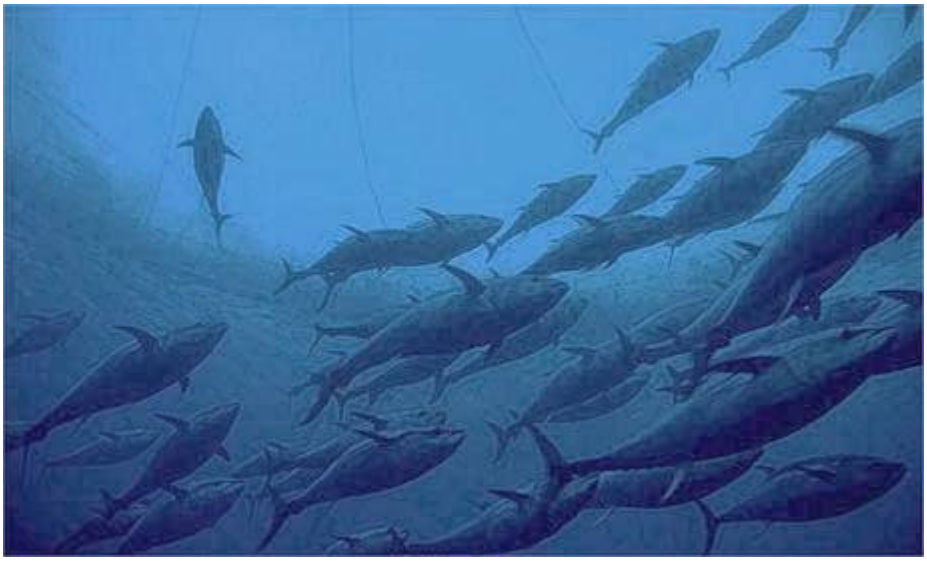
Understanding the role of GPR54 gene in controlling the puberty of high valued commercial fish



Ying Ying Lee
B.Sc. (Animal and Plant Biotechnology)

Submitted in fulfillment of requirements of the degree of Honours

Faculty of Science, Health and Education
University of the Sunshine Coast
May 2009

ISBN: 978-1-925982-42-8

Abstract

The cDNA encoding for G-protein coupled receptor 54 (GPR54) was cloned from the brains of SBT and YTK. The SBT GPR54 has an open reading frame of 1134bp encoding a predicted 378 amino acid peptide, containing seven putative transmembrane domains, a 138 bp 5'UTR and 238 3'UTR. The partial YTK GPR54 cDNA contains 729 bp nucleotide sequence encoding 243 amino acid residues. A RT-qPCR assay was developed for both SBT and YTK GPR54, as confirmed by primer specificity and high R^2 values. The reference gene used, Hprt1, displayed consistent Cq values across most of the different sampling points, however some interaction between the reference gene expression and developmental stage was observed. GPR54 expression levels were determined for two cohorts, an immature group and a pubertal group, at two sampling points. RT-qPCR results showed that YTK brain GPR54 expression levels did not show sexual dimorphism and were significantly higher in the reproductively mature fish at a point past their peak spawning time compared with expression in the immature fish group. The YTK gonad GPR54 expression level was significantly lower in males from the pubertal fish group during peak spawning time. In SBT, GPR54 expression profile in the brain and the gonad did not vary between immature and reproductively advanced fish. Analysis was carried out using both relative gene expression data and raw Cq. Alternative reference genes would need to be examined for the assay to be reliable across tissues and developmental time points.

Table of contents:

List of figures

List of Tables

List of abbreviations

ADP	Anterodorsal preoptic nucleus
AR	Androgen receptor
Arc	Arcuate nucleus
AVPV	Anteroventral periventricular nucleus
bp	Base pair
cDNA	Complementary deoxyribonucleic acid
Cq	Crossing point
DNA	Deoxyribonucleic acid
dNTPs	Dinucleotide triphosphate
E_2	Estradiol 17-β
eEF1A1	Elongation factor 1 alpha
ERα/β	Estrogen receptor α/β
FSH	Follicle-stimulating hormone
GnRH	Gonadotropin-releasing hormone
GPR54/Kiss1r	G-protein-coupled receptor 54
GSI	Gonadosomatic index
Hprt1	Hypoxanthine phosphoribosyltransferase 1
HPG axis	Hypothalamus-pituitary-gonad axis
LH	Luteinizing hormone
mRNA	Messenger ribonucleic acid
NPPv	Nucleus posterioris periventricularis
NVT	Nucleus ventral tuberis
PCR	Polymerase chain reaction
peN	Periventricular nucleus
PKA/C	Protein kinase A/C
RACE-PCR	Rapid amplification of cDNA ends-PCR
RNA	Ribonucleic acid
RPS4	Ribosomal protein S4
RT-PCR	Reverse transcription-PCR
RT-qPCR	Real-time Quantitative RT-PCR
SBT	Southern bluefin tuna

Spd	Spermatids
Spg	Spermatogonia
Spz	Spermatozoa
T	Testosterone
TMDs	Transmembrane domains
UBQ	Ubiquitin
5'or 3' UTR	5'or 3' Untranslated region
YTK	Yellowtail king fish

Acknowledgement

"The root of success is bitter but the fruit is sweet," my colleague Dr. Josephine Nocillado once told me. The words are the true reflection of my honours year. I had experienced all the frustration and excitement in scientific research. It is all the hard work and persistence that made me truly appreciate my honours year. However, I could not complete my journey along without everyone's help. First of all, I would like to sincerely thank my supervisor, Professor Abigail Elizur, for her advice, tremendous help, encouragement and support. I truly value the opportunity she provided me for doing my project. Her passion toward science inspires me enormously. Words cannot describe how grateful I am, it can only be felt by heart.

I sincerely thank Dr. Josephine Nocillado for her massive help on my project, her company with me through my frustration and her huge encouragement. She is more than just a colleague.

Thanks are due to Associate Professor Wayne Knibb for assistance on statistical analysis.

I thank all the members in my laboratory, Kelli Anderson, Anna Kuballa, Eva Hatje, and Rhonda Stokoe, who provided support and help.

Most importantly, I thank my parents. I could not fulfill my dream without their love, support and encouragement.

This project was funded by a Seafood CRC grant to my supervisor, Professor Abigail Elizur. All the fish samples were kindly provided by Cleanseas Tuna.

Ying Ying

Statement of Originality

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

…….
Ying Ying Lee
13th May 2009

1. Introduction

Puberty refers to the developmental period covering the transition from an immature juvenile to a mature adult reproductive system (Schulz and Goos, 1999). In fish, the onset of puberty is characterized by the appearance, for the first time, of spermatocytes in the males and beginning of vitellogenesis in the females, while its endpoint is the first spermiation in males and first ovulation in females (reviewed by Dufour *et al*., 2000).

In teleost fishes, an understanding of the regulation of puberty is important for aquaculture and fish farming (Okuzawa, 2002). The timing of the onset of puberty, which varies between species of fish, is directly linked to economic gain and reproductive management in the aquaculture industry. For instance, the late onset of maturity of the Southern Bluefin Tuna (SBT), *Thunnus maccoyii*, which takes 12 years to mature and at a body weight over 150 kg (Davis *et al*., 1998), is a major bottleneck in its aquaculture. This long generation time results in high cost and high risk associated with maintaining the broodstock in captivity. In this context, the ability to advance the onset of puberty would improve the cost-efficiency of the fish farming operation (Okuzawa, 2002). In contrast, the yellowtail kingfish (YTK), *Seriola lalandi,* undergoes relatively early puberty and can mature as early as 18 months, which is prior to commercial harvest (Clean Seas Tuna, personal communication). During reproductive development, the fish exerts much of its energy towards gonadal development, leading to deterioration of flesh quality and retardation of growth. Hence, one consequence of the early onset of puberty is reduced market value. In this case, the ability to delay the onset of puberty would be beneficial.

As in other vertebrates, the mechanisms underlying the puberty in fish are not fully understood (Okuzawa, 2002; Sisk and Foster, 2004; reviewed by Navarro *et al*., 2007). Until recently, it has been accepted that puberty is controlled by the hypothalamic-pituitary-gonad (HPG) axis (Weltzien *et al*., 2004). Specific neurons from the hypothalamus synthesize GnRH, which when secreted, stimulates the anterior pituitary to release follicle stimulating hormone (FSH) and luteinizing hormone (LH). FSH and LH in turn activate the somatic cells in the gonads to induce the synthesis and release of sex steroids, which control the various stages of gametogenesis.

The discovery that loss of function mutations in G-protein-coupled receptor 54 gene (GPR54=KiSS1r) resulted in the absence of puberty in humans (de Roux *et al*., 2003; Seminara *et al*., 2003) and in other mammalian models (Funes *et al*., 2003; Seminara *et al*., 2003) has added an entirely novel perspective regarding the regulation of puberty in vertebrates. GPR54 is now proposed to be the gatekeeper of puberty (Seminara *et al*., 2003). Gain of function mutations in GPR54 that resulted in precocious puberty in humans further added evidence regarding the critical role of GPR54 (Teles *et al*., 2008). Evidence has now accumulated showing that in mammals, the pathway in which GPR54 modulates the onset of puberty is through the stimulation of GnRH release from hypothalamic GnRH neurons (Messager *et al*., 2005; Plant *et al*., 2006; reviewed by Roa *et al*., 2008).

In contrast to the mammalian systems, research on the GPR54 system in fish is still at its infant stage (reviewed by Elizur 2009). The GPR54 gene and/or transcript has been characterized in several fish species including tilapia (*Oreochromis niloticus;* Parhar *et al.,* 2004), grey mullet (*Mugil cephalus;* Nocillado *et al*., 2007), cobia (*Rachycentron canadum;* Mohamed *et al*., 2007), and fathead minnow (*Pimephales promelas;* Filby *et al*., 2008). Two GPR54 genes have been identified in zebrafish (*Danio rerio;* Biran *et al*., 2008) and goldfish (*Carassius auratus;* Li *et al*., 2009). In addition, two isoforms of GPR54 that have been identified in the Senegalese Sole (*Solea senegalensis;* Mechaly *et al*., 2008).

In fish, GPR54 gene expression has been localized in GnRH neurons (Parhar *et al*., 2004). The expression pattern of GPR54 across puberty in tilapia (Parhar *et al*., 2004), grey mullet (Nocillado., 2007), cobia (Mohammed *et al*., 2007), fathead minnow (Filby *et al*., 2008), Nile tilapia (Martinez-Chavez *et al*., 2008) and zebrafish (Biran *et al*., 2008) suggests that GPR54 is also involved in the initiation of the onset of puberty in fish, as in mammals. However, studies concerning the GPR54 gene in commercially important fish, such as SBT and YTK are still lacking.

