher, JF, Manning, AD, Zammit, BJ, Thomas, PM, Buchanan, J (2011) Effects of postharvest processes on quality of Australian sardines (*Sardinops sagax*) and redbait (*Emmelichthys nitidus nitidus*) for feeding aquacultured southern bluefin tuna (*Thunnus maccoyii*). Aquaculture Nutrition 17: e19-e29.
5. Juan-Jordá, MJ, Mosqueira, I, Cooper, AB, Freire, J, Dulvy, NK (2011) Global population trajectories of tunas and their relatives. Proceedings of the National Academy of Sciences 108: 20650-20655.
6. Abares-Brs (2011). *Australian fisheries statistics 2010,* Canberra.
7. Turchini, GM, Torstensen, BE, Ng, WK (2009) Fish oil replacement in finfish nutrition. Reviews in Aquaculture 1: 10-57.
8. Morais, S, Monroig, O, Zheng, X, Leaver, M, Tocher, D (2009) Highly unsaturated fatty acid synthesis in Atlantic salmon: Characterization of ELOVL5- and ELOVL2-like elongases. Marine Biotechnology 11: 627-639.
9. Mourente, G, Tocher, DR (2009) Tuna nutrition and feeds: Current status and future perspectives. Reviews in Fisheries Science 17: 373-390.

10. Tocher, DR (2003) Metabolism and functions of lipids and fatty acids in teleost fish. Reviews in Fisheries Science 11: 107-184.
11. Perhar, G, Arhonditsis, GB, Brett, MT (2012) Modelling the role of highly unsaturated fatty acids in planktonic food web processes: A mechanistic approach. Environmental Reviews 20: 155-172.
12. Moreno, VJ, De Moreno, JEA, Brenner, RR (1979) Biosynthesis of unsaturated fatty acids in the diatom *Phaeodactylum tricornutum*. Lipids 14: 15-19.
13. Morais, S, Mourente, G, Ortega, A, Tocher, JA, Tocher, DR (2011) Expression of fatty acyl desaturase and elongase genes, and evolution of DHA: EPA ratio during development of unfed larvae of Atlantic bluefin tuna (*Thunnus thynnus* L.). Aquaculture 313: 129-139.
14. Connor, WE (2000) Importance of n-3 fatty acids in health and disease. American Journal of Clinical Nutrition 71: 171S-175S.
15. Calder, PC, Yaqoob, P (2009) Omega 3 polyunsaturated fatty acids and human health outcomes. Biofactors 35: 266-272.
16. Innis, SM (2008) Dietary omega 3 fatty acids and the developing brain. Brain Research 1237: 35-43.
17. Chapkin, RS, Kim, W, Lupton, JR, Mcmurray, DN (2009) Dietary docosahexaenoic and eicosapentaenoic acid: Emerging mediators of inflammation. Prostaglandins, Leukotrienes and Essential Fatty Acids 81: 187-191.
18. Calder, PC (2009) Polyunsaturated fatty acids and inflammatory processes: New twists in an old tale. Biochimie 91: 791-795.
19. Deutsch, L, Gräslund, S, Folke, C, Troell, M, Huitric, M, Kautsky, N, Lebel, L (2007) Feeding aquaculture growth through globalization: Exploitation of marine ecosystems for fishmeal. Global Environmental Change 17: 238-249.

20. Naylor, RL, Hardy, RW, Bureau, DP, Chiu, A, Elliott, M, Farrell, AP, Forster, I, Gatlin, DM, Goldburg, RJ, Hua, K (2009) Feeding aquaculture in an era of finite resources. Proceedings of the National Academy of Sciences 106: 15103-15110.

21. Lam, ME, Pitcher, TJ (2012) Fish commoditization: Sustainability strategies to protect living fish. Bulletin of Science, Technology & Society 32: 31-40.

22. Miller, MR, Nichols, PD, Carter, CG (2008) N-3 Oil sources for use in aquaculture–alternatives to the unsustainable harvest of wild fish. Nutrition Research Reviews 21: 85-96.

23. Parker, RWR, Tyedmers, PH (2012) Uncertainty and natural variability in the ecological footprint of fisheries: A case study of reduction fisheries for meal and oil. Ecological Indicators 16: 76-83.

24. Tacon, AGJ, Metian, M (2008) Global overview on the use of fish meal and fish oil in industrially compounded aquafeeds: Trends and future prospects. Aquaculture 285: 146-158.

25. Crampton, V, Nanton, D, Ruohonen, K, Skjervold, PO, El-Mowafi, A (2010) Demonstration of salmon farming as a net producer of fish protein and oil. Aquaculture Nutrition 16: 437-446.

26. Bendiksen, EÅ, Johnsen, CA, Olsen, HJ, Jobling, M (2011) Sustainable aquafeeds: Progress towards reduced reliance upon marine ingredients in diets for farmed Atlantic salmon (*Salmo salar* L.). Aquaculture 314: 132-139.

27. Bell, JG, Mcevoy, J, Tocher, DR, Mcghee, F, Campbell, PJ, Sargent, JR (2001) Replacement of fish oil with rapeseed oil in diets of Atlantic salmon (*Salmo salar*) affects tissue lipid compositions and hepatocyte fatty acid metabolism. Journal of Nutrition 131: 1535-1543.

28. Bell, J, Henderson, R, Tocher, D, Sargent, J (2004) Replacement of dietary fish oil with increasing levels of linseed oil: Modification of flesh fatty acid

compositions in Atlantic salmon (*Salmo salar*) using a fish oil finishing diet. Lipids 39: 223-232.

29. Gregory, MK, See, VHL, Gibson, RA, Schuller, KA (2010) Cloning and functional characterisation of a fatty acyl elongase from southern bluefin tuna (*Thunnus maccoyii*). Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 155: 178-185.
30. Ghioni, C, Tocher, DR, Bell, MV, Dick, JR, Sargent, JR (1999) Low C18 to C20 fatty acid elongase activity and limited conversion of stearidonic acid, 18:4 (n-3), to eicosapentaenoic acid, 20:5 (n-3), in a cell line from the turbot, *Scophthalmus maximus*. Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids 1437: 170-181.
31. Bell, M, Tocher, D (2009). Biosynthesis of polyunsaturated fatty acids in aquatic ecosystems: General pathways and new directions. *In:* Kainz, M, Brett, MT & Arts, MT (eds.) *Lipids in aquatic ecosystems.* New York: Springer.
32. Zheng, X, Seiliez, I, Hastings, N, Tocher, DR, Panserat, S, Dickson, CA, Bergot, P, Teale, AJ (2004) Characterization and comparison of fatty acyl Δ6 desaturase cDNAs from freshwater and marine teleost fish species. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 139: 269-279.
33. Tocher, DR, Ghioni, C (1999) Fatty acid metabolism in marine fish: Low activity of fatty acyl Δ5 desaturation in gilthead sea bream (*Sparus aurata*) cells. Lipids 34: 433-440.
34. Fountoulaki, E, Vasilaki, A, Hurtado, R, Grigorakis, K, Karacostas, I, Nengas, I, Rigos, G, Kotzamanis, Y, Venou, B, Alexis, MN (2009) Fish oil substitution by vegetable oils in commercial diets for gilthead sea bream (*Sparus aurata* L.); effects on growth performance, flesh quality and fillet fatty acid profile:

Recovery of fatty acid profiles by a fish oil finishing diet under fluctuating water temperatures. Aquaculture 289: 317-326.

35. Tocher, D, Zheng, X, Schlechtriem, C, Hastings, N, Dick, J, Teale, A (2006) Highly unsaturated fatty acid synthesis in marine fish: Cloning, functional characterization, and nutritional regulation of fatty acyl Δ6 desaturase of Atlantic cod (*Gadus morhua* L.). Lipids 41: 1003-1016.
36. Monroig, Ó, Li, Y, Tocher, DR (2011) Delta-8 desaturation activity varies among fatty acyl desaturases of teleost fish: High activity in delta-6 desaturases of marine species. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 159: 206-213.
37. De Antueno, RJ, Knickle, LC, Smith, H, Elliot, ML, Allen, SJ, Nwaka, S, Winther, MD (2001) Activity of human Δ5 and Δ6 desaturases on multiple n-3 and n-6 polyunsaturated fatty acids. FEBS Letters 509: 77-80.
38. Tocher, DR, Carr, J, Sargent, JR (1989) Polyunsaturated fatty acid metabolism in fish cells: Differential metabolism of (n-3) and (n-6) series acids by cultured cells orginating from a freshwater teleost fish and from a marine teleost fish. Comparative Biochemistry and Physiology Part B: Comparative Biochemistry 94: 367-374.
39. Clean seas tuna limited (2012) Clean seas tuna ltd annual report 2012. *Annual report released on the Australian securities exchange 15/08/2012* [Online].
40. Bain, PA, Hutchinson, RG, Marks, AB, Crane, MSJ, Schuller, KA (2013) Establishment of a continuous cell line from southern bluefin tuna (*Thunnus maccoyii*). Aquaculture 376–379: 59-63.
41. Pappenheimer, AM (1917) Experimental studies upon lymphocytes I. The reactions of lymphocytes under various experimental conditions. The Journal of Experimental Medicine 25: 633-650.

42. Repetto, G, Del Peso, A, Zurita, JL (2008) Neutral red uptake assay for the estimation of cell viability/cytotoxicity. Nature Protocols 3: 1125-1131.

43. Hulbert, AJ, Pamplona, R, Buffenstein, R, Buttemer, WA (2007) Life and death: Metabolic rate, membrane composition, and life span of animals. Physiological Reviews 87: 1175-1213.

44. Abdi, H (2007). The Bonferonni and Šidák corrections for multiple comparisons. *In:* Salkind, N (ed.) *Encyclopedia of measurement and statistics.* Thousand Oaks: SAGE Publications.

45. Tocher, DR, Dick, JR (1991) Effect of polyunsaturated fatty acids on the growth of fish cells in culture. Comparative Biochemistry and Physiology. A. Comparative Physiology 100: 461-466.

46. Gutteridge, J (1995) Lipid peroxidation and antioxidants as biomarkers of tissue damage. Clinical Chemistry 41: 1819-1828.

47. Gutteridge, J, Halliwell, B (1990) The measurement and mechanism of lipid peroxidation in biological systems. Trends in Biochemical Sciences 15: 129-135.

48. Zhang, Y, Yasumoto, Y, Changlin, M, Arima, T (2001) Vitamin E inhibits proliferation of primary cultured human mesangial and endothelial cells. Nephron 89: 291-6.

49. Jóźwik, A, Śliwa-Jóźwik, AK (2005) The effect of administration of high doses of selected antioxidants upon the activity of beta-glucuronidase (β-GL) in liver, kidney and blood plasma of mice. Animal Science Papers and Reports 23: 219-227.

50. Nichols, P, Virtue, P, Mooney, B, Elliott, N, Yearsley, G (1998) *Seafood the good food. The oil content and composition of Australian commercial fishes, shellfishes and crustaceans,* Hobart, CSIRO Marine Research.

51. Sprague, M, Dick, JR, Medina, A, Tocher, DR, Bell, J, Mourente, G (2012) Lipid and fatty acid composition, and persistent organic pollutant levels in tissues of migrating Atlantic bluefin tuna (*Thunnus thynnus*, L.) broodstock. Environmental Pollution 171: 61-71.

52. Nakamura, Y-N, Ando, M, Seoka, M, Kawasaki, K-I, Tsukamasa, Y (2007) Changes of proximate and fatty acid compositions of the dorsal and ventral ordinary muscles of the full-cycle cultured Pacific bluefin tuna *Thunnus orientalis* with the growth. Food Chemistry 103: 234-241.

53. Simopoulos, AP (2002) Omega-3 fatty acids in inflammation and autoimmune diseases. Journal of the American College of Nutrition 21: 495-505.

54. Masatomo, M, Osamu, D, Yuzuru, A (1978) Metabolic conversion of polyunsaturated fatty acids in mammalian cultured cells. Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism 530: 153-164.

55. Tocher, DR, Sargent, JR, Frerichs, GN (1988) The fatty acid compositions of established fish cell lines after long-term culture in mammalian sera. Fish Physiology and Biochemistry 5: 219-227.

56. Li, Y, Monroig, O, Zhang, L, Wang, S, Zheng, X, Dick, JR, You, C, Tocher, DR (2010) Vertebrate fatty acyl desaturase with Δ4 activity. Proceedings of the National Academy of Sciences 107: 16840-16845.

57. Morais, S, Castanheira, F, Martinez-Rubio, L, Conceição, LEC, Tocher, DR (2012) Long chain polyunsaturated fatty acid synthesis in a marine vertebrate: Ontogenetic and nutritional regulation of a fatty acyl desaturase with Δ4 activity. Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids 1821: 660-671.

Figures

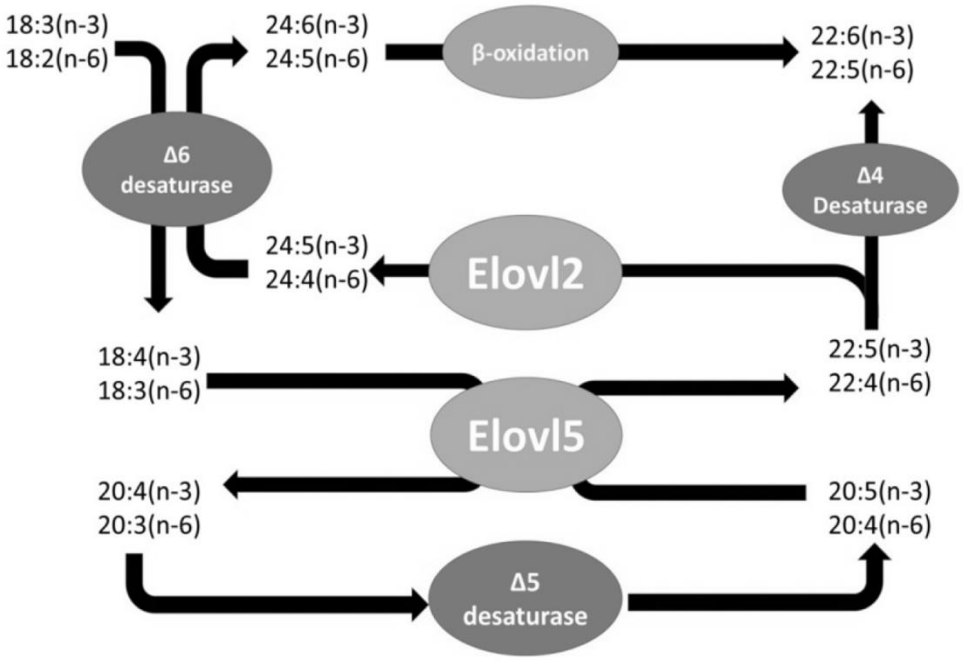

Figure 1: The long-chain polyunsaturated fatty acid biosynthesis pathway in vertebrates. Note: Δ4 fatty acyl desaturase enzyme activity has only been observed in two fish species, the herbivore *Siganus canaliculatus* (56) and the benthic carnivore *Solea senegalensis* (57). Figure adapted from Fig. 5 in Gregory et al. (29).

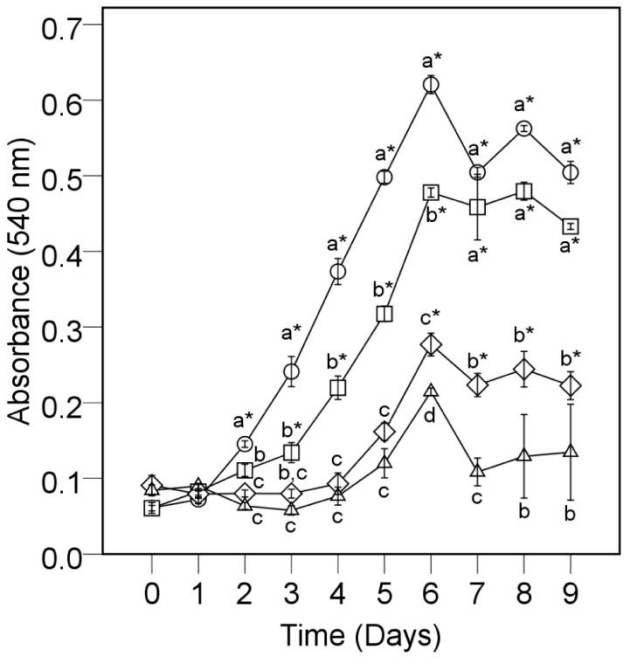

Figure 2: The effects of serum concentration on the proliferation of the SBT-E1 cells in culture. Cells were cultured in 96-well plates at 25°C in 100 µl/well of Leibovitz's L-15 medium supplemented with 15 mM HEPES (pH 7.4) and either 10% (v/v) FBS (○), 5% (v/v) FBS (□), 2% (v/v) FBS (◊) or 1% (v/v) FBS (Δ) for up to 9 days. Data are the mean of 3 replicate wells. Error bars show ± standard error of the mean. The data were analysed using two separate one-way analyses of variance followed by Bonferroni *post-hoc* comparisons. Significant differences between different FBS concentrations on a particular day are indicated by different letters. Significant differences between a particular day and day zero for a particular FBS concentration are indicated by asterisks.

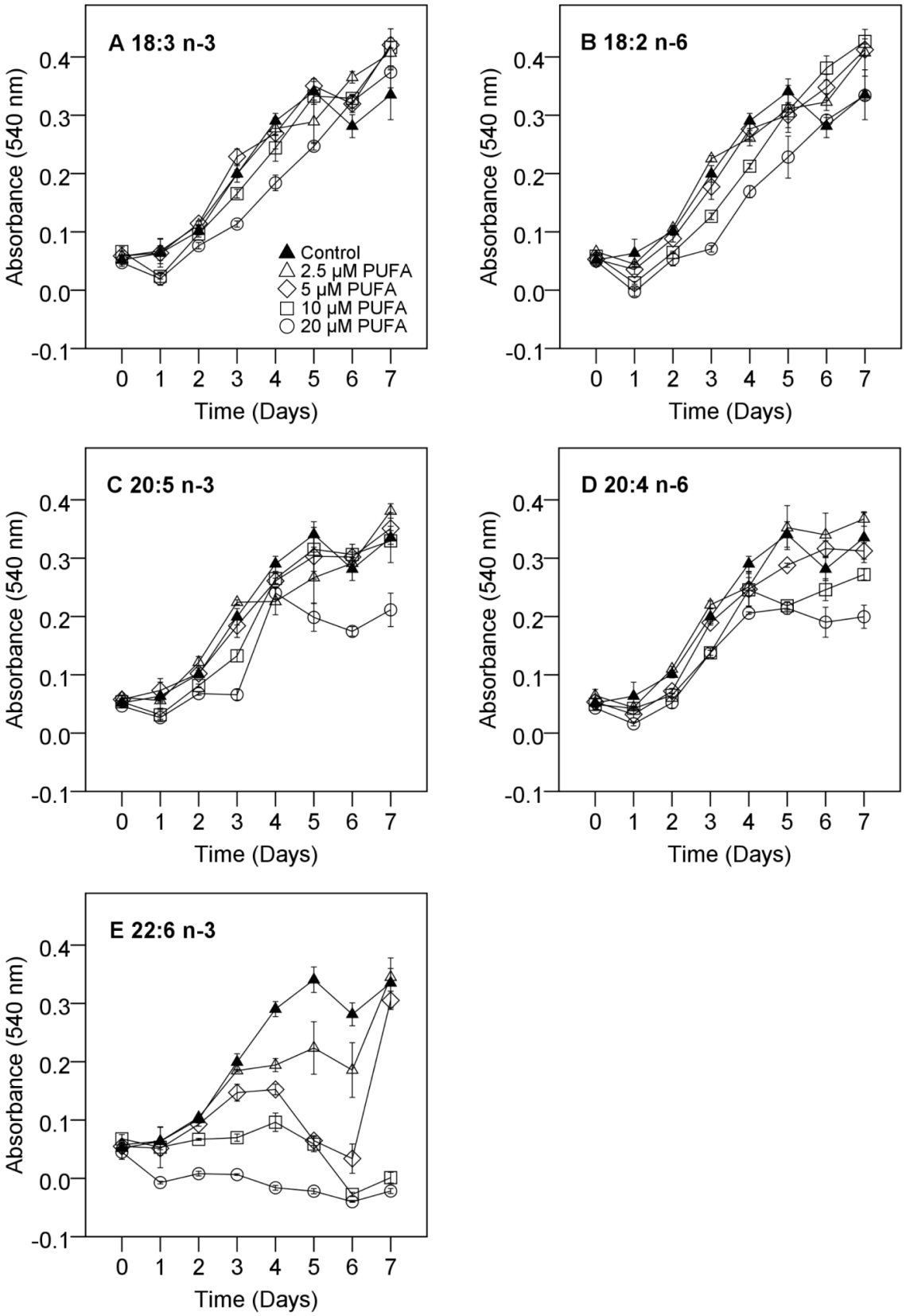

Figure 3: Effects of PUFA supplements on the proliferation of SBT-E1 cells in culture. Cells were cultured in 96-well plates at 25°C in 100 µl/well of Leibovitz's L-15 medium supplemented with 15 mM HEPES (pH 7.4), 2% (v/v) FBS and either

18:3 n-3 (A), 18:2 n-6 (B), 20:5 n-3 (C), 20:4 n-6 (D) or 22:6 n-3 (E) at final concentrations of 0 μM (▲), 2.5 μM (Δ), 5 μM (◊), 10 μM (□) or 20 μM (○). Data are the mean of 3 replicate wells and error bars show ± standard error of the mean. Significant differences between different fatty acid concentrations on a particular day or between a particular day and day zero for a particular fatty acid concentration were determined using two separate one-way analyses of variance followed by Bonferroni *post-hoc* comparisons. Where appropriate, the results of these statistical analyses are given in the text.

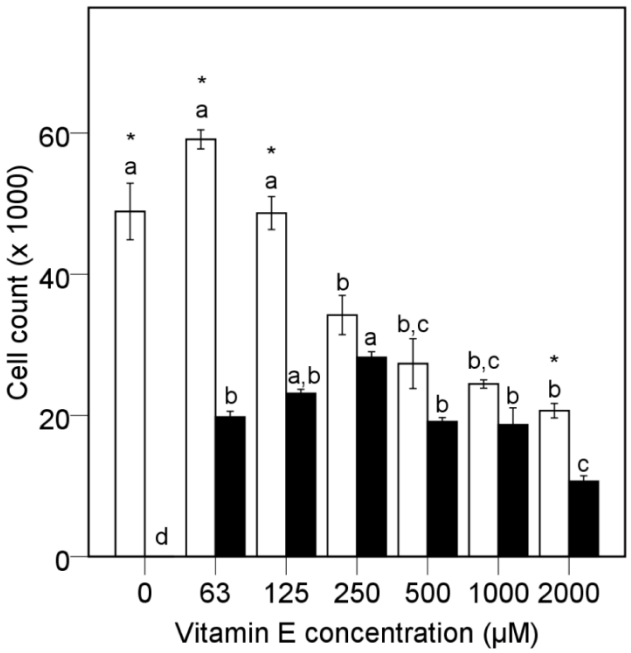

Figure 4: Effects of different vitamin E concentrations on cell number after 4 days of culture of SBT-E1 cells with or without 20 µM DHA. The cells were cultured in 24-well plates at 25°C in 600 µl of Leibovitz's L-15 medium supplemented with 15 mM HEPES (pH 7.4) and either 2% FBS (□) or 2% FBS + 20 µM DHA (■), at a range of vitamin E concentrations from 0-2 mM. Data are the mean of 3 replicate wells and error bars show ± standard error of the mean. Different letters denote significant differences within either the 2% FBS or 2% FBS + 20 µM DHA treatment and asterisks denote significant differences between the plus and minus DHA treatments at a particular vitamin E concentration as determined using two separate one-way analyses of variance followed by Bonferroni *post-hoc* comparisons.

Tables

Table 4: The effect of fatty acid supplementation on the fatty acid profile of the total lipid extracted from the SBT-E1 cells. The cells were cultured for 4 days in Leibovitz's L-15 medium supplemented with 15 mM HEPES (pH 7.4), 125 µM vitamin E and either 2% (v/v) FBS, 10% (v/v) FBS or 2% (v/v) FBS plus 10 µM ALA, LNA, EPA, ARA or DHA. The data are expressed as mole% of total fatty acids and are the mean of 3 replicates. The figures in parentheses are the standard error of the mean. Significant differences between the different fatty acid treatments were determined for each individual fatty acid using one-way analysis of variance followed by Bonferroni *post-hoc* comparisons. Values in the same row with different superscript letters were significantly different from one another ($p < 0.05$).

		Cells						
Fatty acid	Culture medium[1]	2% FBS	2% FBS + ALA	2% FBS + LNA	2% FBS + EPA	2% FBS + ARA	2% FBS + DHA	10% FBS
14:0	2.5(0.5)^{a}	1.3(0.2)^{bc}	1.1(0.1)^{bc}	1.1(0.1)^{bc}	1.5(0.0)^{abc}	1.7(0.1)^{abc}	1.7(0.0)^{ab}	0.7(0.0)^{c}
16:0	24.8(1.0)^{a}	17.7(2.0)^{ab}	15.0(0.6)^{b}	15.1(1.0)^{b}	18.7(0.6)^{ab}	17.9(0.8)^{ab}	17.4(2.7)^{b}	15.9(0.3)^{b}
18:0	7.1(0.4)^{b}	13.0(2.0)^{a}	11.2(0.6)^{ab}	10.7(0.5)^{ab}	12.2(0.6)^{a}	11.0(0.6)^{ab}	12.9(0.1)^{a}	11.1(0.1)^{ab}
Total SFA[2]	54.0(3.9)^{a}	36.7(2.6)^{b}	31.6(0.6)^{b}	31.1(1.6)^{b}	36.5(0.9)^{b}	34.3(1.2)^{b}	36.5(3.4)^{b}	33.4(0.4)^{b}
16:1n-7	2.5(0.3)^{ab}	4.2(0.8)^{a}	2.5(1.0)^{ab}	1.2(0.2)^{b}	2.8(0.5)^{ab}	1.6(0.1)^{ab}	1.6(0.2)^{ab}	3.6(0.1)^{ab}
18:1n-9	12.9(3.3)^{ab}	17.8(1.9)^{a}	12.7(3.6)^{ab}	7.8(0.6)^{b}	12.7(0.4)^{ab}	10.3(0.6)^{ab}	10.7(0.3)^{ab}	20.2(0.3)^{a}
18:1n-7	2.7(0.2)^{ab}	5.2(0.6)^{a}	3.4(1.3)^{ab}	2.1(0.2)^{b}	3.4(0.2)^{ab}	2.7(0.1)^{ab}	2.9(0.2)^{ab}	5.1(0.1)^{a}
Total MUFA[3]	36.4(4.3)^{ab}	30.8(3.2)^{a}	19.9(5.8)^{b}	12.5(1.1)^{c}	20.9(0.8)^{b}	16.1(0.7)^{c}	17.0(0.6)^{b}	32.3(0.3)^{ab}
18:2n-6	2.2(0.4)^{b}	4.1(1.1)^{b}	1.3(0.6)^{b}	36.6(4.5)^{a}	2.6(0.5)^{b}	2.4(0.4)^{b}	2.7(0.5)^{b}	3.4(0.1)^{b}
18:3n-6	0.0(0.0)^{b}	0.3(0.2)^{ab}	0.3(0.1)^{ab}	0.2(0.1)^{ab}	0.4(0.0)^{ab}	0.7(0.1)^{a}	0.6(0.3)^{ab}	0.6(0.0)^{ab}
20:2n-6	0.0(0.0)^{b}	0.4(0.4)^{ab}	0.0(0.0)^{b}	1.1(0.2)^{a}	0.0(0.0)^{b}	0.0(0.0)^{b}	0.0(0.0)^{b}	0.1(0.0)^{b}
20:4n-6	1.8(0.6)^{e}	7.4(0.2)^{bc}	6.1(0.5)^{bcd}	4.9(0.3)^{cd}	4.6(0.6)^{d}	22.3(0.8)^{a}	4.2(0.1)^{de}	8.5(0.1)^{b}
22:4n-6/22:3n-3	0.0(0.0)^{d}	1.1(0.0)^{bc}	1.2(0.0)^{bc}	0.9(0.1)^{bc}	1.0(0.1)^{bc}	11.0(0.3)^{a}	0.7(0.1)^{c}	1.6(0.1)^{b}
Total n-6[4]	6.0(0.1)^{c}	15.2(1.4)^{b}	10.1(0.1)^{bc}	45.4(3.6)^{a}	9.5(1.3)^{bc}	37.7(0.7)^{a}	9.1(0.7)^{bc}	16.7(0.1)^{b}
18:3n-3	0.2(0.1)^{b}	0.0(0.0)^{b}	23.1(4.8)^{a}	0.0(0.0)^{b}	0.0(0.0)^{b}	0.1(0.1)^{b}	0.0(0.0)^{b}	0.1(0.0)^{b}
20:3n-3	0.0(0.0)^{c}	0.0(0.0)^{c}	1.7(0.1)^{a}	0.0(0.0)^{c}	0.0(0.0)^{c}	0.0(0.0)^{c}	0.0(0.0)^{c}	0.2(0.0)^{b}
20:5n-3	1.2(0.6)^{bc}	1.1(0.1)^{bc}	0.8(0.1)^{bc}	0.5(0.1)^{bc}	12.2(0.2)^{a}	0.3(0.0)^{c}	1.7(0.3)^{b}	1.1(0.1)^{bc}
22:5n-3	0.8(0.1)^{f}	4.6(0.2)^{bc}	4.0(0.4)^{bcd}	3.2(0.2)^{cd}	14.1(0.8)^{a}	2.7(0.2)^{de}	1.3(0.1)^{ef}	5.6(0.1)^{b}
22:6n-3	1.7(0.2)^{d}	6.6(0.2)^{bc}	5.4(0.5)^{bc}	4.3(0.5)^{cd}	4.5(0.2)^{cd}	3.4(0.1)^{cd}	31.0(1.6)^{a}	8.2(0.1)^{b}
Total n-3[5]	3.3(0.4)^{d}	12.3(0.4)^{bc}	35.0(3.9)^{a}	8.1(0.8)^{bcd}	30.9(0.5)^{a}	6.6(0.4)^{cd}	34.1(1.8)^{a}	15.3(0.2)^{b}
Total PUFA[6]	10.4(0.4)^{e}	27.9(1.5)^{d}	45.7(4.2)^{ab}	53.6(2.9)^{a}	40.4(0.8)^{bc}	44.3(0.5)^{ab}	43.2(1.2)^{ab}	32.4(0.2)^{cd}
PI[7]	38.0(3.2)^{f}	147.1(3.6)^{de}	156.7(4.5)^{cd}	121.4(4.5)^{e}	222.4(1.1)^{b}	185.3(1.9)^{c}	292.5(13.4)^{a}	158.6(1.1)^{cd}

Abbreviations: SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; PI, peroxidation index.

[1]The culture medium was Leibovitz's L-15 medium supplemented with 15 mM HEPES (pH 7.4) and 5% (v/v) FBS [2]Also includes 8:0, 9:0, 12:0, 17:0 20:0 22:0 and 24:0. [3]Also includes 16:1 n-9, 17:1, 20:1 n-12, 20:1 n-11, 20:1 n-9, 22:1 n-11, 22:1 n-9 and 24:1. [4]Also includes 20:3 n-6, 22:2 n-6 and 22:5 n-6. [5]Also includes 16:2 n-3. [6]Also includes 18:2 n-9, 20:2 n-9, 20:3 n-9.20:3 n-6, 22:2 n-6, 22:5 n-6 and 16:2 n-3. [7]The peroxidation index was calculated using the formula: 0.025 x (% monoenoics) + 1 x (% dienoics) + 2 x (% trienoics) + 4 x (% tetraenoics) + 6 x (% pentaenoics) + 8 x (% hexaenoics) from Hulbert et al. (43).

Appendix B – Published manuscript 2

Reference:

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Dynamics of fatty acid metabolism in a cell line from southern bluefin tuna (*Thunnus maccoyii*)

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Abstract

Bluefin tunas are large predatory marine fish of great commercial value but little is known of their specific nutritional requirements. The three species are farmed in sea cages in Australia, the Mediterranean, Mexico and Japan where they are fed small oily fish sourced from wild-catch fisheries. This may not be sustainable and, therefore, it is important to investigate the possible consequences of the replacement of wild-catch fisheries products (fish oil and fish meal) with alternative oil and meal sources in feeds for these fish. To this end we have studied fatty acid metabolism in a recently developed southern bluefin tuna (SBT, *Thunnus maccoyii*) cell line designated SBT-E1. The predominant fatty acids in the total lipid of the SBT-E1 cells were 16:0, 18:0 and 18:1n-9. There were also substantial amounts of 20:4n-6, 22:5n-3 and 22:6n-3 but only very limited amounts of 18:2n-6, 18:3n-3 or 20:5n-3. The fatty acid composition of the cells reflected that of the culture medium except that 20:4n-6, 22:5n-3 and 22:6n-3 were substantially more abundant in the cells than in the medium. Fatty acid esterification occurred predominantly into phosphatidylcholine (PC) and phosphatidylethanolamine (PE), the two most abundant classes of lipids. The SBT-E1 cells showed very limited Δ6 fatty acyl desaturase (Fads) activity towards either 18:3n-3 or 18:2n-6 but substantial elongation of very long chain fatty acids (Elovl) activity towards 20:5n-3. The latter activity is usually attributable to an Elovl5 enzyme. Surprisingly though, there were much higher levels of Δ6 Fads compared with Elovl5 gene expression in the SBT-E1 cells, suggesting that a different Elovl enzyme may catalyse this reaction in SBT. The cells also showed substantial β-oxidation of 18:3n-3 and 20:5n-3 but much less activity towards 18:0, 18:1n-9 or 18:2n-6. These results may explain the high 22:6n-3 to 20:5n-3 ratios found in the SBT tissues, especially in their phospholipids. The

results are discussed in terms of the presumed nutritional requirements of bluefin tunas given their high trophic level in marine food webs.

Keywords

Bluefin tuna; fatty acid metabolism; esterification; desaturase; elongase; β-oxidation

1. Introduction

Bluefin tunas are large predatory marine fish of great commercial value but little is known of their specific nutritional requirements other than from studies of their natural prey (Itoh et al., 2011; Logan et al., 2011; Masuma et al., 2008; Metian et al., 2014; Miyake et al., 2010; Mylonas et al., 2010; Shimose et al., 2013; Skirtun et al., 2013; Woodhams et al., 2013). The three bluefin tuna species are farmed in sea cages in the Spencer Gulf of South Australia (southern bluefin tuna, *Thunnus maccoyii*), the Mediterranean Sea (Atlantic bluefin tuna, *Thunnus thynnus*) and along the Pacific coast of Mexico and the southern coast of Japan (Pacific bluefin tuna, *Thunnus orientalis*). For the most part, this involves the on-growing and/or fattening of wild-caught juveniles due to the limited success of captive breeding (Bubner et al., 2012; De Metrio et al., 2010; Masuma et al., 2008; Okada et al., 2014; Sawada et al., 2005; Tsuda et al., 2012; Yúfera et al., 2014). The capture of bluefin tunas from the wild is limited by strict catch quotas due to concerns regarding their declining stocks (CCSBT; ICCAT). Therefore, the purpose of bluefin tuna farming is to maximise commercial returns from the limited number of fish available. This is done by on-growing the fish to a larger size, increasing the fat content of their muscle (desired by Japanese sashimi consumers) and holding fish back from the market at times when over supply or currency fluctuations bring sub-optimal prices (Miyake et al., 2010; Mylonas et al., 2010; Skirtun et al., 2013; Woodhams et al., 2013). The main market for bluefin tunas is Japan and the main use is for the production of the Japanese raw fish delicacies sushi and sashimi. On-growing and fattening of bluefin tunas is achieved by feeding them small pelagic fish (e.g. sardines, mackerels, herrings) sourced from wild-catch fisheries (Miyake et al., 2010; Musgrove et al., 2011; Mylonas et al., 2010). This mimics their natural diets and so presumably satisfies

their nutritional requirements but it is not a sustainable practice (Mourente and Tocher, 2009).

In the past 15-20 years, the growth of wild-catch fisheries production has failed to keep pace with the growth of aquaculture and as a result the use of wild-caught fish to feed farmed fish has been called into question (Metian et al., 2014; Naylor et al., 2009; Tacon and Metian, 2013). In other farmed fish species, such as Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*), significant progress has been made in the replacement of wild-catch fisheries products (fish oil and fish meal) with alternatives such as vegetable oils and plant meals but this has not been the case for tunas (Metian et al., 2014; Tacon and Metian, 2013). There are several reasons for this. The first is that Japanese sushi and sashimi consumers prefer wild-caught tunas or, at the very least, farmed tunas that have been fed their natural diet (Ottolenghi, 2008). The second is that classical feeding trials with proper replication have not been possible due to the high commercial value of wild-caught bluefin tunas, the high costs of maintaining them in sea cages or purpose built facilities on land and the limited success of captive breeding programmes (Mourente and Tocher, 2009; Mylonas et al., 2010). The third reason is that replacement of fish oils with vegetable oils in feeds for farmed fish reduces their flesh concentrations of the omega-3 (n-3) long-chain polyunsaturated fatty acids (LC-PUFA) eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3) (Miller et al., 2008; Turchini et al., 2009). These fatty acids have a range of important human health benefits including reduced risk of cardiovascular disease and amelioration of the symptoms of inflammatory disorders such as rheumatoid arthritis (Monteiro et al., 2014; Yates et al., 2014).

Fish oils contain high concentrations of 20:5n-3 and 22:6n-3 whereas vegetable oils completely lack these fatty acids (Turchini et al., 2009). Some vegetable oils, such as linseed or canola oil, contain high concentrations of α-linolenic acid (18:3n-3), the precursor of 20:5n-3 and 22:6n-3. However many fish species, especially marine piscivores, have only limited capacity to convert 18:3n-3 to 20:5n-3 or 22:6n-3 (Ghioni et al., 1999; Tocher, 2003; Tocher and Ghioni, 1999). It is assumed, though it has not been tested, that tunas would also have limited capacity for endogenous production of LC-PUFA from C_{18} PUFA (Mourente and Tocher, 2009).

In vertebrates, including fish, *de novo* synthesis of 20:5n-3 and 22:6n-3 from 18:3n-3 proceeds via a series of reactions catalysed by fatty acyl desaturase (Fads) and elongation of very long chain fatty acids (Elovl) enzymes (Fig. 1). The most commonly observed pathway is as follows. In the first step, 18:3n-3 is desaturated to 18:4n-3 by a Δ6 Fads. In the second step, 18:4n-3 is elongated to 20:4n-3 by an Elovl5. This is followed by desaturation of 20:4n-3 to 20:5n-3 catalysed by a Δ5 Fads and elongation of 20:5n-3 to 22:5n-3 catalysed by the same Elovl5 as mentioned above. Subsequently, 22:5n-3 is elongated to 24:5n-3 by an Elovl2 and 24:5n-3 is desaturated to 24:6n-3 by the same Δ6 Fads as mentioned above. Finally, partial β-oxidation of 24:6n-3 to yield 22:6n-3 occurs in the peroxisomes. It is important to note that there is competition between different intermediates within this pathway for the same enzymes. There is also competition from the corresponding omega-6 (n-6) fatty acids that are abundant in certain vegetable oils such as linoleic acid (18:2n-6) in soybean oil.

Marine fish have an abundance of 20:5n-3 and 22:6n-3 in their natural diets and this probably explains their limited capacity to synthesise these fatty acids *de novo* (Tocher, 2003). Whatever the case may be, and in light of the great commercial value

of bluefin tuna aquaculture, it is important to gain a better understanding of their fatty acid metabolism to support the development of more sustainable artificial feeds. To this end, we have taken advantage of a recently established tuna cell line derived from southern bluefin tuna (*Thunnus maccoyii*) (Bain et al., 2013). Using this cell line, we have investigated the esterification into various lipid classes and the metabolism via desaturation, elongation and/or β-oxidation of a selection of fatty acids known to be quantitatively important in tuna tissues.

2. Materials and methods

2.1 Cell culture

For routine maintenance, the SBT-E1 cell line established from an anterior body cross-section of a captive-bred southern bluefin tuna fingerling was cultured as previously described (Scholefield and Schuller, 2014). The cell line was originally a primary culture and is now presumed to be immortalized. The experiments described in this paper were conducted between passages 43 and 54. The standard culture medium consisted of Leibovitz's L-15 medium supplemented with 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.4) and 10% (v/v) foetal bovine serum (FBS) and the culture temperature was 25°C. The species of origin of the SBT-E1 cells was confirmed by sequencing a fragment of the mitochondrial cytochrome *c* oxidase (*cox1*) gene amplified by polymerase chain reaction as described previously (Bain et al., 2013).

2.2. Lipid class composition of the total lipid from the SBT-E1 cells

The SBT-E1 cells were cultured as described above and total lipid was extracted in chloroform/methanol (2:1, v/v) containing 0.05% (w/v) butylated hydroxytoluene as anti-oxidant (Tocher et al., 1988). Lipid classes were separated by high performance-

thin layer chromatography (HPTLC) as previously described (Bell et al., 1993) and individual classes were visualised by charring at 160^{o}C for 15 min after the HPTLC plate had been sprayed with 3% (w/v) copper acetate in 8% (v/v) phosphoric acid. The relative abundance of each lipid class was determined by scanning densitometry using a ChemiDoc^{TM} MP imaging system (BIO-RAD). The identity of each lipid class was determined by running authentic standards in lanes next to those containing the SBT-E1 cell lipid extracts.

2.3. Fatty acid composition of the total lipid and the various lipid classes from the SBT-E1 cells

The SBT-E1 cells were cultured, total lipid was extracted and the different classes of lipids were separated as described above. The separated lipids were visualised by spraying the plates with fluorescein 5-isothocyanate in methanol and the areas of silica gel corresponding to the different lipid classes were scraped from the plates into separate vials. Fatty acid methyl esters (FAME) were produced from the different lipid classes and analysed by gas chromatography as previously described (Gregory et al., 2011).

2.4 Fatty acid composition of the total lipid and the phospholipids from SBT tissues

Tissue samples were obtained from SBT specimens with fork lengths of 114 – 130 cm during a normal commercial harvest from a tuna farm located near Port Lincoln, South Australia. The fish were gilled and gutted and the tail was removed. Samples of the tail muscle, liver, intestine and kidney were frozen on dry ice. Upon return to the laboratory, total lipid was extracted from the tissues using chloroform/methanol (2:1 v/v) containing 0.005% (w/v) butylated hydroxyanisole using the method described by Bligh and Dyer (1959) with some modifications as described by

Makrides et al. (1996). Subsamples of the total lipid were separated into polar (mostly phospholipids) and neutral lipids using thin layer chromatography (TLC) (Pahl et al., 2010). Briefly, the subsamples were dried under a stream of nitrogen, reconstituted in 150 μl of chloroform/methanol (9:1 v/v) and then applied in streaks to TLC plates. The plates were developed in petroleum spirit/diethyl ether/acetic acid (180:30:2 v/v/v) and the separated lipid classes were visualised by drying the plates, spraying them with fluorescein 5-isothocyanate in methanol and then exposing them to UV light. The bands of silica gel corresponding to the phospholipids were scraped into glass vials containing 2 mL of 1% (v/v) H_2SO_4 in methanol and FAME were produced by heating the vials to 70°C for 3 hours. Finally, the FAME were extracted and analysed as previously described (Gregory et al., 2011).

2.5. Incubation of the SBT-E1 cells with [1-^{14}C]-labelled fatty acids

The ^{14}C-labelled fatty acids were purchased from Perkin Elmer NEN® (Perkin Elmer, Cambridge, UK) at a specific activity of 50-55 mCi mmol^{-1}. Complexes of the labelled fatty acids with fatty acid free-bovine serum albumin (FAF-BSA) in Hanks' Balanced Salt Solution (HBSS) were prepared as described previously (Ghioni et al., 1999). To label the cells, they were cultured in the standard culture medium, harvested and resuspended in the same medium but with the FBS concentration reduced to 5% (v/v). The resuspended cells were seeded at a density of 25,000 cells cm^{-2} into the required number of 75 cm^2 cell culture flasks in a final volume of 15 ml of the culture medium with 5% FBS. Once the cells had adhered to the flasks (after approximately 4 h), 0.25 μCi (0.33 μM) of each [1-^{14}C]-labelled fatty acid complexed with FAF-BSA was added to the appropriate flasks and the cells incubated at 25°C for 72 h.