The present study aimed to isolate the cDNAs that encode the GPR54 in SBT and YTK and to develop and optimize a real-time quantitative RT-qPCR assay, which would be utilized to characterize the expression level of GPR54 during reproductive development. These tools are necessary as a first step towards understanding and

possibly manipulating the onset of puberty in SBT and YTK under commercial aquaculture conditions.

This thesis is submitted in the format of a manuscript and an appendix. The manuscript contains the main part of the work while the appendix provides additional details of the various sections that are beyond the scope of the manuscript and can contribute to the evaluation of the work.

2. Literature review

2.1. Puberty:

Puberty in fish is a complex process which is of interest both scientifically, as a comparative model to mammalian research, and commercially, due to its implication to broodstock management and harvest time. Puberty refers to the developmental period covering the transition from a sexually immature juvenile to a mature adult reproductive system (Schulz and Goos, 1999). In fish, the onset of puberty is characterized by the appearance, for the first time, of spermatocytes in the males and beginning of vitellogenesis in the females, while its end point is the first spermiation in males and first ovulation in females (reviewed by Dufour *et al*., 2000). In teleost fishes, an understanding of the regulation of puberty is important not only for basic science but also for aquaculture and fish farming (Okuzawa, 2002). The timing of the onset of puberty, which varies between species of fish, is directly linked to economic gain and reproductive management in the aquaculture industry.

2.2. Aquaculture of Southern Bluefin Tuna

The late onset of puberty impacts the Southern Bluefin Tuna (SBT) aquaculture in Australia. Southern Bluefin Tuna, *Thunnus maccoyii* is found throughout the southern hemisphere mainly in waters between 30° and 50° south latitude (Mori *et al*., 2001). It can grow up to 2.5m in length and weigh up to 400 kg and is one of the largest bony fish. SBT has very high nutritional and commercial value. It is rich in omega 3 and other beneficial fatty acids. Therefore, SBT is prized in Asian markets as one of the best fish to eat raw, such as sushi and sashimi, and it has become the most desirable food fish in the world. As a consequence, the demand for tuna in the world market is increasing consistently (Primary Industries and Resources SA). The high demand for tuna stimulated overfishing. One of the consequences of intensive tuna fishing is that parent stocks do not have a chance to rebuild. Therefore, there is a need to turn to aquaculture to secure the steady supply of tuna in the future. SBT usually takes 12 years to mature and at a body weight of over 150kg (Davis *et al*., 1998). This long generation time leads to high costs and high risks associated with maintaining the broodstock fish in captivity. In this context, the ability to advance the onset of puberty would improve the cost-efficiency of the fish farming operation (Okuzawa, 2002).

2.3. The impact of early onset of puberty in Yellowtail Kingfish aquaculture

The timing of the onset of puberty also has commercial implications in early maturing fish, such as the Yellowtail Kingfish (YTK), *Seriola lalandi*. YTK can grow up to 1.0 m in length and can weigh up to 10-15 kg (Primary Industries and Resources SA). It is found in tropical and temperate waters of the southern hemisphere. The nutritional value of YTK is very high so that the fish is well accepted in the various market segments and is popular with consumers. As a result, demand for YTK is steadily increasing (Primary Industries and Resources SA). In contrast to SBT, YTK undergoes relatively early puberty and can mature as early as 18 month of age, which is prior to the age when commercial size is achieved (Clean Seas Tuna, personal communication). During reproductive development, the fish exerts much of its energy towards gonadal development, leading to deterioration of flesh quality and retardation of growth. Hence, one consequence of the early onset of puberty is reduced market value. In this case, the ability to delay the onset of puberty would be beneficial.

As in other vertebrates, the mechanisms underlying puberty in fish are not fully understood (Okuzawa, 2002, Sisk and Foster, 2004; reviewed by Navarro *et al*., 2007).

2.4. Regulation of the onset of puberty

Until recently, it has been accepted that puberty is controlled by the hypothalamic-pituitary-gonad (HPG) axis (Fig. 2.1) (Weltzien *et al*., 2004). In the hypothalamus, gonadotropin-releasing hormone (GnRH) is synthesized by GnRH neurons. The secretion of GnRH stimulates the anterior pituitary to release follicle stimulating hormone (FSH) and luteinizing hormone (LH), which in turn activate the somatic cells in the gonads to induce the synthesis and release of sex steroids testosterone (T) and estradiol 17-β (E_2). T and E_2 in turn control the various stages of gametogenesis. In addition, T and E_2 exert both positive and negative feedback into the pituitary and the brain depending on maturational stage (Schulz and Goos., 1999).

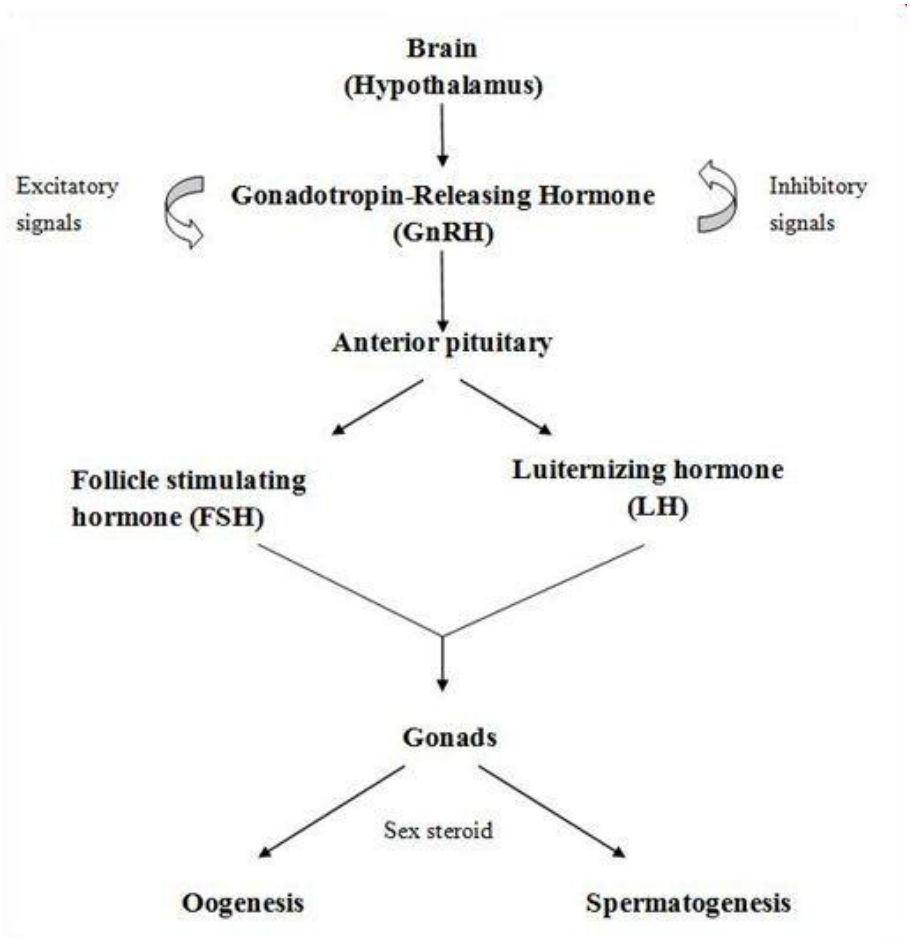


Figure 2.1 Hypothalamus-Pituitary-Gonad (HPG) axis in vertebrates

In vertebrates, The GnRH neurons are also controlled by a complex network of central regulators such as excitatory and inhibitory neuropeptides and neurotransmitters (Roa *et al*., 2006). Activation of GnRH neurons is the key event that initiates the onset of puberty (Matsui *et al*., 2004). In mammals, when approaching puberty, the GnRH neurons become less sensitive to the steroid-mediated inhibition or the ratio between central inhibitory and stimulatory inputs to the GnRH neuron changes (Navarro *et al*., 2007), resulting in a change of the GnRH secretion pattern that triggers an activation of the HPG-axis (Schulz and Goos, 1999). The sex steroids exert their function by binding to their specific receptors, such as androgen receptor (AR) for T and estrogen receptor (ER) for E_2. However, sex steroids do not appear to act directly onto the GnRH neurons (Herbison and Pape, 2001). It is widely believed that other steroid-responsive neurons in the forebrain mediate the predominant actions of estrogen and testosterone in the regulation of GnRH and gonadotropin secretion (Herbison, 1998), suggesting that other steroid sensitive neurons "upstream" of GnRH neurons receive and transmit steroid feedback signals to the HPG axis. Whereas in sexually immature teleosts, sex

steroids exert a positive feedback at all levels of the HPG axis (Huggard *et al*., 1996; Schulz and Goos, 1999), leading to the proposal that in fish one or more components of the HPG axis are not functional prior to puberty (Schulz and Goos 1999).

2.5. "Gatekeeper" of onset of puberty in mammalian vertebrates

A major breakthrough in the field of reproductive physiology occurred in 2003, when two clinical studies reported that loss of function mutations in the G-protein-coupled receptor gene (GPR54=KiSS1r), were associated with the absence of puberty and consequent infertility in humans (de Roux *et al*., 2003; Seminara *et al*., 2003). Patients with mutated GPR54 did not undergo puberty. The physiological consequences of the GPR54 mutations were the failure of the release of endogenous GnRH, effectively blocking the downstream cascade in the HPG axis. The condition in humans was replicated in GPR54 knockout mice, where a phenotype similar to that of humans with GPR54 mutations, i.e., mice not proceeding to puberty and failing to reproduce, was observed (Funes *et al*., 2003; Seminara *et al*., 2003).

On the other hand, gain of function mutations in GPR54 resulted in the opposite phenotype: precocious puberty in humans (Teles *et al*., 2008). Thus, GPR54 is now considered as the gatekeeper of the onset of puberty in mammalian vertebrates (Seminara *et al*., 2003).

GPR54 was initially cloned in rat in 1999 as an orphan receptor with a significant sequence similarity (>40%) with the transmembrane regions of galanin receptors (Lee *et al*., 1999). Subsequently, the human orthologue of GPR54 was cloned (Muir *et al*., 2001; Ohtaki *et al*., 2001). The natural ligands of GPR54 were found to be the kisspeptin peptides, which are encoded by the KiSS-1 gene. Kisspeptin is cleaved to shorter C-terminal peptides: kisspeptin-14, -13, -10 and -54, all of which activate GPR54, with kisspeptin-10 having maximal potency (Kotani *et al*., 2001; Muir *et al*., 2001; Ohtaki *et al*., 2001). Interestingly, the KiSS-1 gene was originally isolated as a tumor metastasis gene, and the peptide product kisspeptin has an ability to suppress metastasis of melanomas (Lee *et al*., 1996).