2.6. Esterification of [1-^{14}C]-labelled fatty acids into individual lipid classes

At the end of the incubation, the culture medium was removed and the cells were washed with phosphate buffered saline (PBS) before being detached using 0.05% (w/v) trypsin/0.02% (w/v) ethylenediaminetetraacetic acid (EDTA) in PBS. The detached cells were pelleted by centrifugation and then washed with 1% (w/v) FAF-BSA in HBSS to remove any residual [1-^{14}C]-labelled fatty acids. Total lipid was extracted as described above and the different classes of lipids were separated on a HPTLC plate developed in methyl acetate/isopropanol/chloroform/methanol/0.25% (w/v) aqueous KCl (25/25/25/10/9, by volume) as described previously (Vitello and Zanetta, 1978). The separated lipids were visualised by placing the plate in a tank saturated with iodine vapour. The areas of silica gel corresponding to the different classes of lipids were scraped from the plate into separate vials and radioactivity was assayed using a TRI-CARB 2000CA scintillation counter (United Technologies Packard, Pangbourne, UK). The transformed spectral index of external standard (tSIE) value was used as a measure of quenching. This value was used to calculate the counting efficiency according to the equation, efficiency = (tSIE x 0.033) + 56 based on a quenching calibration curve. This efficiency value was then used to convert counts per minute (cpm) to disintegrations per minute (dpm) using the equation dpm = (cpm/efficiency) x 100.

2.7. Metabolism of incorporated [1-^{14}C]-labelled fatty acids by desaturation and/or elongation

The cells were incubated with [1-^{14}C]-labelled fatty acids and total lipid extracted as described above. FAME were produced by incubating the total lipid with 1% (v/v) H_2SO_4 in methanol at 55°C for 16 h (Christie, 1993). The FAME were extracted as described previously (Tocher and Harvie, 1988) and dissolved in 100 µl of hexane containing 0.01% (w/v) BHT. The dissolved FAME were separated on a thin-layer

chromatography (TLC) plate (20 x 20 x 0.25 cm) impregnated with 10% (w/v) $AgNO_3$ in acetonitrile as described previously (Ghioni et al., 1999). Areas of silica gel corresponding to individual FAME were detected by autoradiography (Kodak MR2 X-ray film, 16 days exposure at room temperature), scraped from the plate and assayed for radioactivity as described above.

2.8. β-oxidation of [1-^{14}C]-labelled fatty acids

Oxidation of [1-^{14}C]-labelled fatty acids was determined essentially as described previously but with some modifications (Tocher et al., 2004). The cells were incubated with [1-^{14}C]-labelled fatty acids as described above and at the end of the incubation, a 0.5 mL subsample of the medium was taken and set aside. The remainder of the medium was discarded, the cell monolayer washed with PBS and the cells detached with 0.05% (w/v) trypsin/0.02% (w/v) EDTA in PBS. The isolated cells were washed in HBSS containing 1% (w/v) FAF-BSA before being resuspended in HBSS alone and homogenised using an ULTRA-TURRAX® (IKA®) mechanical homogeniser. A subsample (0.5 mL) of the homogenate was taken and set aside. One-hundred microlitres of 6% (w/v) FAF-BSA in water was added to the subsamples of both the culture medium and the homogenised cells before acid-insoluble products were precipitated by the addition of 1.0 mL of ice-cold 4 M $HClO_4$. The samples were centrifuged at 3,500 *g* for 10 min and 0.5 mL of the supernatant was mixed with 4 mL of scintillation fluid and radioactivity was assayed as described above. The data for the culture medium and the cells were combined to give the rate of β-oxidation per 10^6 cells.

2.9. Incubation of the SBT-E1 cells with unlabelled fatty acids

Unlabelled fatty acids were purchased from Cayman Chemical Company in solution in ethanol. The cells were cultured in the standard culture medium (see above) but

with the FBS concentration set at 2, 5 or 10% (v/v). The cells were seeded at a density of 25,000 cells cm^{-2} into the required number of 75 cm^2 cell culture flasks in a final volume of 15 ml of the culture medium. Once the cells had adhered to the flasks (after approximately 4 h), 125 μM vitamin E and 10 μM of each unlabelled fatty acid, complexed with fatty acid free bovine serum albumin, was added to the appropriate flasks as described previously (Scholefield and Schuller, 2014). The cells were then incubated at 25°C for either 24 or 72 h.

2.10. RNA extraction and first-strand cDNA synthesis

RNA was extracted from the SBT-E1 cells incubated with unlabelled fatty acids using an RNeasy® Mini Kit (Qiagen) according to the manufacturer's instructions. One μg of this RNA was then used to synthesise cDNA using a SuperScript® III first-strand synthesis kit (Invitrogen) according to the manufacturer's instructions. Any remaining RNA in the cDNA preparations was digested using *Escherichia coli* RNase H and the cDNA was diluted 5-fold in nuclease free water.

2.11. Quantitative real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using a Platinum® SYBR® Green qPCR SuperMix-UDG kit (Invitrogen) according to the manufacturer's instructions. Each reaction contained the cDNA reverse-transcribed from 50 ng of RNA and 125 nM (each) of one of the pairs of primers in Table 1. Amplicon size and identity was confirmed for each of the primer sets by sequencing and efficiency was experimentally determined using serial dilutions of purified PCR products as template in a qRT-PCR reaction spanning ≥ 5 orders of magnitude. Efficiencies in the range of 0.9-1.0 are considered valid (Kubista et al., 2006). Thus each of the primer combinations were quantitatively validated for qRT-PCR. The

reactions were performed and the data were analysed using a Rotorgene 3000 system (Corbett Research). The fluorescence curves produced for each sample were used to calculate the threshold cycle (Ct) value and the average fold-change in expression compared to a β-actin normalisation gene and the control cells was obtained using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.11. Statistical analyses

The lipid and fatty acid composition data were analysed using one-way analysis of variance (ANOVA) followed by Bonferroni *post-hoc* comparisons. The gene expression data were analysed using one-way analysis of variance (ANOVA) followed by Dunnett's 2-sided multiple comparison test. Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. Lipid class composition of the total lipid from the SBT-E1 cells

The lipid class composition of the total lipid from the SBT-E1 cells cultured in medium containing 5% (v/v) FBS is shown in Table 2. The total lipid consisted of approximately equal proportions of polar and neutral lipids. Amongst the polar lipids, PC was the most abundant (26.2% of total lipid) followed by PE (15.1% of total lipid). In terms of relative abundance, both PC and PE were significantly more abundant than any of the other polar lipid classes and PC was significantly more abundant than PE.

3.2. Fatty acid composition of the total lipid and the various lipid classes from the SBT-E1 cells

Table 3 shows the fatty acid compositions of the total lipid and the various lipid classes from the SBT-E1 cells compared with the fatty acid composition of the total lipid from the culture medium. The culture medium was rich in saturated fatty acids (especially 16:0 and 18:0) and monounsaturated fatty acids (especially 18:1n-9) but poor in polyunsaturated fatty acids (PUFA) (including 18:2n-6 and 18:3n-3) and LC-PUFA (including 20:4n-6, 20:5n-3, 22:5n-3 and 22:6n-3). This was as expected because foetal bovine serum (FBS) was the source of the fatty acids in the culture medium (Scholefield and Schuller, 2014). The fatty acid composition of the total lipid from the SBT-E1 cells reflected that of the culture medium except that there was evidence for enrichment of the cells with 20:4n-6, 22:5n-3 and 22:6n-3. The various fatty acids were distributed differently between the different classes of lipids. Neutral lipids were rich in 16:0 whereas PC was rich in 18:1n-9. PE was rich in the most abundant LC-PUFA 20:4n-6, 22:5n-3 and 22:6n-3. PE also contained the

majority of the 20:5n-3 but the level was very low compared with the other LC-PUFA.

3.3. Fatty acid composition of the total lipid and the phospholipids from SBT tissues

The fatty acid profiles of the total lipid and the phospholipids from the SBT tissues are shown in Table 4. As in the cell line, 22:6n-3 was the most abundant PUFA in the total lipid and in the phospholipids followed by 20:5n-3 but the proportions of these fatty acids (as % of total fatty acids) were greater in the tissues than in the cell line which presumably reflects the dearth of these fatty acids in the cell culture medium. In the tissues, 22:6n-3 was more abundant in the phospholipids than in the total lipid whereas 20:5n-5 was not. This suggested selective retention of 22:6n-3 particularly in the phospholipids. As a result, the 22:6n-3 to 20:5n-3 ratio was greater in the phospholipids than in the total lipid.

3.4. Esterification of [1-^{14}C]-labelled fatty acids into different classes of lipids

The results for the esterification of [1-^{14}C]-labelled fatty acids into different classes of lipids in the SBT-E1 cells are shown in Fig. 2. Most of the fatty acids, other than 20:5n-3, were incorporated predominantly into PC. In the case of 20:5n-3, there was approximately equal incorporation into PC and PE. The label recovered in PC ranged from approximately 50% to a little more than 60% of the total for all of the fatty acids except 20:5n-3. Nearly 80% of the label from 20:5n-3 was recovered in both PC and PE with approximately 40% in each. When the fatty acid esterification data were expressed relative to the lipid class abundance data, there was clear preferential incorporation of 16:0 into PC and SM, 18:1n-9 into PC and PS and 18:2n-6 into PC and PA/CL (data not shown). The results for 18:3n-3 were similar to those for 18:2n-

6 though not as pronounced. This way of expressing the data also showed preferential incorporation of 20:5n-3 into PE over PC.

3.5. Metabolism of [1-^{14}C]-labelled PUFA by desaturation and/or elongation

The results for the desaturation/elongation of incorporated [1-^{14}C]-labelled PUFA by the SBT-E1 cells are shown in Table 5. Cells supplied with [1-^{14}C]18:3n-3 converted 12.8% of this substrate to desaturation/elongation products. The majority of this conversion was to the C_2 elongation product 20:3n-3 (9.4%) but there was also some Δ6 desaturation to yield 18:4n-3 (2.2%) and a small amount of Δ6 desaturation plus C_2 elongation to yield 20:4n-3 (1.2%). In contrast, the cells supplemented with [1-^{14}C]18:2n-6 showed approximately equal amounts of either C_2 elongation (2.8%) or Δ6 desaturation (2.5%) but no detectable Δ6 desaturation plus C_2 elongation and the total amount of substrate conversion (5.3%) was significantly less than for 18:3n-3. In contrast to the above, the cells supplied [1-^{14}C]20:5n-3 showed substantial C_2 elongation (16.8% substrate conversion). Thus, the cells exhibited appreciable fatty acyl elongase activity but only very limited Δ6 Fads activity.

3.6. β-oxidation of [1-^{14}C]-labelled fatty acids

The results for the β-oxidation of incorporated [1-^{14}C]-labelled fatty acids by the SBT-E1 cells are shown in Fig. 3. The rates obtained for 20:5n-3 and 18:3n-3 were approximately 2- and 4-fold greater, respectively, than those obtained for 18:0, 18:1n-9 or 18:2n-6. Thus, the cells showed high rates of β-oxidation of n-3 PUFA, especially 18:3n-3 but also 20:5n-3.

3.7. Effects of FBS concentration and fatty acid supplementation on Δ6 Fads and Elovl5 gene expression in the SBT-E1 cell line

In cell culture, the main source of fatty acids to support cell proliferation is the FBS in the culture medium (Tocher et al, 1988). In Fig. 4A, the SBT-E1 cells were cultured with or without various PUFA in the presence of 5% (v/v) FBS. Addition of the PUFA to the culture medium significantly reduced the expression of Δ6 Fads but not Elovl5. In Fig. 4b, the SBT-E1 cells were cultured either in the presence of 10% (v/v) FBS or with or without various PUFA in the presence of 2% (v/v) FBS. In this case, reducing the FBS concentration from 10% to 2% (v/v) significantly increased the expression of Δ6 Fads and supplementation of the culture medium with the various PUFA in the presence of 2% FBS returned the expression of Δ6 Fads to the low level seen in the presence of 10% (v/v) FBS. Thus, fatty acid starvation (i.e. reduction of the FBS concentration from 10% to 2% (v/v)) increased the expression of Δ6 Fads but this was reversed when the cells were supplied with various PUFA. Similarly, Elovl5 gene expression appeared to be higher in cells cultured in 2% (v/v) FBS compared to 10% (v/v) FBS but there were no significant differences between the different treatments. In Fig. 4C, the absolute expression (transcript copy number relative to β-actin transcript copy number) of Δ6 Fads is compared with that of Elovl5. Interestingly, expression of the Δ6 Fads gene was more than 2 orders of magnitude greater than that of the Elovl5 gene.

4. Discussion

4.1. Lipid class composition of the total lipid from the SBT-E1 cells

In established cell lines from a range of freshwater, anadromous and marine fish species, polar lipids (mostly phospholipids) accounted for 40.5 to 76.1% of the total lipid and the most abundant phospholipid was PC (19.4 to 40.8% of total lipid) followed by PE (17.3 to 23.7% of total lipid) (Tocher et al., 1989; Tocher and Dick, 1999; Tocher and Ghioni, 1999; Tocher et al., 1988). Similarly, in the SBT-E1 cell

line, phospholipids accounted for 59.3% of the total lipid and the most abundant phospholipid was PC (26.2% of total lipid) followed by PE (15.1% of total lipid). Interestingly, PE was less abundant in the SBT-E1 cells compared with the other cells. Studies with fish tissues have shown that PC is almost always the most abundant phospholipid, followed by PE (Morais et al., 2011; Sprague et al., 2012; Tocher, 1995). Phospholipids, predominantly found in cell membranes, are important in maintaining structural integrity and fluidity and are not found in high concentrations elsewhere in the cell. Cold acclimation is associated with increased proportions of PE and decreased proportions of PC in fish lipids (Tocher, 1995). The SBT-E1 cells were cultured at a higher temperature (25°C) than the other cells (22°C) (Tocher et al., 1989; Tocher and Dick, 1999; Tocher and Ghioni, 1999; Tocher et al., 1988). This could perhaps explain the lower proportion of PE in the SBT-E1 cells. Alternatively, it could be due to the fact that tunas are partially endothermic (Graham and Dickson, 2004) with less need to adjust the fluidity of their membranes to cope with lower water temperatures.

The lipid class compositions of tuna tissues have not been studied in detail expect in longtail tuna (*Thunnus tonggol*) and Atlantic bluefin tuna (*Thunnus thynnus*) (Mourente et al., 2002; Saito et al., 2005; Sprague et al., 2012). In “ordinary muscle”, “dark muscle” and liver from longtail tuna, the relative amounts of polar and neutral lipids varied depending upon the tissue but the most abundant polar lipid class was almost always PC (10.5 to 24.3% of total lipid) followed by PE (7.5 to 25% of total lipid) (Saito et al., 2005). Similar results were found for red muscle, white muscle, liver and ovary from female Atlantic bluefin tuna during their reproductive migration to their spawning grounds in the Mediterranean Sea (Mourente et al., 2002; Sprague et al., 2012). Thus, broadly speaking, the SBT-E1 cells retained the characteristics of

their species of origin with respect to the phospholipid composition of their total lipid.

4.2. Fatty acid composition of the total lipid and phospholipids from the SBT-E1 cell line and SBT tissues

The most abundant fatty acids in the total lipid of the SBT-E1 cell line were 16:0, 18:0, 18:1n-9, 20:4n-6, 22:5n-3 and 22:6n-3. This was similar to cell lines from other marine fish species, namely turbot (*Scophthalmus maximus*) and gilthead seabream (*Sparus aurata*) (Ghioni et al., 1999; Tocher and Ghioni, 1999) and also to cell lines from freshwater and anadromous fish species (Tocher et al., 1989; Tocher and Dick, 1999; Tocher et al., 1988). However, there was one major difference and this was the appreciably greater abundance of 22:6n-3 in the tuna cell line (9.2% of total fatty acids) compared with freshwater or anadromous fish cell lines (1.1 – 3.8% of total fatty acids) and also with marine fish cell lines (3.9 – 5.5% of total fatty acids). This suggested selective retention of 22:6n-3 by the tuna cell line because the amount of 22:6n-3 supplied in the culture medium was very low (only 2% of total fatty acids). It has been observed that tunas caught from the wild have unusually high proportions of 22:6n-3 in their lipids and higher ratios of 22:6n-3 to 20:5n-3 than in their prey (Murase and Saito, 1996; Saito et al., 1996). For example, muscle tissues from yellowfin tuna (*Thunnus albacares*) caught off the Pacific coast of Japan contained 3.3 – 5.0% and 22.0 – 36.0% of their total fatty acids as 20:5n-3 and 22:6n-3, respectively, giving 22:6n-3 to 20:5n-3 ratios of 6.7 – 8.6 (Saito et al., 1996). This compared with 8.4% and 22.7% of their total fatty acids as 20:5n-3 and 22:6n-3, respectively, and a 22:6n-3 to 20:5n-3 ratio of only 2.7 for their stomach contents. Similar results have been found for Albacore (*Thunnus alalunga*) and longtail tuna (*Thunnus tonggol*) (Murase and Saito, 1996; Saito et al., 2005). This led to the

proposition that tunas selectively retain 22:6n-3 in their tissues. Selective retention could be due to selective esterification into cellular lipids or to sparing from β-oxidation and high 22:6n-3 to 20:5n-3 ratios could be due to selective metabolism of 20:5n-3 either via elongation to 22:5n-3 or via β-oxidation (Tocher, 2003). The initial reactions in the β-oxidation of 22:6n-3 in mammals involve the peroxisomes and this, presumably, is also true for fish (Madsen et al., 1999; Tocher, 2003). Thus, selective retention of 22:6n-3 could be due to inefficient oxidation as it involves partial oxidation in the peroxisomes followed by transfer to the mitochondria for completion. In the present study we found 22:6n-3 to 20:5n-3 ratios ranging from 2.09 to 2.92 in the total lipid and 2.96 to 5.11 in the phospholipids of the various tissues from the farmed SBT. These values are somewhat lower than those observed for yellowfin tuna (see above) which is due to the higher proportions of 20:5 in the SBT total lipid (6.99 to 9.09% of total fatty acids) as compared with the yellowfin tuna total lipid (3.3 to 5.0% of total fatty acids). This may reflect differences between the diets of wild tunas and farmed tunas (Saito et al., 2005). Overall though, the SBT-E1 cell line appears to have retained the traits of selective retention of 22:6n-3 and high ratios of 22:6n-3 to 20:5n-3 characteristic of tuna tissues.

The main fatty acids in the total lipid of the SBT-E1 cells showed different distributions between the different lipid classes. For example, neutral lipids contained a high proportion of 16:0, almost 40% of the total fatty acids. This compared with a median value of only 25% in other fish cell lines (Tocher et al., 1988). Similarly, PC contained a high proportion of 18:1n-9, approximately 43%. This compared with a median value of only 28% in other fish cell lines (Tocher et al., 1988). The important n-6 LC-PUFA, 20:4n-6, was found predominantly in PE and the combined PI/PA/CL fraction in the SBT-E1 cells. This was similar to several other fish cell lines (Tocher et al., 1988). In contrast, the important n-3 LC-PUFA,

22:6n-3, was found predominantly in PE, PS and SM. This was similar to a cell line from turbot, another marine fish species, but different to cell lines from freshwater and anadromous fish species. In general, LC-PUFA were incorporated predominantly into PE both in the SBT-E1 cell line and in other fish cell lines.

Although it was not particularly abundant, it is important to consider the distribution of 20:5n-3 because of its widely recognized human health benefits as the precursor to eicosanoids which are less pro-inflammatory than those derived from the equivalent n-6 LC-PUFA, 20:4n-6 (Yates et al., 2014). The only lipid class that contained significant amounts of 20:5n-3 was PE. In this lipid class, the 22:6n-3 to 20:5n-3 ratio was 7.04, similar to the ratio of 7.92 found for the total lipid. Again this suggests selective incorporation of 22:6n-3 into the cellular lipids and/or selective metabolism of 20:5n-3.

In tuna tissues, the distribution of different fatty acids between different classes of lipids has not been investigated except to some extent in longtail tuna (Saito et al., 2005). In this species, the distribution of various fatty acids between triacylglycerols (neutral lipids), PC and PE (the two most abundant classes of polar lipids) in “light muscle”, “dark muscle”, liver, pyloric ceca and orbital oil was studied. In all of these tissues 16:0 and 18:1n-9 were most abundant in the triacylglycerol fraction. This was different from the SBT-E1 cells in which 18:1n-9 was most abundant in PC. In the longtail tuna tissues, 20:4n-6 was in roughly equal amounts in PC and PE but in reduced amounts in the triacylglycerol fraction. This was different to what was seen in the SBT-E1 cells in which 20:4n-6 was significantly more abundant in PE than in PC. In the tissues of longtail tuna, 22:6n-3 was most abundant in PC (26.4 to 49.5% of total fatty acids) followed closely by PE (20.6 to 45.4% of total fatty acids) and then triacylglycerols (9.0 to 29.5% of total fatty acids). This was quite different from

the SBT-E1 cells. In the SBT-E1 cells, 22:6n-3 was most abundant in PE, PS and SM and very little was found in either PC or triacylglycerols. In general it can be said that LC-PUFA are more abundant in PE than PC in fish cell lines including the SBT-E1 cell line studied here (Tocher et al., 1988) but this is not the case in tuna tissues in which the predominant LC-PUFA, 22:6n-3, is abundant in both PC and PE (Saito et al., 2005). The fatty acid compositions of the diets of fish and the media used to culture fish cells may influence the distributions of individual fatty acids between the different classes of lipids.

4.4. Esterification of [1-^{14}C] labelled fatty acids into different classes of lipids

In cell lines from Atlantic salmon, rainbow trout and gilthead sea bream, the vast majority (~40-65%) of the label from 18:2n-6 and 18:3n-3 was recovered in PC (Tocher and Ghioni, 1999; Tocher and Sargent, 1990). This was similar to what was found in the SBT-E1 cell line. In contrast, the results for 20:5n-3 were much more variable. In the SBT-E1 cells, approximately equal amounts of label (~40%) from 20:5n-3 were recovered in PC and PE. This compared with ~30% in PC and ~60% in PE in Atlantic salmon and gilthead sea bream cell lines as opposed to ~60% in PC and ~30% in PE in rainbow trout cells. Despite this variation, all three PUFA were incorporated predominantly into either PC or PE (Tocher and Dick, 1999; Tocher and Ghioni, 1999; Tocher and Sargent, 1990). In hepatocytes from Atlantic salmon fed diets containing either fish oil or a vegetable oil blend, a similar trend was observed (Stubhaug et al., 2005). In the polar lipid fraction, label from 16:0, 18:1n-9, 18:2n-6, 18:3n-3, 20:5n-3 and 22:6n-3 was recovered predominantly in PC followed by PE, except in the case of 20:5n-3 where PC was followed by PI. Thus, the cell lines, which have been long established, behaved similarly to primary cultures recently isolated from tissues. Studies of fish tissues have shown higher levels of

16:0 and lower levels of PUFA incorporation into PC as compared with PE and PS (Tocher, 1995). Conversely, they have shown high levels of PUFA (mostly C_{20} and C_{22}) incorporation into PE. The SBT-E1 cell line showed these characteristics as well, especially with respect to the incorporation of 20:5n-3 into PE.