Upon ligand-receptor interaction, the major intracellular signaling systems recruited by GPR54 include activation of phospholipase C and PIP2 hydrolysis, followed by Ca^{2+}

mobilization and phosphorylation of ERK1/2 and p38 MAP kinases (Kotani *et al*., 2001).

2.6. Kisspeptin-GPR54 stimulate GnRH release, an essential event in initiating puberty

The role of the Kisspeptin-GPR54 system in stimulating the release of GnRH is supported by two major studies in mammals: the expression patterns of both kisspeptin and GPR54 and the ability of kisspeptin to stimulate GnRH release.

Gottsch *et al*. (2004) mapped the location of cells expressing KiSS-1 mRNA in mouse by *in situ* hybridization. They found that KiSS-1 mRNA is expressed in cells that reside in the anteroventral periventricular nucleus (AVPV), the periventricular nucleus (peN), the anterodorsal preoptic nucleus (ADP) and the Arcuate nucleus (Arc) in the forebrain (Gottsch *et al*., 2004; Smith *et al*. 2005a).

From its expression patterns, kisspeptin appears to act directly on GnRH neurons in stimulating the secretion of GnRH (Han *et al*., 2005). Areas where kisspeptin neurons reside such as the Arc and AVPV are known to send projections to the medial preoptic area, where there is an abundance of GnRH cell bodies (Canteras *et al*., 1994). Kisspeptin-containing fibers project to these same areas (Brailoiu *et al*., 2005), and kisspeptin fibers appear in close approximation to GnRH neurons (Kinoshita *et al*., 2005). If kisspeptin neurons interact directly with GnRH neurons, one would expect GnRH neurons to express GPR54, the kisspeptin receptor.

Expression studies using double-labeling *in situ* hybridization in rats have shown that the majority (>75%) of GnRH neurons co-express GPR54 mRNA (Irwig *et al*. 2004). It was also shown in mouse and sheep that more than 50% of GnRH neurons express GPR54 (Messager *et al*., 2005; Han *et al*., 2005).

Functional studies conducted in female rats have demonstrated that repeated administration of kisspeptin-10 to immature individuals induced precocious vaginal opening and early activation of the HPG axis (Navarro *et al*., 2004b). Similarly, administration of kisspeptin-10 resulted in release of gonadotropins in adult male mice (Gottsch *et al*., 2004), adult male rats (Irwig *et al*., 2004, Matsui., 2004, Navarro *et al*.,

2004a, Thompson *et al*., 2004), juvenile agonadal male monkeys (Shibata *et al*., 2005), sheep (Messager *et al*., 2005) and human males (Dhillo *et al*., 2005). Moreover, in both rodents and nonhuman primates, the effects of kisspeptin-10 on LH can be completely abrogated by GnRH antagonist, demonstrating that kisspeptin-10 is acting through GnRH neurons to stimulate LH release (Gottsch *et al*., 2004). These results indicate that kisspeptin activation, acting via the receptor, GPR54, is a fundamental driver of GnRH release from hypothalamic neurons, and suggest that the role of the kisspeptin-GPR54 system is in modulating the release of GnRH.

Matsui *et al*., (2004) demonstrated that peripheral administration of kisspeptin to prepubertal, 25-day-old female rats stimulated LH secretion and induced ovulation in the rat. If kisspeptin trigger puberty onset, one would expect to see an increase in KiSS-1 mRNA and/or GPR54 mRNA expression during this time. In both female and male rats, there was a marked increase in KiSS-1 and GPR54 mRNA levels coinciding with the onset of puberty (Navarro *et al*., 2004a). In addition, kisspeptin has also been shown to play a role in triggering the onset of puberty in the primate. First, central injection of kisspeptins stimulates LH in prepubertal, agonadal male monkeys, demonstrating that kisspeptin can override the central inhibition of GnRH secretion characteristic of the prepubertal primate (Shibata *et al*., 2005). Second, hypothalamic content of KiSS-1 mRNA increased across puberty in both the agonadal male and intact female monkeys, suggesting that increased production of kisspeptin could contribute to activating HPG axis at puberty (Shibata *et al*., 2005). Moreover, hypothalamic levels of GPR54 mRNA also increase as a function of pubertal maturation, but only in the intact female monkey (Shibata *et al*., 2005). This suggested that in mammals GnRH neurons become developmentally activated by kisspeptins over the course of puberty.

The activated GnRH neurons in the brain trigger the downstream hormonal cascade events in the HPG axis. Gonadal activity (including hormone production and gametogenesis) is regulated by the brain and pituitary, which are both targets for the feedback control of gonadotropin secretion by sex steroids (T and E_2). Since GnRH neurons do not express sex steroid receptors, the steroids act indirectly to exert their feedback control on GnRH neurons (Herbison, 1998). Smith *et al* (2005b) showed that cells expressing KiSS-1 mRNA also express sex steroid receptors. In male mice, more than 60% of KiSS-1 neurons in the Arc express AR and about 90% express the ERα. In

female mice, nearly all KiSS-1 neurons express ERα, and approximately 30% express ERβ (Smith *et al*., 2005a). Thus, KiSS-1 neurons are direct targets for the action of sex steroids in both the male and female mice. However, the regulation of KiSS-1 neurons by sex steroids is region specific. In the Arc, sex steroids inhibit the expression of KiSS-1, suggesting that these neurons serve as a conduit for the negative feedback regulation of gonadotropin secretion. In the AVPV, sex steroids induce the expression of KiSS-1, implicating a positive regulation of GnRH secretion (Smith *et al*., 2006). In the context of triggering the onset of puberty, in male mice, KiSS-1 mRNA expression, measured by *in situ* hybridization, is stable in the Arc across pubertal development but increases significantly in the AVPV, a region directly implicated in the activation of GnRH neurons (Herbison 1998). This suggests that GnRH neurons are less sensitive to the sex steroids inhibition during onset of puberty.

Both gene expression and functional studies have shown the co-localization of GPR54 with GnRH neurons. The proximity of KiSS-1 neurons with GnRH neurons; the stimulatory effect of kisspeptin directly at the GnRH neurons to regulate the timing of puberty and the link between peripheral sex steroids negative and positive feedback control of GnRH release, all support the functional link between the two. As a whole, kisspeptin and its receptor GPR54 are critically important to the release of GnRH, which triggers the activation of the HPG axis during the onset of puberty.

2.7. Kisspeptin-GPR54 system in fish

The kisspeptin-GPR54 system has also been identified in fish, yet ongoing research in this area in fish is still at the early stages (reviewed by Elizur, 2009). However, the kisspeptin system appears to be functionally conserved in non-mammalian vertebrates and supports the notion that it has a role in pubertal development and reproduction in piscine systems (reviewed by Elizur, 2009).

The GPR54 has been characterized in several fish including tilapia (*Oreochromis niloticus;* Parhar *et al.,* 2004*)*, grey mullet (*Mugil cephalus;* Nocillado *et al*., 2007), cobia (*Rachycentron canadum;* Mohamed *et al*., 2007), and fathead minnow (*Pimephales promelas;* Filby *et al*., 2008). There are two GPR54 genes that have been identified in zebrafish (*Danio rerio;* Biran *et al*., 2008; van Aerle *et al*., 2008) and goldfish (*Carassius auratus;* Li *et al*., 2009). In addition, there are two isoforms of

GPR54, resulting from a mechanism of alternative splicing, that have been identified in the Senegalese Sole (*Solea senegalensis;* Mechaly *et al*., 2008). The isoform arises from intron III retention, and contain a 27 amino acid insert in transmembrane region 4 with two stop codons, leading to a truncated protein.

Expression studies have shown the co-localization of GPR54 with neurons expressing GnRH1, GnRH2, and GnRH3 in tilapia and Atlantic croaker brains (Parhar, 2004; Khan *et al*., 2008). This resembles the co-localization of GPR54 with GnRH neurons in mammals, suggesting the kisspeptin system could be involved with the regulation of all three forms of GnRH (reviewed by Elizur, 2009). Expression of GPR54 in the brain also has been identified in fathead minnow (Filby *et al*., 2008), zebrafish (Biran *et al*., 2008), grey mullet (Nocillado *et al*., 2007), and cobia (Mohamed *et al*., 2007).

Amino acid sequence analysis have shown that there is over 50% sequence homology between the GPR54 from fish and mammals (Parhar *et al*., 2004*,* Nocillado *et al*., 2007, Mohamed *et al*., 2007). Within the fish species, there is more than 90% sequence homology in the GPR54 amino acid sequece (Parhar *et al*., 2004*,* Nocillado *et al*., 2007, Biran *et al*., 2008, Filby *et al*., 2008, and Mohamed *et al*., 2007). One of the two forms of GPR54 which were identified in zebrafish is more similar to other fish GPR54 sequences while the second one is more closely related to the mammalian GPR54 (Biran *et al*., 2008). The goldfish GPR54-1 is more close related to zebrafish GPR54-1 while the goldfish GPR54-2 is more related to second zebrafish GPR54 (Li *et al*., 2009). The high sequence conservation suggests functional similarity of GPR54 between mammals and fish.

Bioinformatics analysis of the genomic sequences of zebrafish, fugu, *Tetraodon*, sea lamprey, goldfish and medaka have led to the identification of KiSS sequences from these species (van Aerle *et al*., 2008; Biran *et al*., 2008; Kanda *et al*., 2008, Li *et al*., 2009). A novel finding in fish is the identification of another KiSS gene, KiSS 2, which has been identified so far in zebrafish, medaka and the European seabass (Kitahashi *et al*., 2008; Felip *et al*., 2008), as well as in goldfish (Li *et al*., 2009).

The spatial distribution of the KiSS1 gene has been determined in the brain of

zebrafish (van Aerle *et al*., 2008), goldfish (Li *et al*., 2009) and medaka (Kanda *et al*., 2008). In medaka, two populations of neurons expressing the KiSS1 gene were identified; both are located in the hypothalamic nuclei, at the nucleus posterioris periventricularis (NPPv) and the nucleus ventral tuberis (NVT). This resembles the situation in mammals, where a number of populations of cells expressing KiSS1 were identified, including in the Arc and AVPV (Smith *et al*., 2005a), suggesting functional similarity of kisspeptin between mammal and fish.

2.8. Kisspeptin-GPR54 and puberty in fish

The expression patterns of the GPR54 and KiSS1 genes across puberty suggest that the system participates in the initiation of the onset of puberty in fish. GPR54 expression levels were significantly higher in brains of cobia males at early puberty compared with immature fish (Mohamed *et al*., 2007). This suggests that, as in mammals, there is a link between gonadal development and GPR54 expression in fish. In addition, GPR54 expression in the brain of female grey mullet was the highest in the early stages of pubertal development, but dropped in the intermediate and advanced stages (Nocillado *et al*., 2007), indicating that in the mullet brain, GPR54 might be required for the onset of puberty.