4.5. Metabolism of [1-^{14}C] labelled PUFA by desaturation and/or elongation

Studies with cell lines from Atlantic salmon, rainbow trout, turbot (*Scophthalmus maximus*) and gilthead seabream (*Sparus aurata*) have shown negligible conversion of 18:3n-3 to 22:6n-3 but some conversion of 18:3n-3 to 20:5n-3 (Ghioni et al., 1999; Tocher and Ghioni, 1999; Tocher and Sargent, 1990). The conversion of 18:3 to 20:5n-3 was greater in freshwater (rainbow trout) and anadromous (Atlantic salmon) fish species than in marine fish species (turbot and gilthead sea bream). In the marine species, label from [1-^{14}C]18:3n-3 accumulated in 18:4n-3 suggesting significant Δ6 Fads activity but limited Elovl5 and/or Δ5 Fads activity. In gilthead seabream, the limitation was found to lie at the Δ5 Fads catalysed desaturation of 20:4n-3 to 20:5n-3 rather than at the Elovl5 catalysed elongation of 18:4n-3 to 20:4n-3 (Tocher and Ghioni, 1999). Clearly there are differences between different fish species depending on their environment and their diet.

The SBT-E1 cell line showed very limited capacity to desaturate 18:3n-3 to 18:4n-3 or 18:2n-6 to 18:3n-6. The apparent rates of conversion were less than 3%. This indicated very limited Δ6 Fads activity. Previous studies, under similar conditions, have shown apparent conversion rates for 18:3n-3 to 18:4n-3 ranging from 12.2 to 64.2% in cell lines from Atlantic salmon, rainbow trout, turbot and gilthead sea bream (Ghioni et al., 1999; Tocher and Ghioni, 1999; Tocher and Sargent, 1990). Thus, the SBT-E1 cell line had by far the lowest conversion rates of any fish cell line studied to date. In contrast, the conversion rate for 20:5n-3 to 22:5n-3 (16.8%) in the

SBT-E1 cells was quite substantial. This rate was towards the upper end of the range (8.1 to 17.5%) found for cell lines from Atlantic salmon, rainbow trout, turbot and gilthead sea bream (Ghioni et al., 1999; Tocher and Ghioni, 1999; Tocher and Sargent, 1990). Thus, the SBT-E1 cell line exhibited substantial fatty acyl elongase activity but very limited Δ6 Fads activity. The substantial fatty acyl elongase activity of the SBT-E1 cells can be seen as at least part of the explanation for the low steady state levels of 20:5n-3 and high steady state levels of 22:5n-3 in the SBT-E1 cellular lipids (discussed above). This high elongase activity coupled with high rates of β-oxidation for 20:5n-3 (discussed below) presumably explains the high 22:6n-3 to 20:5n-3 ratio in the SBT-E1 cells. High 22:6n-3 to 20:5n-3 ratios appear to be characteristic of tuna tissues in general (Morais et al., 2011; Mourente and Tocher, 2009).

The results presented here indicate that the SBT-E1 cell line was essentially completely lacking in Δ6 Fads activity. Previous studies have shown that cell lines from Atlantic salmon and rainbow trout (anadromous and freshwater species) had substantial Δ6 and Δ5 Fads activities but lacked Δ4 Fads activity whereas cell lines from turbot and gilthead sea bream (marine species) had substantial Δ6 Fads activity but a low 18:4n-3 to 20:5n-3 conversion rate (Tocher et al., 1989; Tocher and Sargent, 1990). The low 18:4n-3 to 20:5n-3 conversion rate in the gilthead sea bream cell line was found to be due to low Δ5 Fads activity rather than low Elovl5 activity (Tocher and Ghioni, 1999). Although we have only tested 18:3n-3, 18:2n-6 and 20:5n-3, it appears as if *de novo* LC-PUFA biosynthesis in the SBT-E1 cell line is not limited by Elovl5 activity. This makes it similar to Atlantic salmon and gilthead sea bream. Thus, there is not a straightforward distinction between freshwater, anadromous and marine species. This is also clear when we consider that the cell lines from Atlantic salmon, turbot and gilthead sea bream all exhibited substantial Δ6

Fads activity whereas the SBT-E1 cell line did not. Thus, the SBT-E1 cell line had the least capacity of all of these cell lines for *de novo* synthesis of LC-PUFA from C_{18} PUFA. This is consistent with the high trophic level of bluefin tunas in marine food webs (Mourente and Tocher, 2009).

4.6. β-oxidation [1-^{14}C]-labelled fatty acids

The SBT-E1 cell line showed a high rate of β-oxidation with 20:5n-3, higher than with 18:0, 18:1n-9 or 18:2n-6. This high rate of β-oxidation of 20:5n-3 along with the high rate of elongation of this fatty acid to 22:5n-3 (discussed above) may explain the high 22:6n-3 to 20:5n-3 ratio observed in the SBT-E1 cells and also in tuna tissues (Mourente et al., 2002; Mourente and Tocher, 2009; Saito et al., 2005). This is important because it suggests that selective metabolism of 20:5n-3 rather than, or perhaps in conjunction with, selective esterification of 22:6n-3 into cellular lipids explains the high 22:6n-3 to 20:5n-3 ratios observed in tuna tissues.

In the SBT-E1 cell line, 18:3n-3 was oxidised at nearly twice the rate of 20:5n-3 and at more than 3-fold the rate of 18:0, 18:1n-9 or 18:2n-6. This showed a preference for 18:3n-3 over 20:5n-3 and for these two fatty acids over the other fatty acids tested. In a previous study, 18:3n-3 was oxidised at 5.1- to 8.0-fold the rate of 20:5n-3 in hepatocytes and at 3.5- to 4.8-fold the rate of 20:5n-3 in enterocytes from European sea bass (*Dicentrarchus labrax*) (Mourente et al., 2005). In contrast, in Atlantic salmon hepatocytes, 18:3n-3 and 20:5n-3 were oxidised at similar rates to one another and those rates were significantly lower than for 18:1n-9 and 18:2n-6 (Stubhaug et al., 2005). Thus, it appears as if inclusion of vegetable oils rich in 18:3n-3 at the expense fish oils in feeds for farmed bluefin tunas will be successful in terms of supporting the energy requirements of the animals. However, caution must be exercised when extrapolating from *in vitro* studies to metabolism in whole fish.

For example, feeding studies with juvenile Atlantic salmon have shown that dietary excess of a given fatty acid leads to increased catabolism of that particular fatty acid except in the case of 22:6n-3 (Bell et al., 2002; Bell et al., 2001; Stubhaug et al., 2007; Torstensen and Stubhaug, 2004). Thus, in the intact organism, fatty acid metabolism may be regulated at the level of uptake and incorporation into cellular lipids rather than at the level of β-oxidation. This remains to be investigated in tunas.

4.7 Δ6 Fads and Elovl5 gene expression in the SBT-E1 cell line

It is generally accepted that large predatory fishes, such as tunas, have limited capacity for *de novo* synthesis of LC-PUFA from their C_{18} precursors 18:3n-3 and 18:2n-6 but recently it was reported that unfed Atlantic bluefin tuna (*Thunnus thynnus*) larvae had increasing Δ6 Fads and Elovl5 gene expression with time post hatch (Tocher, 2003, Mourente et al., 2005, Morais et al., 2011). This suggested that nutritional deprivation increases expression of these genes. This is consistent with the results reported here for the SBT-E1 cell line in that reducing the FBS concentration in the cell culture medium from 10% to 2% (v/v) significantly increased the expression of Δ6 Fads. Another interesting observation of this study was that Δ6 Fads gene expression was much greater (20-fold) than Elovl5 gene expression in the SBT-E1 cell line. This was surprising given that the ^{14}C-labelling experiments had shown that Δ6 Fads enzyme activity was much greater than fatty acyl elongase activity towards 20:5n-3 in the SBT-E1 cell line. Functional characterization of a SBT Elovl5 gene in yeast showed that the gene product had substantial activity towards 20:5n-3 (Gregory et al., 2010). Thus Elovl5 was believed to be the main enzyme responsible for the elongation of 20:5n-3 in SBT. The results of the current study, however, suggest that a different fatty acyl elongase enzyme may be involved. Indeed functional characterisation of an Elovl2 from rainbow trout

(*Oncorhynchus mykiss*) has shown that this enzyme has similar levels of activity towards 20:5n-3 as Elovl5 enzymes (Gregory and James 2014). Further work is therefore required to determine whether SBT has other Elovl enzymes.

4.7. Conclusions

In summary, the SBT-E1 cell line was similar to other fish cell lines and tissues in that by far the most abundant phospholipid was PC and quantitatively important saturated fatty acids (16:0, 18:0) and monounsaturated fatty acids (18:1n-9) as well as essential PUFA (18:3n-3 and 18:2n-6) were incorporated predominantly into this lipid. Unlike cell lines from other fish species but similar to tuna tissues, the SBT-E1 cell line exhibited apparent selective retention of 22:6n-3 and a high ratio of 22:6n-3 to 20:5n-3. Major contributors to this were the substantial fatty acyl elongase activity converting 20:5n-3 to 22:5n-3 and the high rate of β-oxidation of 20:5n-3. It is proposed that these factors also contribute to the high 22:6n-3 to 20:5n-3 ratios found in tuna tissues. The SBT-E1 cell line exhibited negligible Δ6 Fads activity towards either 18:3n-3 or 18:2n-6. The low flux of substrates along the n-3 LC-PUFA synthesis pathway is consistent with the expectation that large carnivorous marine fish such as tunas have limited capacity for *de novo* synthesis of n-3 LC-PUFA from their C_{18} precursor presumably related to the abundance of n-3 LC-PUFA in marine food webs. However, an interesting and unexpected finding was that the SBT-E1 cell line had very high levels of expression of a Δ6 Fads gene and low levels of expression of an Elovl5 gene. This was the reverse of what was expected from the ^{14}C-labelling experiments and suggests that there may be other genes involved in *de novo* LC-PUFA biosynthesis in tunas.

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References

Bain, P.A., Hutchinson, R.G., Marks, A.B., Crane, M.S., Schuller, K.A., 2013. Establishment of a continuous cell line from southern bluefin tuna (*Thunnus maccoyii*). Aquaculture 376, 59-63.

Bell, J.G., Dick, J.R., McVicar, A.H., Sargent, J.R., Thompson, K.D., 1993. Dietary sunflower, linseed and fish oils affect phospholipid fatty acid composition, development of cardiac lesions, phospholipase activity and eicosanoid production in Atlantic salmon (*Salmo salar*). Prostaglandins, Leukotrienes and Essential Fatty Acids 49, 665-673.

Bell, J.G., Henderson, R.J., Tocher, D.R., McGhee, F., Dick, J.R., Porter, A., Smullen, R.P., Sargent, J.R., 2002. Substituting fish oil with crude palm oil in the diet of Atlantic salmon (*Salmo salar*) affects muscle fatty acid composition and hepatic fatty acid metabolism. Journal of Nutrition 132, 222-230.

Bell, J.G., McEvoy, J., Tocher, D.R., McGhee, F., Campbell, P.J., Sargent, J.R., 2001. Replacement of fish oil with rapeseed oil in diets of Atlantic salmon (*Salmo salar*) affects tissue lipid compositions and hepatocyte fatty acid metabolism. Journal of Nutrition 131, 1535-1543.

Bligh, E. G., Dyer, W. J., 1959. A rapid method of total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology 37, 911-917.

Bubner, E., Farley, J., Thomas, P., Bolton, T., Elizur, A., 2012. Assessment of reproductive maturation of southern bluefin tuna (*Thunnus maccoyii*) in captivity. Aquaculture 364, 82-95.

CCSBT, Commission for the Conservation of Southern Bluefin Tuna, http://www.ccsbt.org/site/total_allowable_catch.php

Christie, W.W., 1993. Preparation of ester derivatives of fatty acids for chromatographic analysis, in: W.W. Christie (Ed.), Advances in Lipid Methodology - Two. Oily Press, Dundee.

De Metrio, G., Bridges, C.R., Mylonas, C.C., Caggiano, M., Deflorio, M., Santamaria, N., Zupa, R., Pousis, C., Vassallo-Agius, R., Gordin, H., Corriero, A., 2010. Spawning induction and large-scale collection of fertilized eggs in captive Atlantic bluefin tuna (*Thunnus thynnus* L.) and the first larval rearing efforts. Journal of Applied Ichthyology 26, 596-599.

Folch, J., Lees, M., Sloane Stanley, G.H., 1957. A simple method for the isolation and purification of total lipides from animal tissues. J. Biol. Chem. 226, 497-509.

Ghioni, C., Tocher, D.R., Bell, M.V., Dick, J.R., Sargent, J.R., 1999. Low C_{18} to C_{20} fatty acid elongase activity and limited conversion of stearidonic acid, 18:4(*n*-3), to eicosapentaenoic acid, 20:5(*n*-3), in a cell line from the turbot, *Scophthalmus maximus*. Biochimica et Biophysica Acta 1437, 170-181.

Graham, J.B., Dickson, K.A., 2004. Tuna comparative physiology. Journal of Experimental Biology 207, 4015-4024.

Gregory, M.K., James, M.J., 2014. Rainbow trout (*Oncorhynchus mykiss*) Elovl5 and Elovl2 differ in selectivity for elongation of omega-3 docosapentaenoic acid. Biochimica et Biophysica Acta 1841, 1656-1660.

Gregory, M.K., King, H.W., Bain, P.A., Gibson, R.A., Tocher, D.R., Schuller, K.A., 2011. Development of a fish cell culture model to investigate the impact of fish oil replacement on lipid peroxidation. Lipids 46, 753-764.

ICCAT, International Commission for the Conservation of Atlantic Tunas, http://www.iccat.int/en/

Itoh, T., Kemps, H., Totterdell, J., 2011. Diet of young southern bluefin tuna *Thunnus maccoyii* in the southwestern coastal waters of Australia in summer. Fisheries Science 77, 337-344.

Kubista, M., Andrade, J.M., Bengtsson, M., Forootan, A., Jonák, J., Lind, K., Sindelka, R., Sjöback, R., Sjögreen, B., Strömbom, L., Ståhlberg, A., Zoric, N., 2006. The real-time polymerase chain reaction. Molecular Aspects of Medicine 27, 95-125.

Li, Y., Monroig, O., Zhang, L., Wang, S., Zheng, X., Dick, J.R., You, C., Tocher, D.R., 2010. Vertebrate fatty acyl desaturase with Δ4 activity. Proceedings of the National Academy of Sciences 107, 16840-16845.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. Methods 25, 402-408.

Logan, J.M., Rodríguez-Marín, E., Goñi, N., Barreiro, S., Arrizabalaga, H., Golet, W., Lutcavage, M., 2011. Diet of young Atlantic bluefin tuna (*Thunnus thynnus*) in eastern and western Atlantic foraging grounds. Marine Biology 158, 73-85.

Madsen, L., Rustan, A., Vaagenes, H., Berge, K., Dyrøy, E., Berge, R., 1999. Eicosapentaenoic and docosahexaenoic acid affect mitochondrial and peroxisomal fatty acid oxidation in relation to substrate preference. Lipids 34, 951-963.

Masuma, S., Miyashita, S., Yamamoto, H., Kumai, H., 2008. Status of bluefin tuna farming, broodstock management, breeding and fingerling production in Japan. Reviews in Fisheries Science 16, 385-390.

Makrides, M., Neumann, M. A., Gibson, R. A., 1996. Effect of maternal docosahexanoic acid (DHA) supplementation on breast milk composition. European Journal of Clinical Nutrition 50, 352-357.

Metian, M., Pouil, S., Boustany, A., Troell, M., 2014. Farming of bluefin tuna - Reconsidering global estimates and sustainability concerns. Reviews in Fisheries Science and Aquaculture 22, 184-192.

Miller, M.R., Nichols, P.D., Carter, C.G., 2008. n-3 Oil sources for use in aquaculture - alternatives to the unsustainable harvest of wild fish. Nutrition Research Reviews 21, 85-96.

Miyake, M., Guillotreau, P., Sun, C., Ishimura, G., 2010. Recent developments in the tuna industry: Stocks, fisheries, management, processing, trade and markets, FAO Fisheries and Aquaculture Technical Paper. No. 543, Rome.

Monroig, Ó., Li, Y.Y., Tocher, D.R., 2011. Delta-8 desaturation activity varies among fatty acyl desaturases of teleost fish: High activity in delta-6 desaturases of marine species. Comparative Biochemistry and Physiology, Part B 159, 206-213.

Monteiro, J., Leslie, M., Moghadasian, M.H., Arendt, B.M., Allard, J.P., Ma, D.W.L., 2014. The role of n-6 and n-3 polyunsaturated fatty acids in the manifestation of the metabolic syndrome in cardiovascular disease and non-alcoholic fatty liver disease. Food and Function 5, 426-435.

Morais, S., Mourente, G., Ortega, A., Tocher, J.A., Tocher, D.R., 2011. Expression of fatty acyl desaturase and elongase genes, and evolution of DHA:EPA ratio during development of unfed larvae of Atlantic bluefin tuna (*Thunnus thynnus* L.). Aquaculture 313, 129-139.

Mourente, G., Dick, J.R., Bell, J.G., Tocher, D.R., 2005. Effect of partial substitution of dietary fish oil by vegetable oils on desaturation and β-oxidation of [1-^{14}C]18:3n−3 (LNA) and [1-^{14}C]20:5n−3 (EPA) in hepatocytes and enterocytes of European sea bass (*Dicentrarchus labrax* L.). Aquaculture 248, 173-186.

Mourente, G., Megina, C., Díaz-Salvago, E., 2002. Lipids in female northern bluefin tuna (*Thunnus thynnus thynnus* L.) during sexual maturation. Fish Physiology and Biochemistry 24, 351-363.

Mourente, G., Tocher, D.R., 2009. Tuna nutrition and feeds: Current status and future perspectives. Reviews in Fisheries Science 17, 373-390.

Murase, T., Saito, H., 1996. The docosahexaenoic acid content in the lipid of albacore *Thunnus alalunga* caught in two separate localities. Fisheries Science 62, 634-638.

Musgrove, R.J., Carragher, J.F., Manning, A.D., Zammit, B.J., Thomas, P.M., Buchanan, J., 2011. Effects of postharvest processes on quality of Australian sardines (*Sardinops sagax*) and redbait (*Emmelichthys nitidus nitidus*) for feeding aquacultured southern bluefin tuna (*Thunnus maccoyii*). Aquaculture Nutrition 17, E19-E29.

Mylonas, C.C., de la Gándara, F., Corriero, A., Ríos, A.B., 2010. Atlantic bluefin tuna (*Thunnus Thynnus*) farming and fattening in the Mediterranean Sea. Reviews in Fisheries Science 18, 266-280.

Naylor, R.L., Hardy, R.W., Bureau, D.P., Chiu, A., Elliott, M., Farrell, A.P., Forster, I., Gatlin, D.M., Goldburg, R.J., Hua, K., Nichols, P.D., 2009. Feeding aquaculture in an era of finite resources. Proceedings of the National Academy of Sciences 106, 15103-15110.

Okada, T., Honryo, T., Sawada, Y., Agawa, Y., Miyashita, S., Ishibashi, Y., 2014. The cause of death of juvenile Pacific bluefin tuna (*Thunnus orientalis*) reared in sea net cages. Aquacultural Engineering 59, 23-25.

Ottolenghi, F., 2008. Capture-based aquaculture of bluefin tuna, in: A. Lovatelli, P.F. Holthus (Eds.), Capture-based aquaculture. Global Overview. FAO Fisheries Technical Paper. No. 508. FAO, Rome, 169-182.

Pahl, S., Lewis, D., Chen, F., King, K., 2010. Growth dynamics and the proximate biochemical composition and fatty acid profile of the heterotrophically grown diatom *Cyclotella cryptica*, Journal of Applied Phycology 22, 165-171

Saito, H., Ishihara, K., Murase, T., 1996. Effect of prey fish lipids on the docosahexaenoic acid content of total fatty acids in the lipid of *Thunnus albacares* yellowfin tuna. Bioscience, Biotechnology and Biochemistry 60, 962-965.

Saito, H., Seike, Y., Ioka, H., Osako, K., Tanaka, M., Takashima, A., Keriko, J., Kose, S., Souza, J.R., 2005. High docosahexaenoic acid levels in both neutral and polar lipids of a highly migratory fish: *Thunnus tonggol* (Bleeker). Lipids 40, 941-953.

Sawada, Y., Okada, T., Miyashita, S., Murata, O., Kumai, H., 2005. Completion of the Pacific bluefin tuna *Thunnus orientalis* (Temminck et Schlegel) life cycle. Aquaculture Research 36, 413-421.

Scholefield, A.M., Schuller, K.A., 2014. Cell proliferation and long chain polyunsaturated fatty acid metabolism in a cell line from southern bluefin tuna (*Thunnus maccoyii*). Lipids 49, 703-714.

Shimose, T., Watanabe, H., Tanabe, T., Kubodera, T., 2013. Ontogenetic diet shift of age-0 year Pacific bluefin tuna *Thunnus orientalis*. Journal of Fish Biology 82, 263-276.

Skirtun, M., Sahlqvist, P., Vieira, S., 2013. Australian fisheries statistics 2012. Australian Bureau of Agricultural and Resource Economics and Sciences, Canberra.

Sprague, M., Dick, J.R., Medina, A., Tocher, D.R., Bell, J., Mourente, G., 2012. Lipid and fatty acid composition, and persistent organic pollutant levels in tissues of migrating Atlantic bluefin tuna (*Thunnus thynnus*, L.) broodstock. Environmental Pollution 171, 61-71.

Stubhaug, I., Lie, Ø., Torstensen, B.E., 2007. Fatty acid productive value and β-oxidation capacity in Atlantic salmon (*Salmo salar* L.) fed on different lipid sources along the whole growth period. Aquaculture Nutrition 13, 145-155.

Stubhaug, I., Tocher, D.R., Bell, J.G., Dick, J.R., Torstensen, B.E., 2005. Fatty acid metabolism in Atlantic salmon (*Salmo salar* L.) hepatocytes and influence of dietary vegetable oil. Biochimica et Biophysica Acta 1734, 277-288.