GPR54 gene expression in the brain was also examined in the fathead minnow (Filby *et al*., 2008), Nile tilapia (Martinez-Chavez *et al*., 2008), and zebrafish (Biran *et al*., 2008). The expression pattern of GPR54 was similar in these species, one which peaked at the time of onset of ovarian development and declined with reproductive maturity (Filby *et al*., 2008, Martinez-Chavez *et al*., 2008, Biran *et al*., 2008). In male fathead minnow, GPR54 peaked at two different developmental time points: the first, at the time of appearance of type A spermatogonia and the second, at the time when a large increase in testis size and the number of type A spermatogonia was observed, followed by a decline by the final stages of spermatogenesis (Filby *et al*., 2008). In cobia, GPR54 expression was detected from 1-day post-hatching, peaking at day 26 post-hatching. Higher expression levels were also detected in males at the early stages of testis development (Mohamed *et al*., 2007). The GPR54 expression patterns from different fish species suggest the involvement of GPR54 in the regulation of the onset of puberty in fish, as well as additional functions during early development and in other physiological contexts (reviewed by Elizur, 2009).

Gene expression pattern of KiSS1 across puberty has been characterized in zebrafish (Biran *et al*., 2008). Low gene expression levels of KiSS1 were detected in the first weeks post-fertilization. In females, expression levels gradually increased, reaching a peak at 12 weeks post-fertilization, at which time well-developed ovaries with mature oocytes could be observed. In zebrafish males, expression of KiSS1 peaked at 6 weeks post-fertilization and this time coincided with the first stage of spermatogenesis. Thereafter KiSS1 expression levels decreased (Biran *et al*., 2008), suggesting a role for kisspeptin in the regulation of gonadal development.

Functional studies have shown that mammalian kisspeptin-10 injected to early to mid-pubertal fathead minnow, resulted in an increase of GPR54 and GnRH3 expression in the brain (Filby *et al*., 2008). In addition, the amidated form of huKISS10 activated both zebrafish receptor types to stimulate GnRH release. GPR54-1 transduces its activity via the protein kinase C (PKC) pathway, similar to its mammalian counterpart. GPR54-2 transduces via both PKC and PKA pathways (Biran *et al*., 2008). All these results together demonstrate the stimulatory effects of kisspeptin involved in the GPR54-GnRH signaling pathway in fish. Moreover, fish GnRH cells are also direct targets for kisspeptin and kisspeptin is a likely candidate to induce GnRH release at puberty via interactions with GPR54, which is similar to what occurs in mammals.

For the peripheral regulation of GnRH release in medaka, manipulation of sex steroids levels was achieved by ovariectomy and estrogen administration (Kanda *et al*., 2008). As observed in mammals (Smith *et al*., 2005a), the two neuronal populations expressing KiSS1 in medaka exhibited a differential response to changes in steroid level. The number of KiSS1 expressing neurons declined in the NVT in response to ovariectomy, and recovered again in response to estrogen administration, indicating KiSS1 neurons could be involved in the positive feedback of the BPG axis, similar to the mammalian AVPV KiSS1 expressing neurons.

As a whole, the expression profiles of GPR54 in a number of fish species followed closely that of the GnRH forms, consistent with reports in mammalian systems. The timing of GPR54 and KiSS1 expression is consistent with its proposed role in the onset of puberty and reproductive processes (reviewed by Elizur, 2009). Therefore, it can be

concluded that the kisspeptin system plays a critical role in triggering the onset of puberty in both mammals and fish.

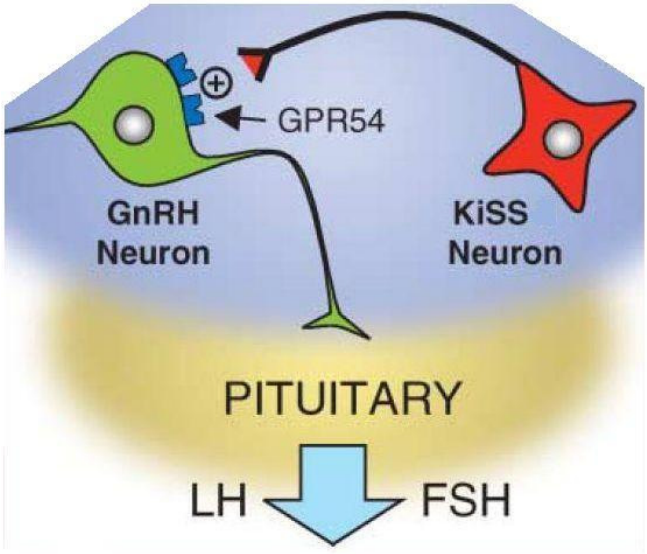


Figure 2.2. Proposed interactions between kisspeptin-secreting neurons and GnRH neurons. KiSS mRNA-expressing neurons make synaptic contact with GnRH neurons. Upon activation of the kisspeptin receptor GPR54, GnRH neurons are stimulated to release GnRH, which in turn stimulates the release of gonadotropins, LH and FSH from the pituitary. (Adapted from Smith *et al*., 2006)

2.9. Knowledge gaps

GPR54 has only been studied in limited number of fish models (Parhar *et al*., 2004, Nocillado *et al*., 2007, Mohamed *et al*., 2007, Filby *et al*., 2008, Biran *et al*., 2008; and van Aerle *et al*., 2008; Li *et al*., 2009, Kitahashi *et al*., 2008, Felip *et al*., 2008, and Mechaly *et al*., 2008; Biran *et al*., 2008, Li *et al*., 2009); and the functional significance of these findings remains to be determined, as well as the generality of multiple GPR54 receptors in other species, including fish and mammal, is yet to be investigated (reviewed by Elizur, 2009. In addition, the importance of the two isoforms of GPR54 involved in puberty regulation in Senegalese Sole (Mechaly *et al*., 2008) also remains to be determined, specifically, the species specific mechanisms involved in RNA splicing resulting in GPR54 isoforms need to be explored. At the gonad level, the role of GPR54 in regulating GnRH release remains unknown, as well as knowledge concerning interaction between brain and gonad in the KiSS system is still lacking. Furthermore, the functional role of the kisspeptin-GPR54 system in fish and its relevance to the induction of puberty onset, as well as the effectiveness of using kisspeptin to manipulate

timing of puberty in teleosts remains to be investigated. Although the kisspeptin-GPR54 system has potential for commercial application, there is very little information about the system in commercially important fish species.

2.10. Aims and objectives of the project

In light of the role of GPR54 as the gatekeeper of reproduction in mammals and its direct link to the control of puberty, the overall aim of the project is to isolate and characterize the GPR54 gene in SBT and YTK as one of the means towards understanding its role during reproductive development in these species.

The specific objectives of this honours project are:

(i) To clone the cDNAs that encode the GPR54 in both SBT and YTK,

(ii) To develop a real-time quantitative RT-qPCR assay for GPR54 that would be utilized to characterize the expression level of GPR54 in both brain and gonad of YTK and SBT. YTK will be used as an experimental model in light of the difficulties in sampling SBT.

2.11. Project significance

Fish are significant non-mammalian vertebrate models for the study of the kisspeptin-GPR54 system, particularly concerning the regulation of the onset of puberty (reviewed by Elizur 2009). The significance of fish models is demonstrated by the case of GnRH and its receptor, whose functional diversity in mammals was re-examined when multiple GnRH genes, with respective cognate receptors, were discovered in teleosts (Powell *et al*, 1994, Lethimonier *et al*., 2004). Therefore, comparative studies of the kisspeptin-GPR54 system in a diverse set of mammalian and non-mammalian species would not only define the phylogeny of this system, but also refine its functional characterization in terms of control of fertility and other biological actions (Roa *et al*., 2006).

The potential commercial applications of being able to manipulate the kisspeptin-GPR54 system in aquaculture are also significant. Understanding the functional and physiological roles of the system in commercially important fish, in this instance, YTK and SBT would contribute to facilitating strategies to manage the early pubertal development in YTK. On the other hand, knowledge gained from the project would also help to develop strategies to advance puberty in SBT, so that the time

required for holding the broodstock will be shortened; the handling stress will be decreased, and open a way for a sustainable production of SBT. As a consequence, the whole economic model associated with tuna farming could be changed and the industry would be able to achieve higher profits.

3. References:

(The format of references follows the specifications of the Journal Aquaculture)

Biran, J., Ben-Dor, S., Levavi-Sivan, B., 2008. Molecular identification and functional characterization of the kisspeptins/kisspeptins receptor system in lower vertebrates. Biology of Reproduction. 79, 776-786.

Brailoiu, G.C., Ohsawa, M., Yin, D., Yang, J., Chang, J.K., Brailoiu, E., Dun, N., 2005. KiSS-1 expression and metastin-like immunoreactivity in the rat brain. Journal of Comparative Neurology. 481**,** 314–329.

Canteras, N.S., Smerly, R.B., Swanson, L.W., 1994. Organization of projections from the ventromedial nucleus of the hypothalamus: a Phaseolus vulgaris-leucoagglutinin study in the rat. Journal of Comparative Neurology. 348**,** 41–79.

Davis, T., Farley, J., Bahar, S., 1998. Size partitioning by depth of southern blufin tuna on the spawning grounds. CCSBT Scientific Committee. Document 9807/5.

de Roux, N., Genin, E., Carel, J.C., Matsuda, F., Chaussain, J.L., Milgrom, E., 2003. Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derive peptide receptor GPR54. Proceedings of the National Academy of Sciences. USA. 100**,** 10972-10976.

Dhillo, W.S., Chaudhri, O.B., Patterson, M., Thompson, E.L., Murphy, K.G., Badman, M.K., McGowan, B.M., Amber, V., Patel, S., Ghatei, M.A., Bloom, S.R., 2005. Kisspeptin-54 stimulates the hypothalamicpituitary gonadal axis in human males. Journal of Clinical Endocrinology and Metabolism. 90**,** 6609–6615.

Dufour, S., Huang, Y.S., Rousseau, K., Sbaihi, M., Le Belle, N., Vidal, B., Marchelidon, J., Querat, B., Burzawa-Gerard, E., Chang, C.F., Schmitz, M., 2000. Puberty in teleosts: new insights into the role of peripheral signals in the stimulation of pituitary gonadotropins. Proc. The 6th International Symposium on the Reproductive Physiology of Fish. 455–461.

Elizur, A., 2009. The KiSS1/GPR54 system in fish. Peptides. 30(1), 164-170.