Tacon, A.G.J., Metian, M., 2013. Fish Matters: Importance of Aquatic Foods in Human Nutrition and Global Food Supply. Reviews in Fisheries Science 21, 22-38.

Tocher, D.R., 1995. Glycerophospholipid metabolism, in: P.W. Hochachka, T.P. Mommsen (Eds.), Biochemistry and Molecular Biology of Fishes. Elsevier, 119-157.

Tocher, D.R., 2003. Metabolism and functions of lipids and fatty acids in teleost fish. Reviews in Fisheries Science 11, 107-184.

Tocher, D.R., Carr, J., Sargent, J.R., 1989. Polyunsaturated fatty acid metabolism in fish cells: Differential metabolism of (n-3) and (n-6) series acids by cultured cells orginating from a freshwater teleost fish and from a marine teleost fish. Comparative Biochemistry and Physiology Part B 94, 367-374.

Tocher, D.R., Dick, J.R., 1999. Polyunsaturated fatty acid metabolism in a cell culture model of essential fatty acid deficiency in a freshwater fish, carp (*Cyprinus carpio*). Fish Physiology and Biochemistry 21, 257-267.

Tocher, D.R., Fonseca-Madrigal, J., Dick, J.R., Ng, W.K., Bell, J.G., Campbell, P.J., 2004. Effects of water temperature and diets containing palm oil on fatty acid desaturation and oxidation in hepatocytes and intestinal enterocytes of rainbow trout (*Oncorhynchus mykiss*). Comparative Biochemistry and Physiology Part B 137, 49-63.

Tocher, D.R., Ghioni, C., 1999. Fatty acid metabolism in marine fish: Low activity of fatty acyl Δ5 desaturation in gilthead sea bream (*Sparus aurata*) cells. Lipids 34, 433-440.

Tocher, D.R., Harvie, D.G., 1988. Fatty acid compositions of the major phosphoglycerides from fish neural tissues; (n−3) and (n−6) polyunsaturated fatty acids in rainbow trout (*Salmo gairdneri*) and cod (*Gadus morhua*) brains and retinas. Fish Physiology and Biochemistry. 5, 229-239.

Tocher, D.R., Sargent, J.R., 1990. Effect of temperature on the incorporation into phospholipid classes and metabolism via desaturation and elongation of n−3 and n−6 polyunsaturated fatty acids in fish cells in culture. Lipids 25, 435-442.

Tocher, D.R., Sargent, J.R., Frerichs, G.N., 1988. The fatty acid compositions of established fish cell lines after long-term culture in mammalian sera. Fish Physiology and Biochemistry 5, 219-227.

Torstensen, B.E., Stubhaug, I., 2004. β-Oxidation of 18:3n−3 in Atlantic salmon (*Salmo salar* L.) hepatocytes treated with different fatty acids. Lipids 39, 153-160.

Tsuda, Y., Sakamoto, W., Yamamoto, S., Murata, O., 2012. Effect of environmental fluctuations on mortality of juvenile Pacific bluefin tuna, *Thunnus orientalis*, in closed life-cycle aquaculture. Aquaculture 330, 142-147.

Turchini, G.M., Torstensen, B.E., Ng, W.K., 2009. Fish oil replacement in finfish nutrition. Reviews in Aquaculture 1, 10-57.

Vitello, F., Zanetta, J.P., 1978. Thin-layer chromatography of phospholipids. Journal of Chromatography A 166, 637-640.

Woodhams, J., Vieira, S., Stobutzki, I., 2013. Fishery status reports 2012. Australian Bureau of Agricultural and Resource Economics and Sciences, Canberra.

Yates, C.M., Calder, P.C., Rainger, G.E., 2014. Pharmacology and therapeutics of omega-3 polyunsaturated fatty acids in chronic inflammatory disease. Pharmacology and Therapeutics 141, 272-282.

Yúfera, M., Ortiz-Delgado, J.B., Hoffman, T., Siguero, I., Urup, B., Sarasquete, C., 2014. Organogenesis of digestive system, visual system and other structures in Atlantic bluefin tuna (*Thunnus thynnus*) larvae reared with copepods in mesocosm system. Aquaculture 426, 126-137.

Figure captions

Fig. 1. Alternative pathways for the *de novo* synthesis of long-chain polyunsaturated fatty acids (LC-PUFA) from their C_{18} precursors in fish (Li et al., 2010; Monroig et al., 2011; Scholefield and Schuller, 2014).

Fig. 2. Incorporation of [1-^{14}C]-labelled fatty acids into different classes of lipids in the SBT-E1 cell line. The data are the mean ± standard error of the mean (n = 3). For each individual fatty acid, significant differences between different classes of lipids are indicated by different letters.

Fig. 3. β-oxidation of [1-^{14}C]-labelled fatty acids by the SBT-E1 cell line. The data are the mean ± standard error of the mean (n = 3). Significant differences between the different fatty acids are indicated by an asterisk.

Fig. 4. The effects of FBS concentration and individual fatty acid supplementation on Δ6 Fads (filled columns) and Elovl5 (cross-hatched columns) gene expression in the SBT-E1 cell line. In Panel A, the cells were incubated under the different conditions for 3 days and the fatty acids were supplied at a concentration of 10 μM. In Panel B, the cells were incubated under the different conditions for 24 hours and the fatty acids were supplied at a concentration of 10 μM. In Panel C, the cells were maintained in culture medium containing 10% (v/v) FBS. In Panels A and B, the data have been normalised using β-actin and are expressed relative to the control, arbitrarily set at a value of 1.0. In Panel C, the transcript abundances for Δ6 Fads and Elovl5 are expressed relative to the transcript abundance for β-actin. The data are the mean ± standard error of the mean (n = 3). Significant differences between the treatments and the control are indicated by an asterisk.

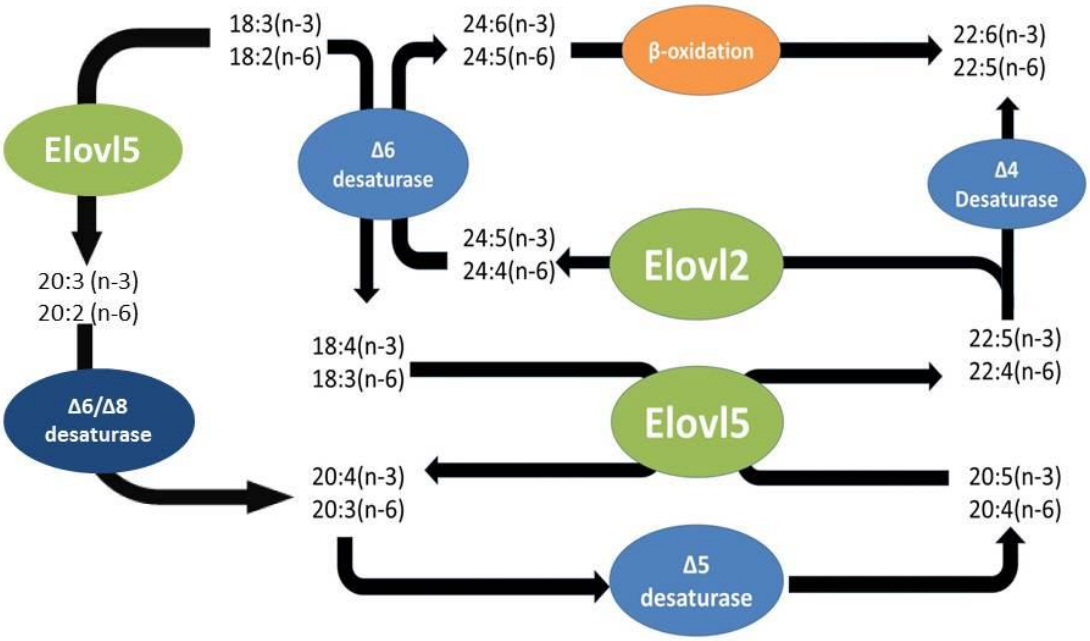
18:3(n-3)
18:2(n-6)
24:6(n-3)
24:5(n-6)
β-oxidation
22:6(n-3)
22:5(n-6)
Elovl5
Δ6
desaturase
Δ4
Desaturase
24:5(n-3)
24:4(n-6)
Elovl2
20:3 (n-3)
20:2 (n-6)
18:4(n-3)
18:3(n-6)
22:5(n-3)
22:4(n-6)
Δ6/Δ8
desaturase
Elovl5
20:4(n-3)
20:3(n-6)
20:5(n-3)
20:4(n-6)
Δ5
desaturase

Figure 1

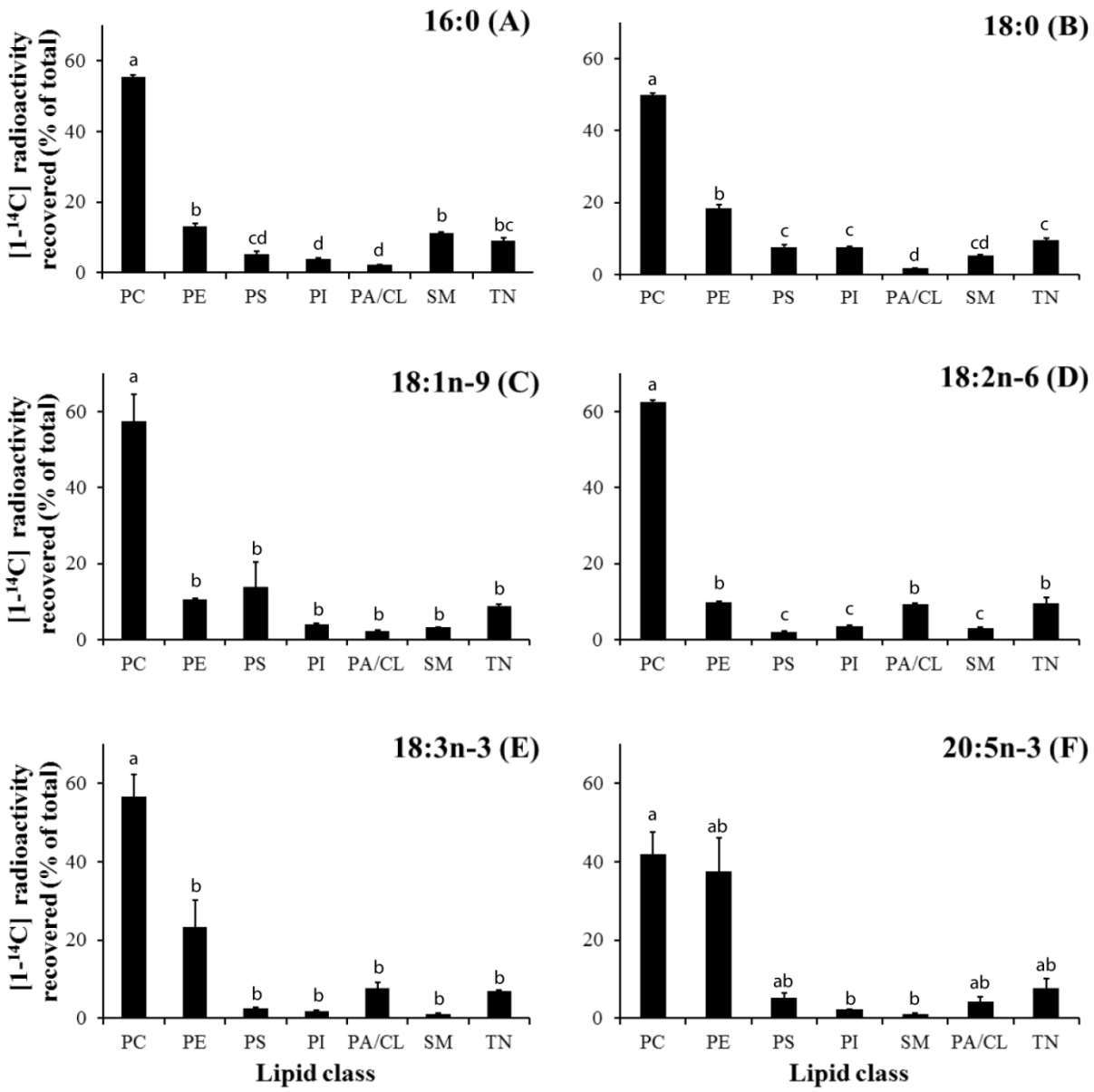
16:0 (A)
18:0 (B)
18:1n-9 (C)
18:2n-6 (D)
18:3n-3 (E)
20:5n-3 (F)
[1-14C] radioactivity recovered (% of total)
PC PE PS PI PA/CL SM TN
Lipid class

Figure 2

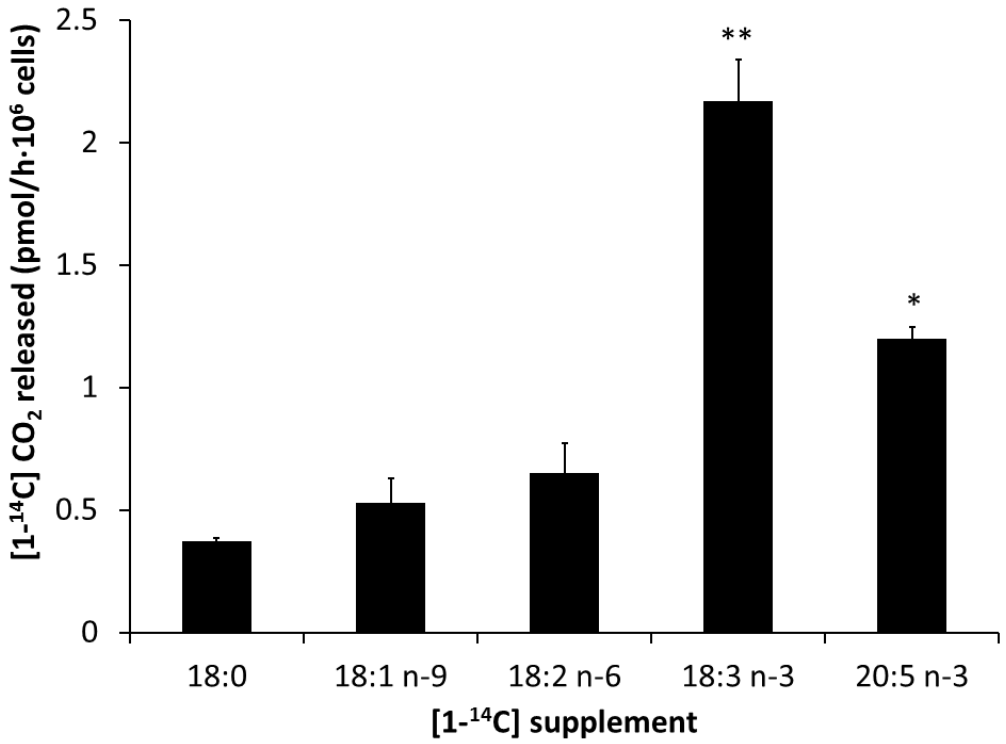
2.5
2
1.5
1
0.5
0
**
*
[1-14C] CO2 released (pmol/h·10^6 cells)
18:0
18:1 n-9
18:2 n-6
18:3 n-3
20:5 n-3
[1-14C] supplement

Figure 3

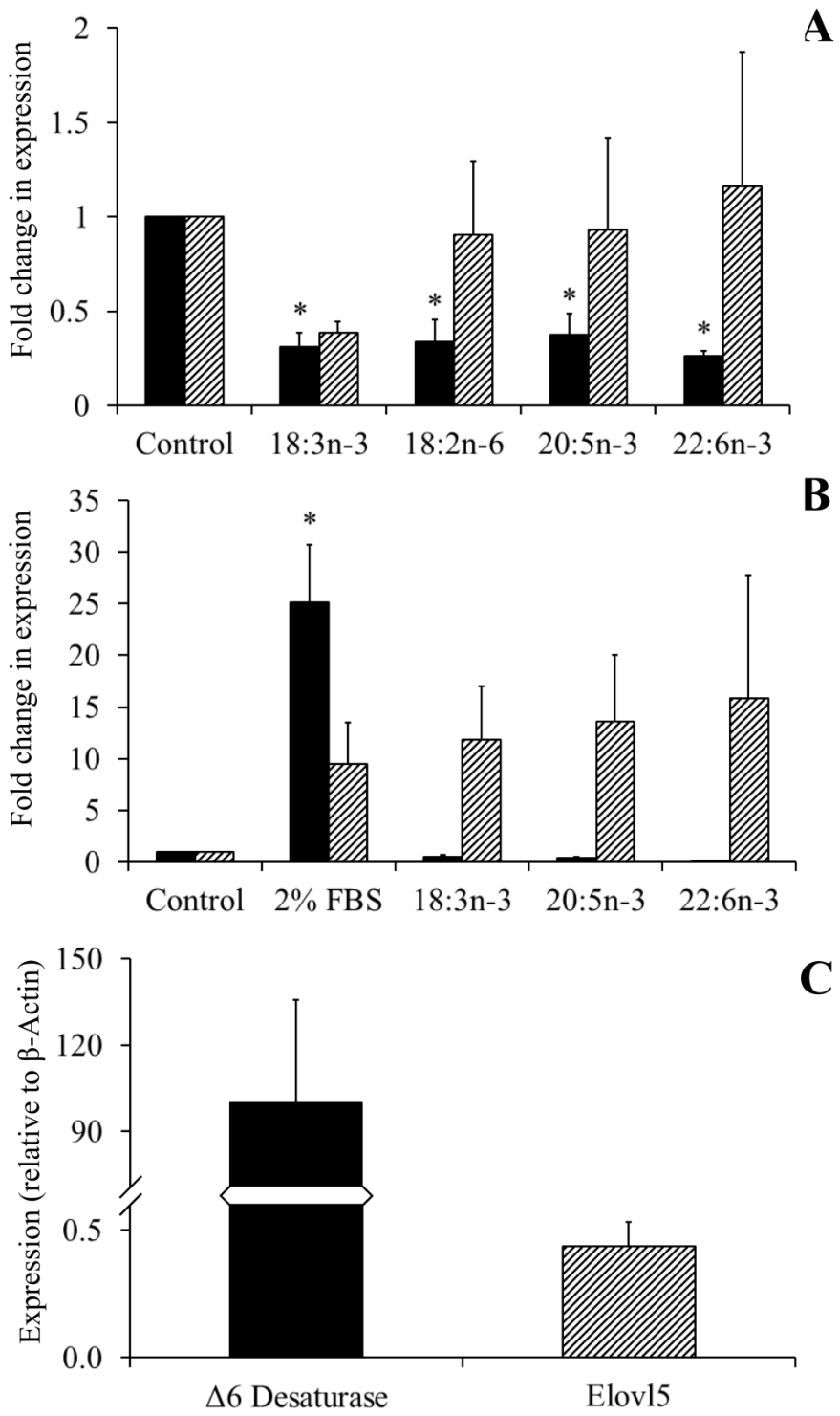
A
Fold change in expression
2
1.5
1
0.5
0
Control
18:3n-3
18:2n-6
20:5n-3
22:6n-3
B
Fold change in expression
35
30
25
20
15
10
5
0
Control
2% FBS
18:3n-3
20:5n-3
22:6n-3
C
Expression (relative to β-Actin)
150
120
90
0.5
0.0
Δ6 Desaturase
Elovl5

Figure 4

Table 1
Primers used for qRT-PCR.

Target gene	Forward primer sequence (5´→ 3´)	Reverse primer sequence (5´→ 3´)	Amplicon size (base pairs)	Efficiency
β-Actin	ACCCACACAGTGCCCATCTA	TCACGCACGATTTCCCTCT	155	0.91
Δ6 Fads	CCGTGCACTGTGTGAGAAAC	CAGTGTAAGCGATAAAATCAGCTG	152	1.00
Elovl5	CCCACCAACATTTGCACTCA	GTCCTGGCAGTAGAAGTTGT	189	0.96

Table 2

Lipid class composition of the total lipid from SBT-E1 cells cultured in medium supplemented with 5% (v/v) FBS. Data are the mean ± SE (n = 3).

Lipid class	Content (% of total lipid)
Phosphatidylcholine	26.19 ± 1.04^{b}
Phosphatidylethanolamine	15.12 ± 0.55^{c}
Phosphatidylserine	4.64 ± 0.39^{de}
Phosphatidylinositol	6.46 ± 0.29^{d}
Phosphatidic acid/cardiolipin	2.14 ± 0.08^{e}
Sphingomyelin	4.75 ± 0.57^{de}
Total neutral lipids	40.70 ± 1.14^{a}

Table 3
Fatty acid compositions of the culture medium, the total lipid from the SBT-E1 cells and the various lipid classes from the SBT-E1 cells. The data are the mean ± SE (n = 3) and are expressed as % of total fatty acids on a mass basis. Different superscript letters within a row indicate significant differences between the different lipid classes ($p < 0.05$). The data were arc-sin transformed prior to statistical analysis using a one-way analysis of variance (ANOVA) followed by Bonferroni *post-hoc* comparisons.