Felip, A., Zanuy, S., Pineda, R., Pinilla, L., Carrillo, M., Tena-Sempere, M., G'omez, A., 2008. Evidence for two distinct KiSS genes in non-placental vertebrates that encode kisspeptins with different gonadatropin-releasing activities in fish and mammals. Molecular and Cellular Endocrinology. DOI: 10.1016/j.mce.2008.11.017.

Filby, A.L., van Aerle, R., Duitman, J. W., Tyler, C.R., 2008. The kisspeptin/gonadotropin-releasing hormone pathway and molecular signaling of puberty in fish. Biology of Reproduction. 78, 278-289.

Funes, S., Hedrick, J. A., Vassileva, G., Markowitz, L., Abbondanzo, S., Golovko, A.

2003. The KiSS-1 receptor GPR54 is essential for the development of the murine reproductive system. Biochemical and Biophysical Research Communications. 312, 1357–63.

Gottsch, M.L., Cunningham, M.J., Smith, J.T., Popa, S.M., Acohido, B.V., Crowley, W.F., *et al*., 2004. A Role for Kisspeptins in the Regulation of Gonadotropin Secretion in the Mouse. Endocrinology. 145**,** 4073-4077.

Han, S.K., Gottsch, M.L., Lee, K.J., Popa, S.M., Smith, J.T., Jakawich, S.K., Clifton, D.K., Steiner, R.A., Herbison, A.E., 2005. Activation of gonadotropin-releasing hormone neurons by kisspeptin as a neuroendocrine switch for the onset of puberty. Journal of Neuroscience. 25**,** 11349–11356.

Herbison, A. E., 1998. Multimodal influence of estrogen upon gonadotropin-releasing hormone neurons. Endocrine Reviews. 19**,** 302–330.

Herbison, A.E., Pape, J. R., 2001. New evidence for estrogen receptors in gonadotropin-releasing hormone neurons. Frontiers in Neuroendocrinology. 22**,** 292–308.

Huggard, D., Khakoo, Z., Kassam, G., Mahmoud, S.S., Habibi, H.R., 1996. Effect of testosterone on maturational gonadotropin subunit messenger ribonucleic acid levels in the goldfish pituitary. Biology of Reproduction. 54, 1184-1191.

Irwig, M.S., Fraley, G.S., Smith, J.T., Acohido, B.V., Popa, S.M,. Cunningham, M.J., Gottsch, M.L., Clifton, D.K., Steiner, R.A., 2004. Kisspeptin activation of gonadotropin releasing hormone neurons and regulation of KiSS-1 mRNA in the male rat. Neuroendocrinology. 80**,** 264–272.

Kanda, S., Akazome, Y., Matsunaga, T., Yamamoto, N., Yamada, S., Tsukamura, H., *et al*., 2008. Identification of KiSS-1 Product Kisspeptin and Steroid-Sensitive Sexually Dimorphic Kisspeptin Neurons in Medaka (Oryzias latipes). Endocrinology. 149**,** 2467-2476.

Khan, I.A., Mohamed, S.J., Kline, R.J., Galima, M.M., 2008. Co-localization of GPR54 with three GnRHs and quantification of GPR54 protein in Atlantic croaker brain. Cybium. 32(3), 27-29.

Kinoshita, M., Tsukamura, H., Adachi, S., Matsui, H., Uenoyama, Y., Iwata, K., Yamada, S., Inoue, K., Ohtaki, T., Matsumoto, H., Maeda, K., 2005. Involvement ofcentral metastin in the regulation of preovulatory luteinizing hormone surge and estrous cyclicity in female rats. Endocrinology.146,4431-4436

Kitahashi, T., Ogawa, S., Pahar, I.S., 2008. Cloning and expression of kiss2 in the zebrafish and medaka. Endocrinology. DOI: 10.1210/en.2008-0940.

Kotani M, Detheux M, Vandenbogaerde A, Communi D, Vanderwinden JM, Le Poul E, Brezillon S, Tyldesley R, Suarez-Huerta N, Vandeput F, Blanpain C,

Schiffmann SN, Vassart G, Parmentier M 2001 The metastasis suppressor gene KiSS-1 encodes kisspeptins, the natural ligands of the orphan G proteincoupled receptor GPR54. Journal of Biological Chemistry. 276:34631-6

Lee, J.H., Miele, M.E., Hick, D.J., Philips, K.K., Trent, J.M., Weissman, B.E., Welch, D.R., 1996. KiSS-1, a novel human malignant melanoma metastasis-suppressor gene. Journal of the National Cancer Insitute. 88, 1731-1737.

Lee, D.K., Nguyen, T., O'Neill, G.P., Chang, R., Liu, Y., Howard, A.D., Coulombe, N., Tan, C.P., Tang-Nguyen, A.T., George, S.R., O'Dowd, B.F., 1999. Discovery of a receptor related to the galanin receptors. FEBS Letters. 446**,** 103–107.

Lethimonier, C., *et al*., 2004. Evolutionary aspects of GnRHs, GnRH neuronal systems and GnRH receptors in teleost fish. General and Comparative Endocrinology. 135**,** 1–16.

Li, S., Zhang, Y., Liu, Y., Huang, X., Huang, W., Lu, D., Zhu, P., Shi, Y., Cheng C.H.K., Liu, X., Lin, H., 2009. Structural and functional multiplicity of the kisspeptin/GPR54 system in goldfish (*Carassius auratus*). Endocrinology. DOI: 10.1677.

Matsui, H., Takatsu, Y., Kumano, S., Matsumoto, H., Ohtaki, T., 2004 Peripheral administration of metastin induces marked gonadotropin release and ovulation inthe rat. Biochemical and Biophysical Research Communications. 320, 383-388.

Martinez-Chavez, C.C., Minghetti, M., Migaud, H., 2008. GPR54 and rGnRH I gene expression during the onset of puberty in Nile tilapia. General and Comparative Endocrinology. 156**,** 224–233.

Mechaly, A.S., Vinas, J., Piferrer, F., 2008. Identification of two isoforms of the kisspeptin-1 receptor (kiss1r) generated by alternative splicing in a modern teleost, the Senegalese sole (*Solea senegalensis*). Biology of Reproduction. DOI: 10.1095/bioreprod.108.072173.

Messager, S., Chatzidaki, E.E., Ma, D., Hendrick, A.G., Zahn, D., Dixon, J., *et al*., 2005. Kisspeptin directly stimulates gonadotropin-releasing hormone release via G protein-coupled receptor 54. Proceedings of the National Academy of Sciences. USA. 102**,** 1761-1766.

Mohamed, J.S., Benninghoff, A.D., Holt, G.J., Khan, I.A., 2007. Developmental expression of the G protein-coupled receptor 54 and three GnRH mRNAs in the teleost fish cobia. Journal of Molecular Endocrinology. 38, 235–244.

Mori, M., Katsukawa, T., Matsuda, H., 2001. Recovery plan for an exploited species, southern bluefin tuna. Population Ecology. 43, 125-132.

Muir, A.I., Chamberlain, L., Elshourbagy, N.A., Michalovich, D., Moore, D.J., Calamari, A., Szekere, P.J., Sarau, H.M., Chambers, J.K., Murdock, P., *et al*.,

2001. AXOR12, a novel human G protein-coupled receptor, activated by the peptide KiSS-1. Journal of Biological Chemistry. 276**,** 28969–28975.

Navarro, V.M., Castellano, J.M., Fernandez-Fernandez, R., Barreiro, M.L., Roa, J., Sanchez-Criado, J.E., Aguilar, E., Dieguez, C., Pinilla, L., Tena-Sempere, M., 2004a. Developmental and hormonally regulated messenger ribonucleic acid expression of KiSS-1 and its putative receptor, GPR54, in rat hypothalamus and potent luteinizing hormone-releasing activity of KiSS-1 peptide. Endocrinology 145, 4565–4574.

Navarro, V. M., Fernandez-Fernandez, R., Castellano, J. M., Roa, J., Mayen, A., Barreiro, M. L., Gaytan, F., Aguilar, E., Pinilla, L., Dieguez, C., 2004b. Advanced vaginal opening and precocious activation of the reproductive axis by KiSS-1 peptide, the endogenous ligand of GPR54. Journal of Physiology 561,379–386.

Navarro, V.M., Castellano, J.M., Garcia-Galiano, D., Tena-Sempere, M., 2007. Neuroendocrine factors in the initiation of puverty: The emergent role of kisseptin. Reviews in Endocrine and Metabolic Disorders. 8, 11-20.

Nocillado, J.N., Levavi-Sivan, B., Carrick, F., Elizur, A., 2007.Temporal expression of G-protein-coupled receptor 54 (GPR54), gonadotropin-releasing hormones (GnRH), and dopamine receptor D2 (drd2) in pubertal female grey mullet, Mugil cephalus. General and Comparative Endocrinology. 150, 278–287.

Ohtaki, T., Shintani, Y., Honda, S., Matsumoto, H., Hori, A., Kanehashi, K., Terao, Y., Kumano, S., Takatsu, Y., Masuda, Y., *et al*., 2001. Metastasis suppressor gene KiSS-1 encodes peptide ligand of a G protein-coupled receptor. Nature. 411**,** 613–617.

Okuzawa K. 2002. Puberty in teleosts. Fish Physiology and Biochemistry 26: 31-41.

Parhar, I.S., Ogawa, S., Sakuma, Y., 2004. Laser-captured single digoxigenin-labeled neurons of gonadotropin-releasing hormone types reveal a novel G protein-coupled receptor (Gpr54) during maturation in cichlid fish. Endocrinology 145, 3613–3618.

Plant, T.M., Ramaswamy, S., Dipietro, M.J., 2006. Repetitive activation of hypothalamic G protein-coupled receptor 54 with intravenous pulses of kisspeptin in the juvenile monkey (Macaca mulatta) elicits a sustained train of gonadotropin-releasing hormone discharges. Endocrinology. 147, 1007-1013.

Powell, J.F.F., Zohar, Y., Elizur, A., Park, M., Fisher, W.H., Craig, A.G., River, J.E., Lovejoy, D.A., Sherwood, N.M., 1994. Three forms of gonadotropin-releasing hormone characterized from brains of one species. Proceeding of the National Academy of Science. USA. 91, 12081-12085.

Primary Industries and Resources SA. Yellowtail kingfish and southern bluefin tuna aquaculture in SA. Fact Sheet. FS No: 26/03. www.pir.sa.gov.au/factseets.

Roa, J., Vigo, E., Castellano, J.M., Navarro, V.M., Fernandez-Fernandez, R., Casanueva, F.F., Dieguez, C., Aguilar, E., Pinilla, L., Tena-Sempere, M., 2006. Hypothalamic expression of KiSS-1 system and gonadotropin-releasing effects of kisspeptin in different reproductive states of the female rat. Endocrinology 147, 2864–2878.

Roa, J., Aguilar, E., Dieguez, C., Pinilla, L. Tena-Sempere, M., 2008. New frontiers in kisspeptin/GPR54 physiology as fundamental gatekeepers of reproductive function. Frontiers in Neuroendocrinology. 29, 48-69.

Schulz, R., Goos, H.J.Th., 1999. Puberty in male fish: concepts and recent developments with special reference to the African catfish (*Clarias gariepinus*). Aquaculture. 177, 5-12.

Seminara, S.B., Messager, S., Chatzidaki, E.E., Thresher, R.R., Acierno, J.S., Shagoury, J.K., Bo-Abbas, Y., Kuohung, W., Schwinof, K.M., Hendrick, A.G., Zahn, D., Dixon, J., Kaiser, U.B., Slaugenhaupt, S.A., Gusella, J.F., O'Rahilly, S., Carlton, M.B., Crowley, W.F., Jr., Aparicio, S.A., Colledge, W.H., 2003. The GPR54 gene as a regulator of puberty. New England Journal of Medicine. 349,1614-1627.

Shibata, M., Gibbs, R.B., Shahab, M., Plant, T.M., 2005. GnRH neurons in peripubertal male rhesus monkey express GPR54: Implication for the control of primate puberty. USA Endocrine Society Meeting. Abstract P1-98.

Sisk, C.L., Foster, D.L., 2004. The neural basis of puberty and adolescence. Nature Neuroscience. 7, 1040-1047.

Smith, J.T., Dungan, H.M., Stoll, E.A., Gottsch, M.L., Braun, R.E., Eacker, S.M., Clifton, D.K., Steiner, R.A., 2005a. Differential regulation of KiSS-1 mRNA expression by sex steroids in the brain of the male mouse. Endocrinology 146, 2976–2984

Smith, J.T., Cunningham, M.J., Rissman, E.F., Clifton, D.K., Steiner, R.A., 2005b. Regulation of Kiss1 gene expression in the brain of the female mouse. Endocrinology 146, 3686–3692.

Smith, J.T., Clifton, D.K., Steiner, R.A., 2006. Regulation of the neuroendocrine reproductive axis by kisspeptin-GPR54 signaling. Reproduction 131, 623–630.

Teles, M.G., Bianco, S.D.C., Brito, N.V., Trarbach, E.B., Kuohung, W., Xu, S., Seminara, S.B., Mendonca, B.B., Kaiser, U.B., Latronico, A.C., 2008. A GPR54-activating mutation in a patient with central precocious puberty. New England Journal of Medicine. 358, 709-715.

Thompson, E.L., Patterson, M., Murphy, K.G., Smith, K.L., Dhillo, W.S., Todd, J.F., Ghatei, M.A., Bloom, S.R., 2004. Central and peripheral administration of kisspeptin-10 stimulates the hypothalamic-pituitary-gonadal axis. Journal of Neuroendocrinology. 16,850-858.

van Aerle, R., Kille, P., Lange, A., Tyler, C.R., 2008. Evidence for the existence of a functional Kiss1/Kiss1 receptor pathway in fish. Peptides 29, 57–64.

Weltzien, F. A., Andersson, E., Andersen, O., Shalchian-Tabrizi, K., Norberg, B., 2004. The brain–pituitary–gonad axis in male teleosts, with special emphasis on XatWsh (*Pleuronectiformes*). Comparative Biochemistry and Physiology A. 137, 447–477.

4.Manuscript:

Title of paper:

Isolation of the G protein-coupled receptor 54 (GPR54) cDNA from Yellowtail kingfish, *Seriola lalandi* and Southern Bluefin tuna, *Thunnus maccoyii*, and development of a quantitative real-time RT-qPCR assay.

Intended journal:
Aquaculture

Authors:

Y. Y. Lee,[1] J. Nocillado,[2] W. Knibb,[3] A. Elizur[4]

Abstract:

The cDNA encoding for G-protein coupled receptor 54 (GPR54) was cloned from the brains of SBT and YTK. The SBT GPR54 has an open reading frame of 1134bp encoding a predicted 378 amino acid peptide, containing seven putative transmembrane domains, a 138 bp 5'UTR and 238 3'UTR. The partial YTK GPR54 cDNA contains 729 bp nucleotide sequence encoding 243 amino acid residues. A RT-qPCR assay was developed for both SBT and YTK GPR54, as confirmed by primer specificity and high R^2 values. The reference gene used, Hprt1, displayed consistent crossing point (Cq) values across most of the different sampling points, however some interactions between the reference gene expression and developmental stage were observed. GPR54 expression levels were determined for two cohorts, an immature group and a pubertal group, at two sampling points. RT-qPCR results showed that YTK brain GPR54 expression levels did not show sexual dimorphism and were significantly higher in the pubertal fish at a point past their peak spawning time compared with expression in the immature fish group. The YTK gonad GPR54 expression level was significantly lower in males from the pubertal fish group during peak spawning time. In SBT, GPR54 expression profile in the brain and the gonad did not vary between immature and reproductively advanced fish. Analysis was carried out using both relative gene expression data and raw Cq. Alternative reference genes would need to be examined for the assay to be reliable across tissues and developmental time points.

---

[1] Ms Ying Ying Lee carried out all the reported experimental works described, and wrote the manuscript.
[2] Dr. Nocillado contributed to the original design of the project and has offered continuous supervision.
[3] Associate Professor Knibb carried out all the statistical analysis associated with this work in conjunction with Ms Lee.
[4] Professor Elizur designed the project and supervised Ms Lee.

Keywords: GPR54; RT-qPCR; Southern Bluefin tuna; Yellowtail Kingfish

1. Introduction

Puberty refers to the developmental period covering the transition from an immature juvenile to a mature adult reproductive system (Schulz and Goos, 1999). In fish, the onset of puberty is characterized by the appearance, for the first time, of spermatocytes in the males and beginning of vitellogenesis in the females, while its endpoint is the first spermiation in males and first ovulation in females (reviewed by Dufour *et al*., 1999).

In teleost fishes, an understanding of the regulation of puberty is important for aquaculture and fish farming (Okuzawa, 2002). The timing of the onset of puberty, which varies between species of fish, is directly linked to economic gain and reproductive management in the aquaculture industry. For instance, the late onset of maturity of the Southern Bluefin Tuna (SBT), *Thunnus maccoyii*, which takes 12 years to mature and at a body weight over 150 kg (Davis *et al*., 1998), is a major bottleneck in its aquaculture. This long generation time results in high cost and high risk associated with maintaining the broodstock in captivity. In this context, the ability to advance the onset of puberty would improve the cost-efficiency of the fish farming operation (Okuzawa, 2002). In contrast, the yellowtail kingfish (YTK), *Seriola lalandi,* undergoes relatively early puberty and can mature as early as 18 months, which is prior to commercial harvest (Clean Seas Tuna, personal communication). During reproductive development, the fish exerts much of its energy towards gonadal development, leading to deterioration of flesh quality and retardation of growth. Hence, one consequence of the early onset of puberty is reduced market value. In this case, the ability to delay the onset of puberty would be beneficial.

As in other vertebrates, the mechanisms underlying the puberty in fish are not fully understood (Okuzawa, 2002; Sisk and Foster, 2004; reviewed by Navarro *et al*., 2007). Until recently, it has been accepted that puberty is controlled by the hypothalamic-pituitary-gonad (HPG) axis (Weltzien *et al*., 2004). Specific neurons from the hypothalamus synthesize GnRH, which when secreted, stimulates the anterior pituitary to release follicle stimulating hormone (FSH) and luteinizing hormone (LH). FSH and LH in turn activate the somatic cells in the gonads to induce the synthesis and

release of sex steroids, which control the various stages of gametogenesis.

The discovery that loss of function mutations in G-protein-coupled receptor 54 gene (GPR54=KiSS1r) resulted in the absence of puberty in humans (de Roux *et al*., 2003; Seminara *et al*., 2003) and in other mammalian models (Funes *et al*., 2003; Seminara *et al*., 2003) has added an entirely novel perspective regarding the regulation of puberty in vertebrates. GPR54 is now proposed to be the gatekeeper of puberty (Seminara *et al*., 2003). Gain of function mutations in GPR54 that resulted in precocious puberty in humans further added evidence regarding the critical role of GPR54 (Teles *et al*., 2008). Evidence has now accumulated showing that in mammals, the pathway in which GPR54 modulates the onset of puberty is through the stimulation of GnRH release from hypothalamic GnRH neurons (Messager *et al*., 2005; Plant *et al*., 2006; reviewed by Roa *et al*., 2008).

In contrast to the mammalian systems, research on the GPR54 system in fish is still at its infant stage (reviewed by Elizur 2009). The GPR54 gene and/or transcript has been characterized in several fish species including tilapia (*Oreochromis niloticus;* Parhar *et al.,* 2004), grey mullet (*Mugil cephalus;* Nocillado *et al*., 2007), cobia (*Rachycentron canadum;* Mohamed *et al*., 2007), and fathead minnow (*Pimephales promelas;* Filby *et al*., 2008). Two GPR54 genes have been identified in zebrafish (*Danio rerio;* Biran *et al*., 2008) and goldfish (*Carassius auratus;* Li *et al*., 2009). In addition, two isoforms of GPR54 that have been identified in the Senegalese Sole (*Solea senegalensis;* Mechaly *et al*., 2008).

In fish, GPR54 gene expression has been localized in GnRH neurons (Parhar *et al*., 2004). The expression pattern of GPR54 across puberty in tilapia (Parhar *et al*., 2004), grey mullet (Nocillado., 2007), cobia (Mohammed *et al*., 2007), fathead minnow (Filby *et al*., 2008), Nile tilapia (Martinez-Chavez *et al*., 2008) and zebrafish (Biran *et al*., 2008) suggests that GPR54 is also involved in the initiation of the onset of puberty in fish, as in mammals. However, studies concerning the GPR54 gene in commercially important fish, such as SBT and YTK are still lacking.

The present study aimed to isolate the cDNAs that encode the GPR54 in SBT and YTK and to develop and optimize a real-time quantitative RT-qPCR assay, which would be utilized to characterize the expression level of GPR54 during reproductive

development. These tools are necessary as a first step towards understanding and possibly manipulating the onset of puberty in SBT and YTK under commercial aquaculture conditions.