Fatty Acid	Culture[1] medium	Total lipid[2]	Lipid class[3]					
			PC	PE	PS	PI/PA/CL	SM	TN
14:0	2.14 ± 0.42	0.55 ± 0.07	0.79 ± 0.06^{b}	2.59 ± 1.14^{b}	2.22 ± 0.62^{b}	2.29 ± 0.89^{b}	2.06 ± 0.03^{b}	8.46 ± 0.16^{a}
16:0	23.95 ± 0.90	14.21 ± 0.74	12.94 ± 0.22^{b}	15.70 ± 6.01^{b}	16.20 ± 2.02^{ab}	14.86 ± 5.82^{b}	16.19 ± 3.77^{ab}	38.87 ± 1.44^{a}
18:0	7.53 ± 0.42	10.47 ± 0.19	6.05 ± 0.05^{c}	15.36 ± 0.59^{ab}	19.83 ± 2.57^{ab}	21.43 ± 0.32^{a}	8.16 ± 2.37^{bc}	12.01 ± 0.40^{bc}
Total SFA[4]	57.12 ± 1.51	30.41 ± 0.80	20.80 ± 0.31^{b}	38.90 ± 7.94^{b}	44.47 ± 1.17^{ab}	41.53 ± 6.45^{b}	35.97 ± 6.89^{b}	70.53 ± 1.57^{a}
16:1n-7	2.39 ± 0.31	3.06 ± 0.18	7.16 ± 0.12^{a}	1.15 ± 0.04^{c}	0.82 ± 0.07^{c}	0.77 ± 0.03^{c}	2.16 ± 0.35^{b}	1.38 ± 0.12^{bc}
18:1n-9	13.63 ± 3.52	21.90 ± 0.34	43.01 ± 0.29^{a}	9.41 ± 0.37^{b}	9.41 ± 1.17^{b}	10.48 ± 0.70^{b}	7.50 ± 2.19^{bc}	4.27 ± 0.35^{c}
18:1n-7	2.84 ± 0.21	5.52 ± 0.13	9.96 ± 0.05^{a}	5.25 ± 0.61^{b}	1.38 ± 0.18^{c}	2.67 ± 0.25^{c}	2.04 ± 0.57^{c}	1.13 ± 0.13^{c}
Total MUFA[5]	22.56 ± 3.55	33.56 ± 0.54	61.70 ± 0.32^{a}	17.00 ± 0.79^{b}	13.60 ± 0.81^{b}	14.80 ± 0.89^{b}	12.47 ± 2.24^{b}	11.37 ± 0.90^{b}
18:2n-6/19:0	2.27 ± 0.38	3.32 ± 0.15	2.92 ± 0.02^{a}	1.32 ± 0.04^{b}	0.95 ± 0.08^{b}	0.92 ± 0.19^{b}	0.00 ± 0.00^{c}	1.15 ± 0.18^{b}
18:3n-6	0.00 ± 0.00	0.60 ± 0.02	0.50 ± 0.01^{a}	0.18 ± 0.04^{ab}	0.00 ± 0.00^{c}	0.20 ± 0.10^{ab}	0.00 ± 0.00^{c}	0.07 ± 0.01^{b}
20:2n-6	0.00 ± 0.00	0.14 ± 0.01	0.17 ± 0.01	0.22 ± 0.12	0.44 ± 0.22	0.25 ± 0.25	0.99 ± 0.53	0.41 ± 0.20
20:4n-6	2.70 ± 0.09	8.57 ± 0.25	1.81 ± 0.06^{c}	11.02 ± 1.18^{b}	2.14 ± 0.34^{c}	16.10 ± 1.72^{a}	0.00 ± 0.00^{d}	1.15 ± 0.14^{c}
22:4n-6/22:3n-3	0.00 ± 0.00	2.00 ± 0.19	0.27 ± 0.01^{b}	0.93 ± 0.08^{a}	1.18 ± 0.21^{a}	0.18 ± 0.09^{bc}	0.00 ± 0.00^{c}	0.16 ± 0.03^{bc}
Total n-6 PUFA[6]	5.72 ± 0.39	17.18 ± 0.38	7.43 ± 0.23^{b}	15.97 ± 1.77^{a}	8.47 ± 1.65^{b}	23.13 ± 2.73^{a}	5.97 ± 0.73^{b}	4.40 ± 0.17^{b}
18:3n-3	0.20 ± 0.10	0.14 ± 0.01	0.06 ± 0.03	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.02 ± 0.02
20:3n-3	0.00 ± 0.00	0.17 ± 0.01	0.09 ± 0.05^{ab}	0.10 ± 0.10^{ab}	0.00 ± 0.00^{b}	0.15 ± 0.15^{ab}	0.00 ± 0.00^{b}	0.52 ± 0.03^{a}
20:5n-3	0.62 ± 0.13	1.14 ± 0.04	0.40 ± 0.01^{b}	1.61 ± 0.17^{a}	0.08 ± 0.08^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}
22:5n-3	1.03 ± 0.13	6.26 ± 0.04	1.27 ± 0.03^{b}	6.48 ± 0.75^{a}	6.03 ± 1.24^{a}	1.40 ± 0.13^{b}	0.00 ± 0.00^{c}	0.49 ± 0.09^{b}
22:6n-3	2.03 ± 0.21	9.03 ± 0.24	1.84 ± 0.04^{b}	11.33 ± 1.07^{a}	8.15 ± 0.94^{a}	3.19 ± 0.51^{b}	9.29 ± 1.27^{a}	1.20 ± 0.19^{b}

Total n-3 PUFA[7]	3.89 ± 0.42	16.82 ± 0.24	3.67 ± 0.13[d]	19.53 ± 1.88[a]	14.30 ± 2.12[ab]	4.73 ± 0.50[cd]	9.30 ± 1.30[bc]	2.23 ± 0.32[d]
22:6n-3:20:5n-3 ratio	3.43 ± 0.44	7.96 ± 0.48	4.55 ± 0.10	7.07 ± 0.20	n.a.[8]	n.a.[8]	n.a.[8]	n.a.[8]

[1]The culture medium contained 5% (v/v) FBS which was the source of the fatty acids. [2]The cells were cultured in the presence of 10% (v/v) FBS. [3]The cells were cultured in the presence of 5% (v/v) FBS. [4]Also includes 8:0, 9:0, 10:0, 11:0, 12:0, 13:0, 15:0, 17:0, 20:0, 22:0 and 24:0. [5]Also includes 11:1, 13:1, 14:1, 15:1, 16:1n-9, 17:1, 19:1, 22:1n-9 and 24:1. [6]Also includes 20:3 n-6, 22:2n-6 and 22:5n-6. [7]Also includes 18:4n-3. [8]A 22:6n-3:20:5n-3 ratio was not able to be obtained from these samples due to a zero value for 20:5n-3 in one or more samples.

Table 4
Fatty acid composition of total lipid and phospholipids from SBT liver, muscle, intestine and kidney. The data are the mean ± SE (n = 3) expressed as % of total fatty acids on a mass basis.

Fatty Acid	Total lipid				Phospholipids			
	Liver	Muscle	Intestine	Kidney	Liver	Muscle	Intestine	Kidney
14:0	2.54 ± 0.83	3.75 ± 0.39	3.95 ± 0.19	4.36 ± 0.16	1.22 ± 0.35	1.34 ± 0.09	1.33 ± 0.12	2.26 ± 0.56
16:0	19.26 ± 1.24	19.93 ± 0.97	19.54 ± 0.51	19.26 ± 0.27	22.34 ± 1.06	21.07 ± 1.05	18.44 ± 0.64	24.19 ± 0.58
18:0	8.24 ± 1.31	7.62 ± 1.04	7.49 ± 0.73	5.84 ± 0.49	12.76 ± 1.60	13.39 ± 0.83	18.33 ± 1.01	10.81 ± 0.66
Total SFA[1]	31.84 ± 1.63	33.37 ± 1.80	33.05 ± 0.77	31.31 ± 0.80	38.08 ± 0.43	37.31 ± 0.65	39.91 ± 0.68	39.32 ± 1.86
16:1n-7	2.54 ± 0.48	3.97 ± 0.35	4.56 ± 0.49	4.93 ± 0.44	1.01 ± 0.16	1.52 ± 0.08	1.43 ± 0.10	2.28 ± 0.70
18:1n-9	15.65 ± 6.38	13.87 ± 0.20	14.43 ± 1.30	15.50 ± 1.02	6.11 ± 2.10	8.20 ± 0.46	6.66 ± 0.34	9.94 ± 1.74
18:1n-7	3.38 ± 0.45	2.11 ± 1.02	3.30 ± 0.17	3.39 ± 0.16	2.05 ± 0.35	2.89 ± 0.13	2.48 ± 0.11	3.60 ± 0.38
Total MUFA[2]	34.42 ± 5.09	31.54 ± 4.18	30.88 ± 3.64	32.27 ± 2.66	14.69 ± 1.47	17.57 ± 0.93	16.27 ± 0.92	21.01 ± 2.02
18:2n-6/19:0	1.82 ± 0.24	1.92 ± 0.12	1.79 ± 0.19	1.77 ± 0.15	1.05 ± 0.04	1.69 ± 0.05	2.11 ± 0.17	1.45 ± 0.21
18:3n-6	0.24 ± 0.01	0.20 ± 0.01	0.02 ± 0.00	0.22 ± 0.00	0.09 ± 0.01	0.12 ± 0.00	0.18 ± 0.02	0.17 ± 0.01
20:2n-6	0.43 ± 0.06	0.34 ± 0.01	0.29 ± 0.01	0.30 ± 0.02	0.31 ± 0.05	0.35 ± 0.03	0.31 ± 0.03	0.34 ± 0.01
20:4n-6	1.48 ± 0.22	1.38 ± 0.18	1.44 ± 0.09	1.37 ± 0.10	3.67 ± 0.38	2.37 ± 0.07	3.15 ± 0.17	3.86 ± 0.49
22:4n-6/22:3n-3	0.13 ± 0.00	0.15 ± 0.02	0.15 ± 0.02	0.15 ± 0.02	0.09 ± 0.02	0.20 ± 0.01	0.22 ± 0.03	0.20 ± 0.02
Total n-6 PUFA[3]	4.85 ± 0.32	4.82 ± 0.44	4.61 ± 0.46	4.59 ± 0.26	6.02 ± 0.29	5.94 ± 0.10	7.16 ± 0.31	6.92 ± 0.38
18:3n-3	1.00 ± 0.16	0.96 ± 0.05	1.03 ± 0.16	1.14 ± 0.15	0.32 ± 0.03	0.41 ± 0.02	0.55 ± 0.06	0.53 ± 0.19
20:3n-3	0.20 ± 0.02	0.14 ± 0.00	0.13 ± 0.01	0.14 ± 0.01	0.13 ± 0.02	0.12 ± 0.01	0.10 ± 0.01	0.16 ± 0.00
20:5n-3	7.61 ± 1.33	6.99 ± 0.31	8.33 ± 0.34	9.09 ± 0.28	6.73 ± 1.06	6.41 ± 0.44	8.45 ± 0.15	7.66 ± 0.77
22:5n-3	2.14 ± 0.21	1.73 ± 0.05	1.49 ± 0.05	1.75 ± 0.01	1.30 ± 0.01	1.66 ± 0.09	1.23 ± 0.09	1.34 ± 0.06
22:6n-3	17.30 ± 1.91	20.14 ± 2.35	20.18 ± 2.54	18.93 ± 1.69	32.41 ± 0.44	30.43 ± 0.74	26.08 ± 1.23	22.82 ± 3.04
Total n-3 PUFA[4]	28.25 ± 3.34	29.96 ± 2.02	31.16 ± 2.81	31.05 ± 1.70	40.89 ± 1.33	38.99 ± 0.26	36.40 ± 1.22	32.51 ± 3.67

22:6n-3/20:5n-3 ratio	2.36 ± 0.31	2.92 ± 0.47	2.43 ± 0.30	2.09 ± 0.23	5.11 ± 0.93	4.81 ± 0.46	3.09 ± 0.17	2.96 ± 0.11

[1]Also includes 8:0, 9:0, 10:0, 11:0, 12:0, 13:0, 15:0, 17:0, 20:0, 22:0 and 24:0. [2]Also includes 11:1, 13:1, 14:1, 15:1, 16:1n-9, 17:1, 19:1, 22:1n-9 and 24:1. [3]Also includes 20:3 n-6, 22:2n-6 and 22:5n-6.
[4]Also includes 18:4n-3.

Table 5
Metabolism of [1-^{14}C] PUFA by desaturation and/or elongation in the SBT-E1 cells. The data are the mean ± standard error of the mean (n = 3) and are expressed as % of total radioactivity recovered for each of the PUFA supplied. n.d. = not detected. The asterisk indicates that the conversion of 18:3n-3 to 20:3n-3 was significantly greater than its conversion to either 18:4n-3 or 20:4n-3. Different superscript letters indicate significant differences between the different supplied PUFA. The data were analysed by one-way ANOVA followed by Bonferroni *post-hoc* comparisons. Differences are considered significant when $p < 0.05$.

	[1-^{14}C] PUFA supplied		
Fatty acid	18:3n-3	18:2n-6	20:5n-3
18:3n-3	87.19 ± 0.96	n.d.	n.d.
18:4n-3	2.18 ± 0.08	n.d.	n.d.
20:3n-3	9.43 ± 0.76*	n.d.	n.d.
20:4n-3	1.20 ± 0.12	n.d.	n.d.
20:5n-3	n.d.	n.d.	83.20 ± 0.77
22:5n-3	n.d.	n.d.	16.80 ± 0.77
18:2n-6	n.d.	94.67 ± 0.35	n.d.
18:3n-6	n.d.	2.51 ± 0.27	n.d.
20:2n-6	n.d.	2.82 ± 0.08	n.d.
Total conversion	12.81 ± 0.96^{b}	5.33 ± 0.35^{c}	16.80 ± 0.77^{a}

References

CCSBT. "Commission for the Conservation of Southern Bluefin Tuna." from http://www.ccsbt.org/site/total_allowable_catch.php.

CST, 2011. "Tuna breeding program update." *Company announcement released on the Australian Securities Exchange 28/06/2011 at http://www.asx.com.au*.

CST, 2012. "Clean seas tuna ltd annual report 2012." *Annual report released on the Australian securities exchange 15/08/2012 at http://www.asx.com.au*.

ICCAT. "International Commission for the Conservation of Atlantic Tunas." from http://www.iccat.int/en/.

Abdi, H. 2007. The Bonferonni and Šidák corrections for multiple comparisons. *Encyclopedia of measurement and statistics*. N. Salkind (Ed.). Thousand Oaks, SAGE Publications.

Agawa, Y., Honryo, T., Ishii, A., Kobayashi, T., Oku, H. and Sawada, Y. 2012. "Molecular identification and tissue distribution of peroxisome proliferators activated receptor gamma transcript in cultured *Thunnus orientalis*." *Aquaculture Research* 43(8): 1145-1158.

Alp, P. R., Newsholme, E. A. and Zammit, V. A. 1976. "Activities of citrate synthase and NAD+-linked and NADP+-linked isocitrate dehydrogenase in muscle from vertebrates and invertebrates." *Biochemical Journal* 154: 689-700.

Altringham, J. D. and Block, B. A. 1997. "Why do tuna maintain elevated slow muscle temperatures? Power output of muscle isolated from endothermic and ectothermic fish." *Journal of Experimental Biology* 200(20): 2617-2627.

Andersson, U. and Scarpulla, R. C. 2001. "PGC-1-Related Coactivator, a Novel, Serum-Inducible Coactivator of Nuclear Respiratory Factor 1-Dependent Transcription in Mammalian Cells." *Molecular and cellular biology* 21(11): 3738-3749.

Bain, P. A., Hutchinson, R. G., Marks, A. B., Crane, M. S. J. and Schuller, K. A. 2013. "Establishment of a continuous cell line from southern bluefin tuna (*Thunnus maccoyii*)." *Aquaculture* 376–379: 59-63.

Baldán, Á., Relat, J., Marrero, P. F. and Haro, D. 2004. "Functional interaction between peroxisome proliferator-activated receptors-α and Mef-2C on human

carnitine palmitoyltransferase 1β (CPT1β) gene activation." *Nucleic Acids Research* 32(16): 4742-4749.

Barrera, G., Toaldo, C., Pizzimenti, S., Cerbone, A., Pettazzoni, P., Dianzani, M. U. and Ferretti, C. 2008. "The Role of PPAR Ligands in Controlling Growth-Related Gene Expression and their Interaction with Lipoperoxidation Products." *PPAR Research* 2008(524671).

Bell, J. G., Dick, J. R., McVicar, A. H., Sargent, J. R. and Thompson, K. D. 1993. "Dietary sunflower, linseed and fish oils affect phospholipid fatty acid composition, development of cardiac lesions, phospholipase activity and eicosanoid production in Atlantic salmon (*Salmo salar*)." *Prostaglandins, Leukotrienes and Essential Fatty Acids* 49(3): 665-673.

Bell, J. G., Henderson, R. J., Tocher, D. R., McGhee, F., Dick, J. R., Porter, A., Smullen, R. P. and Sargent, J. R. 2002. "Substituting fish oil with crude palm oil in the diet of Atlantic salmon (*Salmo salar*) affects muscle fatty acid composition and hepatic fatty acid metabolism." *The Journal of Nutrition* 132(2): 222-230.

Bell, J. G., McEvoy, J., Tocher, D. R., McGhee, F., Campbell, P. J. and Sargent, J. R. 2001. "Replacement of fish oil with rapeseed oil in diets of Atlantic salmon (*Salmo salar*) affects tissue lipid compositions and hepatocyte fatty acid metabolism." *Journal of Nutrition* 131(5): 1535-1543.

Bell, M. and Tocher, D. 2009. Biosynthesis of polyunsaturated fatty acids in aquatic ecosystems: General pathways and new directions. *Lipids in aquatic ecosystems*. M. Kainz, M. T. Brett and M. T. Arts (Ed.). New York, Springer**:** 211-236.

Bermejo-Nogales, A., Calduch-Giner, J. and Pérez-Sánchez, J. 2014. "Tissue-specific gene expression and functional regulation of uncoupling protein 2 (UCP2) by hypoxia and nutrient availability in gilthead sea bream (Sparus aurata): implications on the physiological significance of UCP1–3 variants." *Fish Physiology and Biochemistry* 40(3): 751-762.

Black, B. L. and Olson, E. N. 1998. "Transcriptional control of muscle development by myocyte enhancer factor-2 (MEF2) proteins." *Annual Review of Cell and Developmental Biology* 14(1): 167-196.

Blennemann, P., Moon, Y. K. and Freake, H. C. 1992. "Tissue-specific regulation of fatty acid synthesis by thyroid hormone." *Endocrinology* 130(2): 637-643.

Block, B. A. 1994. "Thermogenesis in Muscle." *Annual Review of Physiology* 56(1): 535-577.

Block, B. A. and Finnerty, J. R. 1994. "Endothermy in fishes: a phylogenetic analysis of constraints, predispositions, and selection pressures." *Environmental Biology of Fishes* 40(3): 283-302.

Block, B. A., Finnerty, J. R., Stewart, A. F. R. and Kidd, J. 1993. "Evolution of endothermy in fish: Mapping physiological traits on a molecular phylogeny." *Science* 260(5105): 210-214.

Brand, M. D. 2000. "Uncoupling to survive? The role of mitochondrial inefficiency in ageing." *Experimental Gerontology* 35(6–7): 811-820.

Brand, M. D. and Esteves, T. C. 2005. "Physiological functions of the mitochondrial uncoupling proteins UCP2 and UCP3." *Cell Metabolism* 2(2): 85-93.

Bremer, K., Monk, C. T., Gurd, B. J. and Moyes, C. D. 2012. "Transcriptional regulation of temperature-induced remodeling of muscle bioenergetics in goldfish." *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* 303(2): R150-R158.

Bremer, K. and Moyes, C. D. 2011. "Origins of variation in muscle cytochrome c oxidase activity within and between fish species." *The Journal of Experimental Biology* 214(11): 1888-1895.

Brill, R. W. 1996. "Selective advantages conferred by the high performance physiology of tunas, billfishes, and dolphin fish." *Comparative Biochemistry and Physiology Part A: Physiology* 113(1): 3-15.

Brill, R. W. and Bushnell, P. G. 2001. The cardiovascular system of tunas. *Fish Physiology*. B. Barbara and E. Stevens (Ed.), Academic Press. Volume 19**:** 79-120.

Bubner, E., Farley, J., Thomas, P., Bolton, T. and Elizur, A. 2012. "Assessment of reproductive maturation of southern bluefin tuna (*Thunnus maccoyii*) in captivity." *Aquaculture* 364–365(0): 82-95.

Calder, P. C. 2009. "Polyunsaturated fatty acids and inflammatory processes: New twists in an old tale." *Biochimie* 91(6): 791-795.

Calder, P. C. and Yaqoob, P. 2009. "Omega 3 polyunsaturated fatty acids and human health outcomes." *Biofactors* 35(3): 266-272.

Cech, J. J., Laurs, R. M. and Graham, J. B. 1984. "Temperature-induced changes in blood gas equilibria in the albacore, *Thunnus alalunga*, a warm-bodied tuna." *Journal of Experimental Biology* 109(1): 21-34.

Chan, M. C. and Arany, Z. 2014. "The many roles of PGC-1α in muscle — recent developments." *Metabolism - Clinical and Experimental* 63(4): 441-451.

Chapkin, R. S., Kim, W., Lupton, J. R. and McMurray, D. N. 2009. "Dietary docosahexaenoic and eicosapentaenoic acid: Emerging mediators of inflammation." *Prostaglandins, Leukotrienes and Essential Fatty Acids* 81(2–3): 187-191.

Chidakel, A., Mentuccia, D. and Celi, F. S. 2005. "Peripheral Metabolism of Thyroid Hormone and Glucose Homeostasis." *Thyroid* 15(8): 899-903.

Chinsomboon, J., Ruas, J., Gupta, R. K., Thom, R., Shoag, J., Rowe, G. C., Sawada, N., Raghuram, S. and Arany, Z. 2009. "The transcriptional coactivator PGC-1α mediates exercise-induced angiogenesis in skeletal muscle." *Proceedings of the National Academy of Sciences* 106(50): 21401-21406.

Chomczynski, P. and Mackey, K. 1995. "Short technical reports. Modification of the TRI reagent procedure for isolation of RNA from polysaccharide- and proteoglycan-rich sources." *Biotechniques* 19(6): 942-945.

Christie, W. W., Ed. 1993. *Preparation of ester derivatives of fatty acids for chromatographic analysis*. Advances in Lipid Methodology - Two. Dundee, Oily Press.

Connor, W. E. 2000. "Importance of n-3 fatty acids in health and disease." *American Journal of Clinical Nutrition* 71(1): 171S-175S.

Corton, J. C. and Brown-Borg, H. M. 2005. "Peroxisome Proliferator-Activated Receptor γ Coactivator 1 in Caloric Restriction and Other Models of Longevity." *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences* 60(12): 1494-1509.

Dalziel, A. C., Moore, S. E. and Moyes, C. D. 2005. "Mitochondrial enzyme content in the muscles of high-performance fish: evolution and variation among fiber types." *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* 288(1): R163-R172.

de Antueno, R. J., Knickle, L. C., Smith, H., Elliot, M. L., Allen, S. J., Nwaka, S. and Winther, M. D. 2001. "Activity of human Δ5 and Δ6 desaturases on multiple n-3 and n-6 polyunsaturated fatty acids." *FEBS Letters* 509(1): 77-80.

De Metrio, G., Bridges, C. R., Mylonas, C. C., Caggiano, M., Deflorio, M., Santamaria, N., Zupa, R., Pousis, C., Vassallo-Agius, R., Gordin, H. and Corriero, A. 2010. "Spawning induction and large-scale collection of fertilized eggs in captive Atlantic bluefin tuna (*Thunnus thynnus* L.) and the first larval rearing efforts." *Journal of Applied Ichthyology* 26(4): 596-599.

Dewar, H., Graham, J. and Brill, R. 1994. "Studies of tropical tuna swimming performance in a large water tunnel - thermoregulation." *The Journal of Experimental Biology* 192(1): 33-44.

Dickson, K. A. and Graham, J. B. 2004. "Evolution and Consequences of Endothermy in Fishes." *Physiological and Biochemical Zoology* 77(6): 998-1018.

Duplus, E., Glorian, M. and Forest, C. 2000. "Fatty acid regulation of gene transcription." *Journal of Biological Chemistry* 275(40): 30749-30752.

Faizan, M., Stubhaug, I., Menoyo, D., Esatbeyoglu, T., Wagner, A. E., Struksnæs, G., Koppe, W. and Rimbach, G. 2013. "Dietary Alpha-Tocopherol Affects Tissue Vitamin E and Malondialdehyde Levels but Does not Change Antioxidant Enzymes and Fatty Acid Composition in Farmed Atlantic Salmon (*Salmo salar* L.)." *International Journal for Vitamin and Nutrition Research* 83(4): 238-245.

Farley, J. H. and Davis, T. L. O. 1998. "Reproductive dynamics of southern bluefin tuna, *Thunnus maccoyii*." *Fishery Bulletin* 96(2): 223-236.

Farley, J. H., Davis, T. L. O., Gunn, J. S., Clear, N. P. and Preece, A. L. 2007. "Demographic patterns of southern bluefin tuna, *Thunnus maccoyii*, as inferred from direct age data." *Fisheries Research* 83(2–3): 151-161.

Fernández-Palacios, H., Izquierdo, M. S., Robaina, L., Valencia, A., Salhi, M. and Vergara, J. 1995. "Effect of n − 3 HUFA level in broodstock diets on egg quality of gilthead sea bream (Sparus aurata L.)." *Aquaculture* 132(3–4): 325-337.

Fijan, N., Sulimanović, D., Bearzotti, M., Muzinić, D., Zwillenberg, L. O., Chilmonczyk, S., Vautherot, J. F. and de Kinkelin, P. 1983. "Some properties of the Epithelioma papulosum cyprini (EPC) cell line from carp *cyprinus carpio*." *Annales de l'Institut Pasteur / Virologie* 134(2): 207-220.

Fountoulaki, E., Vasilaki, A., Hurtado, R., Grigorakis, K., Karacostas, I., Nengas, I., Rigos, G., Kotzamanis, Y., Venou, B. and Alexis, M. N. 2009. "Fish oil substitution by vegetable oils in commercial diets for gilthead sea bream (*Sparus aurata* L.); effects on growth performance, flesh quality and fillet fatty acid profile: Recovery of fatty acid profiles by a fish oil finishing diet under fluctuating water temperatures." *Aquaculture* 289(3-4): 317-326.

Freon, P., Cury, P., Shannon, L. and Roy, C. 2005. "Sustainable Exploitation of Small Pelagic Fish Stocks Challenged by Environmental and Ecosystem Changes: A Review." *Bulletin of Marine Science* 76(2): 385-462.

Fritsche, K. L., Cassity, N. A. and Huang, S.-C. 1992. "Dietary (n-3) Fatty Acid and Vitamin E Interactions in Rats: Effects on Vitamin E Status, Immune Cell Prostaglandin E Production and Primary Antibody Response." *Journal of Nutrition* 122(4): 1009-1018.

Gao, J., Koshio, S., Ishikawa, M., Yokoyama, S., Mamauag, R. E. P. and Han, Y. 2012. "Effects of dietary oxidized fish oil with vitamin E supplementation on growth performance and reduction of lipid peroxidation in tissues and blood of red sea bream *Pagrus major*." *Aquaculture* 356–357: 73-79.

Gao, J., Lin, H., Wang, X., Song, Z. and Jiao, H. 2010. "Vitamin E supplementation alleviates the oxidative stress induced by dexamethasone treatment and improves meat quality in broiler chickens." *Poultry Science* 89(2): 318.

Gayral, P., Weinert, L., Chiari, Y., Tsagkogeorga, G., Ballenghien, M. and Galtier, N. 2011. "Next-generation sequencing of transcriptomes: a guide to RNA isolation in nonmodel animals." *Molecular Ecology Resources* 11(4): 650-661.

Ghioni, C., Tocher, D. R., Bell, M. V., Dick, J. R. and Sargent, J. R. 1999. "Low C18 to C20 fatty acid elongase activity and limited conversion of stearidonic acid, 18:4 (n-3), to eicosapentaenoic acid, 20:5 (n-3), in a cell line from the turbot, *Scophthalmus maximus*." *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids* 1437(2): 170-181.

Ghioni, C., Tocher, D. R. and Sargent, J. R. 1997. "The effect of culture on morphology, lipid and fatty acid composition, and polyunsaturated fatty acid metabolism of rainbow trout (*Oncorhynchus mykiss*) skin cells." *Fish Physiology and Biochemistry* 16(6): 499-513.

Graham, J. B. and Dickson, K. A. 2004. "Tuna comparative physiology." *Journal of Experimental Biology* 207(23): 4015-4024.

Gregory, M. K. and James, M. J. 2014. "Rainbow trout (Oncorhynchus mykiss) Elovl5 and Elovl2 differ in selectivity for elongation of omega-3 docosapentaenoic acid." *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* 1841(12): 1656-1660.

Gregory, M. K., See, V. H. L., Gibson, R. A. and Schuller, K. A. 2010. "Cloning and functional characterisation of a fatty acyl elongase from southern bluefin tuna (*Thunnus maccoyii*)." *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* 155(2): 178-185.

Guan, H.-P., Ishizuka, T., Chui, P. C., Lehrke, M. and Lazar, M. A. 2005. "Corepressors selectively control the transcriptional activity of PPARγ in adipocytes." *Genes & Development* 19(4): 453-461.

Guderley, H. and Johnston, I. A. 1996. "Plasticity of fish muscle mitochondria with thermal acclimation." *The Journal of Experimental Biology* 199(6): 1311-1317.

Guerriero, G. and Ciarcia, G. 2001. "Free radicals and vitamin E antioxidant defence in the aquacultured sea bass reproduction." *Free Radical Biology and Medicine* 31: 6.

Gunn, J. S., Clear, N. P., Carter, T. I., Rees, A. J., Stanley, C. A., Farley, J. H. and Kalish, J. M. 2008. "Age and growth in southern bluefin tuna, *Thunnus maccoyii* (Castelnau): Direct estimation from otoliths, scales and vertebrae." *Fisheries Research* 92(2–3): 207-220.

Gutteridge, J. 1995. "Lipid peroxidation and antioxidants as biomarkers of tissue damage." *Clinical Chemistry* 41(12): 1819-1828.

Gutteridge, J. and Halliwell, B. 1990. "The measurement and mechanism of lipid peroxidation in biological systems." *Trends in Biochemical Sciences* 15(4): 129-135.

Halliwell, B. 2007. "Biochemistry of oxidative stress." *Biochemical Society Transactions* 35(5): 1147-1150.

Hardewig, I., van Dijk, P. L. M., Moyes, C. D. and Pörtner, H. O. 1999. *Temperature-dependent expression of cytochrome-c oxidase in Antarctic and temperate fish*.

Herzig, R. P., Scacco, S. and Scarpulla, R. C. 2000. "Sequential Serum-dependent Activation of CREB and NRF-1 Leads to Enhanced Mitochondrial Respiration through the Induction of Cytochrome c." *Journal of Biological Chemistry* 275(17): 13134-13141.

Hulbert, A. J., Pamplona, R., Buffenstein, R. and Buttemer, W. A. 2007. "Life and death: Metabolic rate, membrane composition, and life span of animals." *Physiological Reviews* 87(4): 1175-1213.

Ihsan, M., Watson, G. and Abbiss, C. 2014. "PGC-1α Mediated Muscle Aerobic Adaptations to Exercise, Heat and Cold Exposure." *Cellular and Molecular Exercise Physiology* 3(1): e7.

Innis, S. M. 2008. "Dietary omega 3 fatty acids and the developing brain." *Brain Research* 1237(0): 35-43.

Itoh, T., Kemps, H. and Totterdell, J. 2011. "Diet of young southern bluefin tuna *Thunnus maccoyii* in the southwestern coastal waters of Australia in summer." *Fisheries Science* 77(3): 337-344.

Izquierdo, M. S. 1996. "Essential fatty acid requirements of cultured marine fish larvae." *Aquaculture Nutrition* 2(4): 183-191.

Jóźwik, A. and Śliwa-Jóźwik, A. K. 2005. "The effect of administration of high doses of selected antioxidants upon the activity of beta-glucuronidase (β-GL) in liver, kidney and blood plasma of mice." *Animal Science Papers and Reports* 23(4): 219-227.

Jubrias, S. A., Vollestad, N. K., Gronka, R. K. and Kushmerick, M. J. 2008. "Contraction coupling efficiency of human first dorsal interosseous muscle." *The Journal of Physiology* 586(Pt 7): 1993-2002.

Khalimonchuk, O. and Rödel, G. 2005. "Biogenesis of cytochrome c oxidase." *Mitochondrion* 5(6): 363-388.

Kiessling, K. H. and Kiessling, A. 1993. "Selective utilization of fatty acids in rainbow trout (*Oncorhynchus mykiss* Walbaum) red muscle mitochondria." *Canadian Journal of Zoology* 71(2): 248-251.

Kim-Han, J. S. and Dugan, L. L. 2005. "Mitochondrial Uncoupling Proteins in the Central Nervous System." *Antioxidants & Redox Signaling* 7(9-10): 1173-1181.

Kirchhoff, N. T., Leef, M. J., Ellis, D., Purser, J. and Nowak, B. F. 2011. "Effects of the first two months of ranching on the health of southern bluefin tuna *Thunnus maccoyii*." *Aquaculture* 315(3-4): 207-212.

Kliewer, S. A., Forman, B. M., Blumberg, B., Ong, E. S., Borgmeyer, U., Mangelsdorf, D. J., Umesono, K. and Evans, R. M. 1994. "Differential expression and activation of a family of murine peroxisome proliferator-activated receptors." *Proceedings of the National Academy of Sciences of the United States of America* 91(15): 7355-7359.

Koressaar, T. and Remm, M. 2007. "Enhancements and modifications of primer design program Primer3." *Bioinformatics* 23(10): 1289-1291.

Kubista, M., Andrade, J. M., Bengtsson, M., Forootan, A., Jonák, J., Lind, K., Sindelka, R., Sjöback, R., Sjögreen, B., Strömbom, L., Ståhlberg, A. and Zoric, N. 2006. "The real-time polymerase chain reaction." *Molecular Aspects of Medicine* 27(2–3): 95-125.

Kubo, T., Sakamoto, W., Murata, O. and Kumai, H. 2008. "Whole-body heat transfer coefficient and body temperature change of juvenile Pacific bluefin tuna *Thunnus orientalis* according to growth." *Fisheries Science* 74(5): 995-1004.

Larkin, M., Blackshields, G., Brown, N., Chenna, R., McGettigan, P., McWilliam, H., Valentin, F., Wallace, I., Wilm, A. and Lopez, R. 2007. "Clustal W and Clustal X version 2.0." *Bioinformatics* 23(21): 2947-2948.

Leaver, M. J., Boukouvala, E., Antonopoulou, E., Diez, A., Favre-Krey, L., Ezaz, M. T., Bautista, J. M., Tocher, D. R. and Krey, G. 2005. "Three Peroxisome Proliferator-Activated Receptor Isotypes from Each of Two Species of Marine Fish." *Endocrinology* 146(7): 3150-3162.

Lebold, K. M., Jump, D. B., Miller, G. W., Wright, C. L., Labut, E. M., Barton, C. L., Tanguay, R. L. and Traber, M. G. 2011. "Vitamin E Deficiency Decreases Long-Chain PUFA in Zebrafish (*Danio rerio*)." *Journal of Nutrition* 141(12): 2113-2118.

LeMoine, C. M. R., Genge, C. E. and Moyes, C. D. 2008. "Role of the PGC-1 family in the metabolic adaptation of goldfish to diet and temperature." *Journal of Experimental Biology* 211(9): 1448-1455.

LeMoine, C. R., Craig, P., Dhekney, K., Kim, J. and McClelland, G. 2010a. "Temporal and spatial patterns of gene expression in skeletal muscles in response to swim training in adult zebrafish (Danio rerio)." *Journal of Comparative Physiology B* 180(1): 151-160.

LeMoine, C. R., Lougheed, S. and Moyes, C. 2010b. "Modular Evolution of PGC-1α in Vertebrates." *Journal of Molecular Evolution* 70(5): 492-505.

Li, Y., Monroig, O., Zhang, L., Wang, S., Zheng, X., Dick, J. R., You, C. and Tocher, D. R. 2010. "Vertebrate fatty acyl desaturase with Δ4 activity." *Proceedings of the National Academy of Sciences* 107(39): 16840-16845.

Liang, H. and Ward, W. F. 2006. "PGC-1α: a key regulator of energy metabolism." *Advances in Physiology Education* 30(4): 145-151.

Lin, J., Handschin, C. and Spiegelman, B. M. 2005. "Metabolic control through the PGC-1 family of transcription coactivators." *Cell Metabolism* 1(6): 361-370.

Lin, J., Puigserver, P., Donovan, J., Tarr, P. and Spiegelman, B. M. 2002. "Peroxisome Proliferator-activated Receptor γ Coactivator 1β (PGC-1β), A Novel PGC-1-related Transcription Coactivator Associated with Host Cell Factor." *Journal of Biological Chemistry* 277(3): 1645-1648.

Little, A. G., Lougheed, S. C. and Moyes, C. D. 2010. "Evolutionary affinity of billfishes (Xiphiidae and Istiophoridae) and flatfishes (Plueronectiformes): Independent and trans-subordinal origins of endothermy in teleost fishes." *Molecular Phylogenetics and Evolution* 56(3): 897-904.

Liu, C. and Lin, J. D. 2011. "PGC-1 coactivators in the control of energy metabolism." *Acta Biochimica et Biophysica Sinica* 43(4): 248-257.

Livak, K. J. and Schmittgen, T. D. 2001. "Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method." *Methods* 25(4): 402-408.

Logan, J., Rodríguez-Marín, E., Goñi, N., Barreiro, S., Arrizabalaga, H., Golet, W. and Lutcavage, M. 2011. "Diet of young Atlantic bluefin tuna (*Thunnus thynnus*) in eastern and western Atlantic foraging grounds." *Marine Biology* 158(1): 73-85.

Luquet, S., Gaudel, C., Holst, D., Lopez-Soriano, J., Jehl-Pietri, C., Fredenrich, A. and Grimaldi, P. A. 2005. "Roles of PPAR delta in lipid absorption and metabolism: a new target for the treatment of type 2 diabetes." *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease* 1740(2): 313-317.

Madsen, L., Rustan, A., Vaagenes, H., Berge, K., Dyrøy, E. and Berge, R. 1999. "Eicosapentaenoic and docosahexaenoic acid affect mitochondrial and peroxisomal fatty acid oxidation in relation to substrate preference." *Lipids* 34(9): 951-963.

Magnuson, J. J. 1972. "Comparative study of adaptations for continuous swimming and hydrostatic equilibrium of scombroid and xiphoid fishes." *Fishery Bulletin* 71(2): 337-356.

Mark, F. C., Lucassen, M. and Pörtner, H. O. 2006. "Thermal sensitivity of uncoupling protein expression in polar and temperate fish." *Comparative Biochemistry and Physiology Part D: Genomics and Proteomics* 1(3): 365-374.

Masuma, S., Miyashita, S., Yamamoto, H. and Kumai, H. 2008. "Status of Bluefin Tuna Farming, Broodstock Management, Breeding and Fingerling Production in Japan." *Reviews in Fisheries Science* 16(1-3): 385-390.

Mazière, C., Conte, M.-A., Degonville, J., Ali, D. and Mazière, J.-C. 1999. "Cellular Enrichment with Polyunsaturated Fatty Acids Induces an Oxidative Stress and Activates the Transcription Factors AP1 and NFκB." *Biochemical and Biophysical Research Communications* 265(1): 116-122.

McGee, S. L. and Hargreaves, M. 2004. "Exercise and Myocyte Enhancer Factor 2 Regulation in Human Skeletal Muscle." *Diabetes* 53(5): 1208-1214.

Metian, M., Pouil, S., Boustany, A. and Troell, M. 2014. "Farming of Bluefin Tuna–Reconsidering Global Estimates and Sustainability Concerns." *Reviews in Fisheries Science & Aquaculture* 22(3): 184-192.

Miller, M. R., Nichols, P. D. and Carter, C. G. 2008. "N-3 Oil sources for use in aquaculture–alternatives to the unsustainable harvest of wild fish." *Nutrition Research Reviews* 21(2): 85-96.

Mirebeau-Prunier, D., Le Pennec, S., Jacques, C., Gueguen, N., Poirier, J., Malthiery, Y. and Savagner, F. 2010. "Estrogen-related receptor α and PGC-1-related coactivator constitute a novel complex mediating the biogenesis of functional mitochondria." *FEBS Journal* 277(3): 713-725.

Miura, S., Kai, Y., Kamei, Y. and Ezaki, O. 2008. "Isoform-Specific Increases in Murine Skeletal Muscle Peroxisome Proliferator-Activated Receptor-γ Coactivator-1α (PGC-1α) mRNA in Response to β2-Adrenergic Receptor Activation and Exercise." *Endocrinology* 149(9): 4527-4533.

Miyake, M., Guillotreau, P., Sun, C.-H. and Ishimura, G. 2010. "Recent developments in the tuna industry: stocks, fisheries, management, processing, trade and markets." *FAO Fisheries and Aquaculture Technical paper* No. 543, Rome: 125p.

Monroig, Ó., Li, Y. and Tocher, D. R. 2011. "Delta-8 desaturation activity varies among fatty acyl desaturases of teleost fish: High activity in delta-6 desaturases of marine species." *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* 159(4): 206-213.

Monroig, Ó., Rotllant, J., Sánchez, E., Cerdá-Reverter, J. M. and Tocher, D. R. 2009. "Expression of long-chain polyunsaturated fatty acid (LC-PUFA) biosynthesis genes during zebrafish *Danio rerio* early embryogenesis." *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* 1791(11): 1093-1101.

Monteiro, J., Leslie, M., Moghadasian, M. H., Arendt, B. M., Allard, J. P. and Ma, D. W. L. 2014. "The role of n - 6 and n - 3 polyunsaturated fatty acids in the manifestation of the metabolic syndrome in cardiovascular disease and non-alcoholic fatty liver disease." *Food & Function* 5(3): 426-435.

Morais, S., Castanheira, F., Martinez-Rubio, L., Conceição, L. E. C. and Tocher, D. R. 2012. "Long chain polyunsaturated fatty acid synthesis in a marine vertebrate: Ontogenetic and nutritional regulation of a fatty acyl desaturase with Δ4 activity." *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* 1821(4): 660-671.

Morais, S., Monroig, O., Zheng, X., Leaver, M. and Tocher, D. 2009. "Highly Unsaturated Fatty Acid Synthesis in Atlantic Salmon: Characterization of ELOVL5- and ELOVL2-like Elongases." *Marine Biotechnology* 11(5): 627-639.

Morais, S., Mourente, G., Ortega, A., Tocher, J. A. and Tocher, D. R. 2011. "Expression of fatty acyl desaturase and elongase genes, and evolution of DHA:EPA ratio during development of unfed larvae of Atlantic bluefin tuna (*Thunnus thynnus* L.)." *Aquaculture* 313: 129-139.

Moreno, V. J., de Moreno, J. E. A. and Brenner, R. R. 1979. "Biosynthesis of unsaturated fatty acids in the diatom *Phaeodactylum tricornutum*." *Lipids* 14(1): 15-19.

Mori, T., Kondo, H., Hase, T., Tokimitsu, I. and Murase, T. 2007. "Dietary Fish Oil Upregulates Intestinal Lipid Metabolism and Reduces Body Weight Gain in C57BL/6J Mice." *The Journal of Nutrition* 137(12): 2629-2634.

Mourente, G., Bell, J. and Tocher, D. 2007. "Does dietary tocopherol level affect fatty acid metabolism in fish?" *Fish Physiology and Biochemistry* 33(3): 269-280.

Mourente, G., Diaz-Salvago, E., Bell, J. and Tocher, D. 2002a. "Increased activities of hepatic antioxidant defence enzymes in juvenile gilthead sea bream (*Sparus aurata* L.) fed dietary oxidised oil: attenuation by dietary vitamin E." *Aquaculture* 214(1-4): 343-361.

Mourente, G., Diaz-Salvago, E., Tocher, D. and Bell, J. 2000. "Effects of dietary polyunsaturated fatty acid/vitamin E (PUFA/tocopherol ratio on antioxidant defence mechanisms of juvenile gilthead sea bream (*Sparus aurata* L., Osteichthyes, Sparidae)." *Fish Physiology and Biochemistry* 23(4): 337-351.

Mourente, G., Dick, J. R., Bell, J. G. and Tocher, D. R. 2005. "Effect of partial substitution of dietary fish oil by vegetable oils on desaturation and β-oxidation of [1-^{14}C]18:3n−3 (LNA) and [1-^{14}C]20:5n−3 (EPA) in hepatocytes and enterocytes of European sea bass (*Dicentrarchus labrax* L.)." *Aquaculture* 248(1–4): 173-186.

Mourente, G., Megina, C. and Díaz-Salvago, E. 2002b. "Lipids in female northern bluefin tuna (*Thunnus thynnus thynnus* L.) during sexual maturation." *Fish Physiology and Biochemistry* 24(4): 351-363.

Mourente, G. and Tocher, D. R. 2009. "Tuna nutrition and feeds: Current status and future perspectives." *Reviews in Fisheries Science* 17(3): 373-390.

Moyes, C. D. 2003. "Controlling muscle mitochondrial content." *Journal of Experimental Biology* 206(24): 4385-4391.

Moyes, C. D., Mathieu-Costello, O. A., Brill, R. W. and Hochachka, P. W. 1992. "Mitochondrial metabolism of cardiac and skeletal muscles from a fast (*Katsuwonus pelamis*) and a slow (*Cyprinus carpio*) fish." *Canadian Journal of Zoology* 70(6): 1246-1253.

Murase, T., Kondo, H., Hase, T., Tokimitsu, I. and Saito, M. 2001. "Abundant expression of uncoupling protein-2 in the small intestine: up-regulation by dietary fish oil and fibrates." *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* 1530(1): 15-22.

Murase, T. and Saito, H. 1996. "The docosahexaenoic acid content in the lipid of albacore *Thunnus alalunga* caught in two separate localities." *Fisheries Science* 62(4): 634-638.