2. Materials and methods

2.1. Tissue sources

Brain and gonad tissues were collected from two cohorts of YTK (six males and six females per cohort/month) in December 2008 (Sampling 1) and in January 2009 (Sampling 2). Cohort1 were 24 months old and sexually mature, or pubertal. Cohort2 were 10 months old, and sexually immature. They will be referred to as the pubertal or immature group throughout the manuscript. Fish were obtained from CleanSeas Tuna (CST) commercial YTK cage farm at Arno Bay, South Australia, following the animal ethics guidelines approved by the Department of Primary Industries and Fisheries, Queensland (approval number AN/A/08/43). The dissected tissues were immediately stored in RNA*Later* (Ambion, Austin, TX) for 24h at 4°C and subsequently at -80°C until used. SBT samples were obtained from CleanSeas Tuna (CST) farms at Arno Bay or Port Lincoln, South Australia. SBT brain and gonad tissues were collected from broodstock mortalities during 2007 and 2008. In addition, SBT gonad tissues were also collected from production fish during February 2009. The dissected tissues were immediately stored in RNA*Later* (Ambion, Austin, TX) and subsequently at -80°C until used.

2.2. Gonadal histology

YTK and SBT gonadal development was determined by histological analysis. Gonadal tissue samples were fixed in 10% buffered formalin solution, dehydrated in ethanol series, and embedded in paraffin wax. Sections (6μm) were stained with hematoxylin and eosin, and analysed under light microscopy at 100-400X magnification. Histological sections for YTK were prepared by the Histotechnology Unit, UQ-Queensland Institute of Medical Research and for SBT by the Flinders University Histology Unit.

2.3. RNA extraction

Total RNA was extracted from SBT and YTK brain with TriZol Reagent (Invitrogen, Carlsbad, CA). Whole brain was homogenized in proportional amount of TriZol Reagent, i.e., 100mg of tissue in 1ml of TriZol reagent. Aliquots (1ml) of the homogenized tissue stock were stored at -80°C. Total RNA was extracted from a 1ml aliquot. The RNA was treated with DNAse (Promega) according to the manufacturer's instructions. The concentration of the RNA was determined using a spectrophotometer (Genequant ProTM, Biochrom, Cambridge, UK). The integrity of the RNA extracts was assessed by visualizing on denaturing formaldehyde gel. RNA was stored in -80°C until use.

Total RNA were extracted from gonadal tissue samples (15-20mg) in accordance with the Illustra RNA spin Mini-prep (GE healthcare, Catalogue # 25-0500-71, UK) total RNA extraction kit protocol.

2.4. Amplification of GPR54 cDNA

2.4.1. Degenerate primer design

Degenerate PCR primers were designed from conserved regions of GPR54 sequences available from five fish species: cobia (*Rachycentron canadum* DQ790001), grey mullet (*Mugil cephalus* DQ683737), tilapia (*Oreochromis niloticus* AB162143*)*, zebrafish (*Danio rerio* EU047917) and fathead minnow (*Pimephales promelas*). The GPR54 cDNA sequences for these species were aligned using Clustal W2 (http://www.ebi.ac.uk/) and the designed primers (F6-F8, R5-R7) are presented in Table 1.

2.4.2. First-strand cDNA synthesis, PCR amplification and cloning of partial GPR54 cDNA sequence

First-strand cDNA was synthesized from 1µg RNA template using QuantiTect® Reverse Transcription Kit (Qiagen, Catalogue # 205311, Victoria, Australia). The kit also includes a step for the removal of genomic DNA.

Polymerase Chain Reaction (PCR) reaction using the first-strand cDNA as a template was performed by using different combinations of the GPR54 degenerate primers (Table 1). The 25µl PCR amplification mix for a single reaction tube contained 2.5 µl 10x PCR buffer, 2µl $MgCl_2$ (25mM), 0.5µl dNTPs (10mM), 0.5µl forward primer (10mM), 0.5µl reverse primer (10mM), 0.1µl Taq DNA polymerase (Fisher Biotec, Australia) and 1µl of cDNA template. A negative control was included in all PCR experiments, which is a standard reaction mix with the template substituted for nuclease-free water. The cycling parameters were as follows: (1) an initial denaturation at 94°C for 2 minutes; followed by (2) 35 cycles of denaturation at 94°C for 30 seconds, (3) annealing at 48°C for 30 seconds, and extension at 72°C for 1 minute; and final extension at 72°C for 10 minutes. PCR products were then visualized on a 2% agarose gel. Products of the expected sizes were purified with QIAquick PCR purification columns (Qiagen, Victoria, Australia). The purified PCR product was ligated into pGEM®-T easy vector (Promega, New South Wales, Australia). An aliquot of the ligation reaction was used to transform JM109 competent cells (Promega, New South Wales, Australia). Positive transformants were identified by PCR amplification using gene-specific primer pairs (Table 1) and were sent for sequencing. Sequencing was done by the Australian Genome Research Facility (AGRF, Brisbane, QLD, Australia). Sequences were analysed using Basic Local Alignment Tool (BLAST) searches (http://ncbi.nml.nih.gov/BLAST/) and Sequencher software (Version 4.8; Gene Codes Corporation, Ann Arbour, ME, USA). The deduced amino acids sequences were aligned with other sequences using Clustal W2 (http://www.ebi.ac.uk/Tools/clustalw2/).

2.4.3. Isolation of full length GPR54 cDNA sequence using RACE (Rapid Amplification of cDNA Ends) PCR

To facilitate the PCR amplification of the full SBT and YTK cDNA sequences, gene-specific primers were designed from the partial cDNA sequence obtained for SBT and YTK using Primer3 software (http://wi.mit.edu). PCR reactions utilizing 5' and 3' NUP primer with the corresponding gene-specific primers were carried out (Table 1). Products obtained from the 5' and 3' RACE-PCRs were purified, sub-cloned and sequenced following the protocol described above.

2.5. Development of a Real-time Quantitative RT-PCR (RT-qPCR) for SBT and YTK GPR54

Development of the RT-qPCR assay followed the MIQE guidelines (Bustin *et al*., 2009). Quantification of relative expression levels was determined by an efficiency-corrected relative expression method that takes into account variations in reaction efficiencies of both target and reference genes (Pfaffl, 2001). First-strand cDNA was synthesized from 1µg total RNA using QuantiTect® Reverse Transcription Kit (Qiagen, Catalogue # 205311, Victoria, Australia). The cDNA synthesized from total brain and gonad RNA from broodstock SBT and YTK were used as calibrator to adjust for assay variation.

Normalisation of the data with a single internal reference gene, Hypoxanthine phosphoribosyltransferase 1 (Hprt1) was designed to facilitate the comparison of expression levels of the target gene within tissues at two different sampling points from two different fish cohorts. The gene-specific primers used for the amplification of target and reference genes are shown in Table 2. Primers were designed (using Primer 3 software http://wi.mit.edu) to span predicted intron/exon junctions and with yield expected amplicon size of 50-200 bp. Standard PCR amplifications using the designed RT-qPCR primers were conducted in order to confirm the absence of contaminating genomic DNA.

RT-qPCR amplifications were conducted on RotorGene 6000 (Corbett Research, Sydney, Australia) using SYBR green I as fluorescent label. Assays were optimized by adjusting either the final concentration of $MgCl_2$ or primers in the reaction, as well as the annealing temperature. Validation assays for target gene, GPR54 and reference gene, Hprt1 were conducted from each tissue using serially diluted cDNA as template. Reaction efficiencies were automatically calculated by RotoGene's software (version 6-0-22) based on the second derivative maximum ($E = 10^{-(1/\text{slope})}$). Threshold is determined automatically by the detection system of the RotorGene 6000 (Corbett Research, Sydney, Australia) machine, based on a point where the background noise is excluded and the reaction has an exponential increase phase. Triplicate reactions were performed for the cDNA template and their corresponding negative controls, both for target and reference genes, from the twelve fish (six males and six females) collected per sampling point per cohort.

The 10µl reaction volume consisted of 5µl of 2X Platinum SYBR Green qPCR SuperMix UDG (Invitrogen), 200nM of the forward and reverse primers and 3µl diluted

cDNA template and nuclease free water in one 0.1ml clear strip tube (Qiagen, Victoria, Australia). Cycling parameters were as follows: (1) an initial hold at 50°C for 2 minutes, (2) a second hold at 94°C for 2 minutes, (3) 40 cycles of 94°C for 25 seconds, (3) 60°C for 15 seconds, and 72°C for 25 seconds. Melt curve analysis was conducted from 72°C to 99°C and was held for 5 seconds at every increment of 1°C. Products were further visualized on ethidium bromide-stained agarose gel and their identities were confirmed by sequencing. A negative control with sterile water as template was included in order to check for reagent contamination.

2.6. Statistical analysis

All statistical analyses were performed using the SPSS v13 Software (SPSS, Chicago, IL, USA). Testing for normality was performed using the Kolmogorov-Smirnov test. Kurskal-Wallis, K-independent non-parametric test was used in the analysis of GPR54 relative expression levels, GPR54 Cq, Hprt1 Cq, body weight and gonadosomatic index (GSI). Data are shown as means ± standard error of mean (SEM) and mean with their standard deviation (SD). Differences were accepted as significant at $P<0.05$.

3. Results

3.1. Isolation of GPR54 cDNA

The nucleotide sequence of the isolated SBT GPR54 cDNA included an open reading frame of 1134 bp encoding a predicted 378 amino acid peptide, a 138 bp 5'UTR and 238 bp 3'UTR (Bankit 1218262). The partially isolated YTK cDNA contains 729 bp nucleotide sequence encoding 243 amino acid residues (Bankit 1218311). The SBT GPR54 shared more than 90% homology to YTK, 79-89% homology to zebrafish GPR54-1 and mullet GPR54, 37.35% homology to zebrafish GPR54-2, 76% homology to bullfrog GPR54 and 70-71% homology to its rat and human homologues. Phylogram analysis revealed that SBT and YTK GPR54 genes are more closely related than GPR54 gene from other species (Fig. 3.1).

Analysis of the predicted amino acid sequences of SBT GPR54 revealed seven putative transmembrane (TM) domains. TM1-7 showed 82-100% identity to other fish species. Comparisons with the TM domain of amphibian, GPR54 revealed 71-93% identity in TM1-7. SBT GPR54 TM1-7 shared only 43-86% homology with mammalian

GPR54.

3.2. Average body weight and gonadosomatic index of sampled fish

The average body weight, gonad weight and average GSI of the two cohorts of YTK sampled in the present study are shown in Table 3, Table 4 and Table 5, respectively. The average body weight of the pubertal fish group was significantly higher ($P<0.05$) than that the immature fish group. Within each fish group, the mean body weights of male and female fish did not have significant difference ($P>0.05$) between the two sampling points (Table 3). The GSI in the immature group could not be determined in sampling 1 due to the small size of the gonad. In sampling 2, GSI were determined to be 0.04 for the males and 0.14±0.02 for the females (Table 5). Based on GSI, the gonad from the pubertal fish group was much more developed than that of the immature group. Within the pubertal fish group, the female gonad from sampling 1 was slightly more developed ($P>0.05$) than gonads from sampling 2. Male GSI from sampling 1 was significantly higher ($P<0.05$) than that from sampling 2 (Table 5).