Musgrove, R. J., Carragher, J. F., Manning, A. D., Zammit, B. J., Thomas, P. M. and Buchanan, J. 2011. "Effects of postharvest processes on quality of Australian sardines (*Sardinops sagax*) and redbait (*Emmelichthys nitidus nitidus*) for feeding aquacultured southern bluefin tuna (*Thunnus maccoyii*)." *Aquaculture Nutrition* 17(2): e19-e29.

Mylonas, C. C., De La Gándara, F., Corriero, A. and Ríos, A. B. 2010. "Atlantic Bluefin Tuna (*Thunnus Thynnus*) Farming and Fattening in the Mediterranean Sea." *Reviews in Fisheries Science* 18(3): 266-280.

Nakamura, Y., Ando, M., Seoka, M., Kawasaki, K. and Tsukamasa, Y. 2007. "Changes of proximate and fatty acid compositions of the dorsal and ventral ordinary muscles of the full-cycle cultured Pacific bluefin tuna *Thunnus orientalis* with the growth." *Food Chemistry* 103(1): 234-241.

Nicholas, K. B., Nicholas, H. B. and Deerfield, D. W. 1997. "GeneDoc: analysis and visualization of genetic variation." *EMBNEW. NEWS* 4: 14.

Nichols, P., Virtue, P., Mooney, B., Elliott, N. and Yearsley, G. 1998. *Seafood the good food. The oil content and composition of Australian commercial fishes, shellfishes and crustaceans*. Hobart, CSIRO Marine Research.

Nicholson, B. L. and Byrne, C. 1973. "An Established Cell Line from the Atlantic Salmon (Salmo salar)." *Journal of the Fisheries Research Board of Canada* 30(7): 913-916.

O'Brien, K. M. 2011. "Mitochondrial biogenesis in cold-bodied fishes." *The Journal of Experimental Biology* 214(2): 275-285.

Oberkofler, H., Esterbauer, H., Linnemayr, V., Strosberg, A. D., Krempler, F. and Patsch, W. 2002. "Peroxisome Proliferator-activated Receptor (PPAR) γ Coactivator-1 Recruitment Regulates PPAR Subtype Specificity." *Journal of Biological Chemistry* 277(19): 16750-16757.

Okada, T., Honryo, T., Sawada, Y., Agawa, Y., Miyashita, S. and Ishibashi, Y. 2014. "The cause of death of juvenile Pacific bluefin tuna (*Thunnus orientalis*) reared in sea net cages." *Aquacultural Engineering* 59: 23-25.

Oppedal, F., Vågseth, T., Dempster, T., Juell, J.-E. and Johansson, D. 2011. "Fluctuating sea-cage environments modify the effects of stocking densities on production and welfare parameters of Atlantic salmon (*Salmo salar* L.)." *Aquaculture* 315(3–4): 361-368.

Orczewska, J. I., Hartleben, G. and O'Brien, K. M. 2010. "The molecular basis of aerobic metabolic remodeling differs between oxidative muscle and liver of threespine sticklebacks in response to cold acclimation." *American Journal of Physiology* 299(1): R352-R364.

Ottolenghi, F., Ed. 2008. *Capture-based aquaculture of bluefin tuna*. Capture-based aquaculture. Global overview. *FAO Fisheries Technical Paper*. Rome, FAO Fisheries Technical Paper.

Patterson, T. A., Evans, K., Carter, T. I. and Gunn, J. S. 2008. "Movement and behaviour of large southern bluefin tuna (*Thunnus maccoyii*) in the Australian region determined using pop-up satellite archival tags." *Fisheries Oceanography* 17(5): 352-367.

Perhar, G., Arhonditsis, G. B. and Brett, M. T. 2012. "Modelling the role of highly unsaturated fatty acids in planktonic food web processes: A mechanistic approach." *Environmental Reviews* 20(3): 155-172.

Poli, B. M., Parisi, G., Scappini, F. and Zampacavallo, G. 2005. "Fish welfare and quality as affected by pre-slaughter and slaughter management." *Aquaculture International* 13(1-2): 29-49.

Poyton, R. O., Trueblood, C. E., Wright, R. M. and Farrell, L. E. 1988. "Expression and Function of Cytochrome c Oxidase Subunit Isologues." *Annals of the New York Academy of Sciences* 550(1): 289-307.

Proctor, C. H., Thresher, R. E., Gunn, J. S., Mills, D. J., Harrowfield, I. R. and Sie, S. H. 1995. "Stock structure of the southern bluefin tuna *Thunnus maccoyii*: an investigation based on probe microanalysis of otolith composition." *Marine Biology* 122(4): 511-526.

Pucci, E., Chiovato, L. and Pinchera, A. 2000. "Thyroid and lipid metabolism." *International Journal of Obesity and Related Metabolic Disorders* 24(S2): S109-112.

Puigserver, P., Wu, Z., Park, C. W., Graves, R., Wright, M. and Spiegelman, B. M. 1998. "A Cold-Inducible Coactivator of Nuclear Receptors Linked to Adaptive Thermogenesis." *Cell* 92(6): 829-839.

Radonić, A., Thulke, S., Mackay, I. M., Landt, O., Siegert, W. and Nitsche, A. 2004. "Guideline to reference gene selection for quantitative real-time PCR." *Biochemical and Biophysical Research Communications* 313(4): 856-862.

Ramsden, D. B., Ho, P. W. L., Ho, J. W. M., Liu, H.-F., So, D. H. F., Tse, H.-M., Chan, K.-H. and Ho, S.-L. 2012. "Human neuronal uncoupling proteins 4 and 5 (UCP4 and UCP5): structural properties, regulation, and physiological role in protection against oxidative stress and mitochondrial dysfunction." *Brain and Behavior* 2(4): 468-478.

Repetto, G., Del Peso, A. and Zurita, J. L. 2008. "Neutral red uptake assay for the estimation of cell viability/cytotoxicity." *Nature Protocols* 3(7): 1125-1131.

Ricquier, D. and Bouillaud, F. 2000. "The uncoupling protein homologues: UCP1, UCP2, UCP3, StUCP and AtUCP." *Biochemical Journal* 345(Pt 2): 161-179.

Ridgway, K. R. and Condie, S. A. 2004. "The 5500-km-long boundary flow off western and southern Australia." *Journal of Geophysical Research* 109: 1-18.

Rodnick, K. J. and Sidell, B. D. 1994. "Cold acclimation increases carnitine palmitoyltransferase I activity in oxidative muscle of striped bass." *American Journal of Physiology* 266(2 Pt 2): R405-412.

Romano, P., Manniello, A., Aresu, O., Armento, M., Cesaro, M. and Parodi, B. 2009. "Cell Line Data Base: structure and recent improvements towards molecular authentication of human cell lines." *Nucleic acids research* 37(suppl 1): D925-D932.

Ruas, Jorge L., White, James P., Rao, Rajesh R., Kleiner, S., Brannan, Kevin T., Harrison, Brooke C., Greene, Nicholas P., Wu, J., Estall, Jennifer L., Irving, Brian A., Lanza, Ian R., Rasbach, Kyle A., Okutsu, M., Nair, K. S., Yan, Z., Leinwand, Leslie A. and Spiegelman, Bruce M. 2012. "A PGC-1α Isoform Induced by Resistance Training Regulates Skeletal Muscle Hypertrophy." *Cell* 151(6): 1319-1331.

Sadana, P. and Park, Edwards A. 2007. "Characterization of the transactivation domain in the peroxisome-proliferator-activated receptor γ co-activator (PGC-1)." *Biochemical Journal* 403(Pt 3): 511-518.

Saito, H., Ishihara, K. and Murase, T. 1996. "Effect of prey fish lipids on the docosahexaenoic acid content of total fatty acids in the lipid of *Thunnus albacares* yellowfin tuna." *Bioscience, Biotechnology and Biochemistry* 60(6): 962-965.

Saito, H., Seike, Y., Ioka, H., Osako, K., Tanaka, M., Takashima, A., Keriko, J., Kose, S. and Souza, J. R. 2005. "High docosahexaenoic acid levels in both neutral

and polar lipids of a highly migratory fish: *Thunnus tonggol* (Bleeker)." *Lipids* 40(9): 941-953.

Salem, M., Silverstein, J., Rexroad, C. E. and Yao, J. 2007. "Effect of starvation on global gene expression and proteolysis in rainbow trout (*Oncorhynchus mykiss*)." *Bmc Genomics* 8: 328.

Sargent, J., Bell, G., McEvoy, L., Tocher, D. and Estevez, A. 1999. "Recent developments in the essential fatty acid nutrition of fish." *Aquaculture* 177(1-4): 191-199.

Sawada, Y., Okada, T., Miyashita, S., Murata, O. and Kumai, H. 2005. "Completion of the Pacific bluefin tuna *Thunnus orientalis* (Temminck et Schlegel) life cycle." *Aquaculture Research* 36(5): 413-421.

Scarpulla, R. C. 1997. "Nuclear Control of Respiratory Chain Expression in Mammalian Cells." *Journal of Bioenergetics and Biomembranes* 29(2): 109-119.

Scarpulla, R. C., Vega, R. B. and Kelly, D. P. 2012. "Transcriptional integration of mitochondrial biogenesis." *Trends in Endocrinology & Metabolism* 23(9): 459-466.

Schoonjans, K., Staels, B. and Auwerx, J. 1996. "The peroxisome proliferator activated receptors (PPARs) and their effects on lipid metabolism and adipocyte differentiation." *Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism* 1302(2): 93-109.

Schreiber, S. N., Emter, R., Hock, M. B., Knutti, D., Cardenas, J., Podvinec, M., Oakeley, E. J. and Kralli, A. 2004. "The estrogen-related receptor α (ERRα) functions in PPARγ coactivator 1α (PGC-1α)-induced mitochondrial biogenesis." *Proceedings of the National Academy of Sciences of the United States of America* 101(17): 6472-6477.

Sears, I. B., MacGinnitie, M. A., Kovacs, L. G. and Graves, R. A. 1996. "Differentiation-dependent expression of the brown adipocyte uncoupling protein gene: regulation by peroxisome proliferator-activated receptor gamma." *Molecular and cellular biology* 16(7): 3410-3419.

Seebacher, F. 2009. "Responses to temperature variation: integration of thermoregulation and metabolism in vertebrates." *Journal of Experimental Biology* 212(18): 2885-2891.

Sepulveda, C. A., Dickson, K. A., Frank, L. R. and Graham, J. B. 2007. "Cranial endothermy and a putative brain heater in the most basal tuna species, Allothunnus fallai." *Journal of Fish Biology* 70(6): 1720-1733.

Shimose, T., Watanabe, H., Tanabe, T. and Kubodera, T. 2013. "Ontogenetic diet shift of age-0 year Pacific bluefin tuna *Thunnus orientalis*." *Journal of Fish Biology* 82(1): 263-276.

Simopoulos, A. P. 2002. "Omega-3 fatty acids in inflammation and autoimmune diseases." *Journal of the American College of Nutrition* 21(6): 495-505.

Sprague, M., Dick, J. R., Medina, A., Tocher, D. R., Bell, J. and Mourente, G. 2012. "Lipid and fatty acid composition, and persistent organic pollutant levels in tissues of migrating Atlantic bluefin tuna (*Thunnus thynnus*, L.) broodstock." *Environmental Pollution* 171: 61-71.

Sprecher, H. 2000. "Metabolism of highly unsaturated n-3 and n-6 fatty acids." *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* 1486(2–3): 219-231.

St-Pierre, J., Lin, J., Krauss, S., Tarr, P. T., Yang, R., Newgard, C. B. and Spiegelman, B. M. 2003. "Bioenergetic Analysis of Peroxisome Proliferator-activated Receptor γ Coactivators 1α and 1β (PGC-1α and PGC-1β) in Muscle Cells." *Journal of Biological Chemistry* 278(29): 26597-26603.

Stephan, M. and Hobsbawn, P. 2014. *Australian fisheries and aquaculture statistics 2013*. ABARES, Canberra, Fisheries Research and Development Corporation project 2010/208.

Stramma, L., Johnson, G. C., Sprintall, J. and Mohrholz, V. 2008. "Expanding Oxygen-Minimum Zones in the Tropical Oceans." *Science* 320(5876): 655-658.

Stramma, L., Schmidtko, S., Levin, L. A. and Johnson, G. C. 2010. "Ocean oxygen minima expansions and their biological impacts." *Deep Sea Research Part I: Oceanographic Research Papers* 57(4): 587-595.

Stuart, J. A., Harper, J. A., Brindle, K. M. and Brand, M. D. 1999. "Uncoupling protein 2 from carp and zebrafish, ectothermic vertebrates." *Biochimica et Biophysica Acta (BBA) - Bioenergetics* 1413(1): 50-54.

Stubhaug, I., Lie, Ø. and Torstensen, B. E. 2007. "Fatty acid productive value and β-oxidation capacity in Atlantic salmon (*Salmo salar* L.) fed on different lipid sources along the whole growth period." *Aquaculture Nutrition* 13(2): 145-155.

Stubhaug, I., Tocher, D. R., Bell, J. G., Dick, J. R. and Torstensen, B. E. 2005. "Fatty acid metabolism in Atlantic salmon (*Salmo salar* L.) hepatocytes and influence of dietary vegetable oil." *Biochimica et Biophysica Acta* 1734(3): 277-288.

Tacon, A. G. J. and Metian, M. 2013. "Fish Matters: Importance of Aquatic Foods in Human Nutrition and Global Food Supply." *Reviews in Fisheries Science* 21(1): 22-38.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S. 2011. "MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods." *Molecular biology and evolution* 28(10): 2731-2739.

Timmiss, T. 2011. "Letter to SBT concession holders regarding SBT TAC's for 2011/12 and 2012/13." *Australian Fisheries Management Authority*.

Tiranti, V., Rossi, E., Rocchi, M., DiDonato, S., Zuffardi, O. and Zeviani, M. 1995. "The Gene (NFE2L1) for Human NRF-1, an Activator Involved in Nuclear-Mitochondrial Interactions, Maps to 7q32." *Genomics* 27(3): 555-557.

Tocher, D. R. 1995. Glycerophospholipid metabolism. *Biochemistry and Molecular Biology of Fishes*. P. W. Hochachka and T. P. Mommsen (Ed.), Elsevier. 4**:** 119-157.

Tocher, D. R. 2003. "Metabolism and functions of lipids and fatty acids in teleost fish." *Reviews in Fisheries Science* 11(2): 107-184.

Tocher, D. R., Carr, J. and Sargent, J. R. 1989. "Polyunsaturated fatty acid metabolism in fish cells: Differential metabolism of (n-3) and (n-6) series acids by cultured cells orginating from a freshwater teleost fish and from a marine teleost fish." *Comparative Biochemistry and Physiology Part B* 94(2): 367-374.

Tocher, D. R. and Dick, J. R. 1991. "Effect of polyunsaturated fatty acids on the growth of fish cells in culture." *Comparative Biochemistry and Physiology. A. Comparative Physiology* 100(2): 461-466.

Tocher, D. R. and Dick, J. R. 1999. "Polyunsaturated fatty acid metabolism in a cell culture model of essential fatty acid deficiency in a freshwater fish, carp (*Cyprinus carpio*)." *Fish Physiology and Biochemistry* 21(3): 257-267.

Tocher, D. R., Fonseca-Madrigal, J., Dick, J. R., Ng, W.-K., Bell, J. G. and Campbell, P. J. 2004. "Effects of water temperature and diets containing palm oil on fatty acid desaturation and oxidation in hepatocytes and intestinal enterocytes of rainbow trout (*Oncorhynchus mykiss*)." *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* 137(1): 49-63.

Tocher, D. R. and Ghioni, C. 1999. "Fatty acid metabolism in marine fish: Low activity of fatty acyl Δ5 desaturation in gilthead sea bream (*Sparus aurata*) cells." *Lipids* 34(5): 433-440.

Tocher, D. R. and Sargent, J. R. 1990. "Effect of temperature on the incorporation into phospholipid classes and metabolism via desaturation and elongation of n−3 and n−6 polyunsaturated fatty acids in fish cells in culture." *Lipids* 25(8): 435-442.

Tocher, D. R., Sargent, J. R. and Frerichs, G. N. 1988. "The fatty acid compositions of established fish cell lines after long-term culture in mammalian sera." *Fish Physiology and Biochemistry* 5(4): 219-227.

Tocher, D. R., Zheng, X., Schlechtriem, C., Hastings, N., Dick, J. R. and Teale, A. J. 2006. "Highly unsaturated fatty acid synthesis in marine fish: Cloning, functional characterization, and nutritional regulation of fatty acyl Δ6 desaturase of Atlantic cod (*Gadus morhua* L.)." *Lipids* 41(11): 1003-1016.

Torstensen, B. E., Nanton, D. A., Olsvik, P. A., Sundvold, H. and Stubhaug, I. 2009. "Gene expression of fatty acid-binding proteins, fatty acid transport proteins (cd36 and FATP) and β-oxidation-related genes in Atlantic salmon (*Salmo salar* L.) fed fish oil or vegetable oil." *Aquaculture Nutrition* 15(4): 440-451.

Torstensen, B. E. and Stubhaug, I. 2004. "β-Oxidation of 18:3n−3 in Atlantic salmon (*Salmo salar* L.) hepatocytes treated with different fatty acids." *Lipids* 39(2): 153-160.

Tseng, Y.-C., Chen, R.-D., Lucassen, M., Schmidt, M. M., Dringen, R., Abele, D. and Hwang, P.-P. 2011. "Exploring Uncoupling Proteins and Antioxidant Mechanisms under Acute Cold Exposure in Brains of Fish." *PLoS ONE* 6(3): e18180.

Tsuda, Y., Sakamoto, W., Yamamoto, S. and Murata, O. 2012. "Effect of environmental fluctuations on mortality of juvenile Pacific bluefin tuna, Thunnus orientalis, in closed life-cycle aquaculture." *Aquaculture* 330–333(0): 142-147.

Turchini, G. M., Torstensen, B. E. and Ng, W. K. 2009. "Fish oil replacement in finfish nutrition." *Reviews in Aquaculture* 1(1): 10-57.

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B. C., Remm, M. and Rozen, S. G. 2012. "Primer3—new capabilities and interfaces." *Nucleic acids research* 40(15): e115.

Vercauteren, K., Pasko, R. A., Gleyzer, N., Marino, V. M. and Scarpulla, R. C. 2006. "PGC-1-Related Coactivator: Immediate Early Expression and Characterization of a CREB/NRF-1 Binding Domain Associated with Cytochrome c Promoter Occupancy and Respiratory Growth." *Molecular and cellular biology* 26(20): 7409-7419.

Vitello, F. and Zanetta, J. P. 1978. "Thin-layer chromatography of phospholipids." *Journal of Chromatography A* 166(2): 637-640.

Walter, I. and Seebacher, F. 2007. "Molecular mechanisms underlying the development of endothermy in birds (*Gallus gallus*): a new role of PGC-1α?" *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* 293(6): R2315-R2322.

Wang, X. and Quinn, P. J. 1999. "Vitamin E and its function in membranes." *Progress in Lipid Research* 38(4): 309-336.

Ward, R. D., Zemlak, T. S., Innes, B. H., Last, P. R. and Hebert, P. D. N. 2005. "DNA barcoding Australia's fish species." *Philosophical Transactions of the Royal Society B: Biological Sciences* 360(1462): 1847-1857.

Wiegand, G. and Remington, S. J. 1986. "Citrate Synthase: Structure, Control, and Mechanism." *Annual Review of Biophysics and Biophysical Chemistry* 15(1): 97-117.

Wikström, M., Morgan, J. E. and Verkhovsky, M. I. 1997. "Proton and electrical charge translocation by cytochrome-c oxidase." *Biochimica et Biophysica Acta (BBA) - Bioenergetics* 1318(1–2): 299-306.

Winton, J., Batts, W., DeKinkelin, P., LeBerre, M., Bremont, M. and Fijan, N. 2010. "Current lineages of the epithelioma papulosum cyprini (EPC) cell line are contaminated with fathead minnow, Pimephales promelas, cells." *Journal of Fish Diseases* 33(8): 701-704.

Woodhams, J., Vieira, S. and Stobutzki, I. 2013. Fishery status reports 2012. Canberra, Australian Bureau of Agricultural and Resource Economics and Sciences.

Wu, Z., Puigserver, P., Andersson, U., Zhang, C., Adelmant, G., Mootha, V., Troy, A., Cinti, S., Lowell, B., Scarpulla, R. C. and Spiegelman, B. M. 1999. "Mechanisms Controlling Mitochondrial Biogenesis and Respiration through the Thermogenic Coactivator PGC-1." *Cell* 98(1): 115-124.

Yates, C. M., Calder, P. C. and Ed Rainger, G. 2014. "Pharmacology and therapeutics of omega-3 polyunsaturated fatty acids in chronic inflammatory disease." *Pharmacology & Therapeutics* 141(3): 272-282.

Yu, Y. T., Breitbart, R. E., Smoot, L. B., Lee, Y., Mahdavi, V. and Nadal-Ginard, B. 1992. "Human myocyte-specific enhancer factor 2 comprises a group of tissue-restricted MADS box transcription factors." *Genes & Development* 6(9): 1783-1798.

Yúfera, M., Ortiz-Delgado, J. B., Hoffman, T., Siguero, I., Urup, B. and Sarasquete, C. 2014. "Organogenesis of digestive system, visual system and other structures in Atlantic bluefin tuna (*Thunnus thynnus*) larvae reared with copepods in mesocosm system." *Aquaculture* 426–427(0): 126-137.

Zhang , J. and Lazar, M. A. 2000. "The Mechanism of Action of Thyroid Hormones." *Annual Review of Physiology* 62(1): 439-466.

Zhang, Y., Huypens, P., Adamson, A. W., Chang, J. S., Henagan, T. M., Boudreau, A., Lenard, N. R., Burk, D., Klein, J., Perwitz, N., Shin, J., Fasshauer, M., Kralli, A. and Gettys, T. W. 2009. "Alternative mRNA Splicing Produces a Novel Biologically Active Short Isoform of PGC-1α." *Journal of Biological Chemistry* 284(47): 32813-32826.

Zhang, Y., Yasumoto, Y., Changlin, M. and Arima, T. 2001. "Vitamin E inhibits proliferation of primary cultured human mesangial and endothelial cells." *Nephron* 89(3): 291-296.

Zheng, X., Seiliez, I., Hastings, N., Tocher, D. R., Panserat, S., Dickson, C. A., Bergot, P. and Teale, A. J. 2004. "Characterization and comparison of fatty acyl Δ6 desaturase cDNAs from freshwater and marine teleost fish species." *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* 139(2): 269-279.