The average body weight and GSI of three SBT broodstock fish are shown in Table 6 and 7 respectively. The body weights of the three broodstock fish ranged from 126.9 to 128.0 Kg. The GSI were 0.64 and 0.11 for fish 2 and 3, respectively.

3.3. Histological analysis of the gonads of two cohorts of YTK

Gonadal histology of the immature YTK fish group revealed that both males and females were reproductively undeveloped, i.e immature. The female gonads had oocyes at chromatin nucleolar and perinucleolar stages (Fig. 3.2A and 3.2B). The male gonads showed spermatogonia, spermatocytes and spermatids (Fig. 3.3A and 3.3B). In contrast, fish in the pubertal group had developing gonads. Male and female fish from sampling 1 were more developed than those from sampling 2. Histological analysis showed that female gonads from sampling 1 contained developing oocytes, with few oocytes at the vitellogenic stage (Fig. 3.2C). Female gonads from sampling 2 had gonads at mature resting stage, with cortical alveoli oocytes being predominant (Fig. 3.2D). For the male fish at sampling 1, gonads predominantly contained spermatozoa (Fig. 3.3C). In sampling 2, the male fish gonad predominantly contained spermatids (Fig. 3.3).

Gonadal histology of three female SBT individuals revealed that they were at immature stage with oocytes containing single large nucleus (Fig. 3.4).

3.4. RT-qPCR validation assays

The amplification efficiencies obtained from the validation assays using serially diluted YTK and SBT brain and gonad first-strand cDNA as template ranged from 1.86 to 2.00, with all R^2 values at 0.99 (Table 8 and Figures 3.5 & 3.6). A single peak was obtained from both target and reference gene during melt curve analysis, demonstrating specificity of the primers (Fig. 3.7). For YTK Hprt1, brain Cq did not show sexual dimorphism at each of the sampling points in either the immature or the pubertal fish groups. Within the pubertal fish group, YTK Hprt1 Cq varied by 3 cycles in males between sampling 1 and 2 ($P<0.05$). The male YTK Hprt1 Cq also varied ($P<0.05$) by 3 cycles between immature and pubertal fish at sampling 1 (Fig 3.8a).

The gonad YTK Hprt1 Cq is constant between sexes and sampling points within the immature fish group. In pubertal fish group, the gonad YTK Hprt1 Cq was significantly ($P<0.05$) lower in females (3 cycles). The female gonad YTK Hprt1 Cq also fluctuated ($P<0.05$) between fish group and sampling points. YTK Hprt1 Cq is significantly different ($P<0.05$) between brain and gonad (8 cycles) (Fig 3.8b).

SBT brain Hprt1 Cq appeared similar in three maturing broodstock samples. No brain samples were available for the immature fish. SBT gonad Hprt1 Cq appear to vary 4 cycles between individuals from immature group and 3 cycles between individuals from the maturing broodstock (Fig 3.8 c, e, d)

3.5. YTK GPR54 gene expression analysis

When utilizing the efficiency-corrected relative expression method (Pfaffl, 2001) to calculate the relative expression levels, YTK brain GPR54 transcript levels showed significant differences ($P<0.05$) between immature females at the two sampling points (Fig. 3.9a). The males from the immature group showed no significant differences ($P>0.05$) in relative expression levels between the sampling points.

Within the pubertal fish group, the brain GPR54 expression level was almost 2-fold higher ($P<0.05$) in sampling 2 relative to sampling 1 from the immature group, in both male and female fish (Fig. 3.9a).

In the gonad of the immature fish group, there were no significant differences between males and females at sampling 1, however at sampling 2 the GPR54 relative expression levels in the female was significantly higher than that in the males ($P<0.05$), but the same as in sampling 1 (Fig. 3.9b). For the pubertal group, the female GPR54 relative expression levels from sampling 1 was significantly higher ($P<0.05$) than that in

the male from sampling 1, but the same as sampling 2. The male relative expression levels were significantly different ($P<0.05$) between sampling points (Fig 3.9b).

When comparing relative expression levels of GPR54 between the immature and pubertal groups, male and female expression levels were higher in sampling point 1 in the immature fish compared with the pubertal fish. In sampling point 2, pubertal males show higher relative expression levels compared with the immature males, while females from both groups showed the same relative expression levels (Fig. 3.9b)

As the Hprt1 reference gene did not appear to have a constant gene expression between the different sampling points, sex, and tissue types, its usefulness as a normalizing gene was compromised, and therefore the raw data were also analysed for pattern of gene expression. These data are presented as Cq.

In the brain, there was no significant difference ($P>0.05$) between males and females at each of the sampling points, for both the immature and pubertal group (Fig. 3.10a). There were also no significant differences between the females at all sampling points. The immature males at sampling 1 had a significantly higher ($P<0.05$) Cq than the pubertal males at the two sampling points. The pubertal males from sampling 2 had a significantly ($P<0.05$) higher Cq than the males from sampling 1.

Examining the raw data for the gonad, all samples had the same Cq (Fig. 3.10b)with the exception of the pubertal female in sampling 1 that had a significantly lower ($P<0.05$) Cq than all the other samples.

3.6. SBT GPR54 gene expression analysis

The efficiency-corrected relative expression method (Pfaffl, 2001) was utilised to determine the relative expression levels of SBT GPR54 in gonad from three immature and three maturing broodstock individual fish and three brain tissues from maturing fish (Fig. 3.11). The gonadal GPR54 relative expression level was similar between immature and maturing broodstock fish and the brain GPR54 relative expression did not vary between maturing broodstock individual. However n was too low to obtain statistically significant results.

Due to the variations in Hprt1 Cq and the results obtained for YTK, which indicated Hprt1 might be compromised as a reference gene, the raw, Cq for the SBT was also examined (Fig. 3.12). For the SBT brain from maturing fish, the Cq did not vary between individual fish (Fig. 3.12a), while for the gonad, Cq varied by 3 cycles between fish 1 and fish 2 & 3 (Fig 3.12b). For the SBT immature gonad samples, Cq varied by 4

cycles between fish 1 and fish 2 & 3 (Fig 3.12c).

4. Discussion

The GPR54 gene has been identified as a gatekeeper in triggering the onset of puberty in mammals (reviewed by Seminara, 2005; Murphy, 2005; Dungan *et al*., 2006). However, knowledge concerning the role of GPR54 in initiating puberty in fish is limited. Although GPR54 cDNA sequences have been isolated and their expression profile throughout puberty have been studied in several fish species, there is very little information about the system in commercially important fish species such as the SBT and YTK.

We report here the isolation of full length brain SBT GPR54 cDNA and partial brain YTK GPR54 cDNA sequences. We further report the development of a RT-qPCR assay for the measurement of GPR54 gene expression in both brain and gonad tissues and discussion the potential risk of using Hprt1 as a reference gene for normalizing GPR54 expression levels.

Analysis of the deduced predicted amino acid sequence of SBT GPR54 cDNA sequence revealed seven putative transmembrane domains (TMDs), characteristic of membrane-bound G-protein-coupled receptors (Muir *et al*., 2001). The high sequence homology in the TMDs across species suggests that the receptors use the cavity between the TMDs as the binding site of their corresponding ligands (Baldwin, 1994). Unlike its mammalian counterpart, teleosts lack a C-terminal tail in GPR54, a feature which could affect their regulation, as the C-terminal tail of GPR54 in mammals was found to have a role in its signaling (Evans *et al*., 2008).

For gene expression studies, two cohorts of YTK were used. Based on histological analysis, in the immature fish group females contained oocytes with single nucleus and males contained spermatogonia and few spermatids. In the immature group, there was no significant histological profile change in either females or males between the December sampling (1) and the January sampling (2). In the pubertal group, at the December sampling (1), females possessed vitellogenic oocytes and males showed spermatozoa. These observations indicate the pubertal fish group was undergoing spawning. However, in the second sampling, pubertal females contained oocytes at mature resting stage while males displayed predominantly spermatids, indicating that the pubertal fish from January began to reproductively regress. For SBT, only a limited sample size was available, and those fish showed previtellogenic oocytes, suggesting

the fish were reproductively inactive at the time.

In order to measure gene expression, a real-time quantitative RT-qPCR was developed. The RT-qPCR validation assays have shown that the target, a region of the GPR54 gene, has been successfully amplified in both brain and gonad tissues from SBT and YTK. The specificity of the primers was confirmed by the single peak shown during the melt curve analysis. The expression levels of the target was determined by using an efficiency-corrected method that takes into account variations in reaction efficiencies of both target and reference gene (Pfaffl, 2001). All the R^2 values from the validation assays were 0.99, suggesting a strong linear relationship between cDNA concentration and Cq values (Pfaffl, 2001). The expression of GPR54 gene was normalised to the mRNA expression of the reference gene, Hprt1. A reference gene is expected to be stable and should not to vary with experimental conditions or treatments (Weltzien *et al*., 2005; Bustin *et al*., 2009). Finding the correct reference gene that indeed would not change in response to the experimental procedures is a significant challenge, and Hprt1 was chosen as the reference gene in this case following a study by Filby and Tyler (2007), who identified Hprt1 to be stable in response to varying estrogen levels in fish. Considering the two experimental groups in this study, i.e the immature and pubertal fish were expected to have different levels of circulating estrogens, Hprt1 was thought to be an appropriate reference gene. However, it was determined that Hprt1 Cq varied between YTK brain and gonad samples. Within the tissues, Hprt1 Cq also varied 2-3 cycles between male pubertal and immature fish, indicating that gene expression of Hprt1 could be affected by the tissue, sex and reproductive stage in YTK. Therefore in addition to the relative expression levels, the raw (Cq) data were also analysed. While these raw data do not allow for correction of RNA loading, it assumes that such errors would be random, considering exactly the same amount of starting material of RNA was used in all samples and a pattern of expression could be determined and statistically analysed.

The expression profile of brain GPR54 from YTK was compared between the immature and pubertal fish groups using both efficiency-correlated relative expression method (Pfaffl, 2001), and analysis of the raw data. Relative expression analysis revealed that YTK brain GPR54 expression levels were significantly higher in the pubertal fish at the point when gonadal regression was taking place compared with its expression during the peak of the reproductive season or in the immature fish (Fig. 3.9a). The raw data analysis supported these results only for males, while female expression

levels were not significantly different from the other groups (Fig. 3.10a). The YTK brain GPR54 raw expression pattern is in agreement with that described for cobia (Mohamed *et al*., 2007), where expression in early puberty males was significantly higher than that found in females. The YTK brain expression levels appear to be higher in the pubertal fish compared with the immature fish. However, no YTK at early puberty were available during this study, to examine for possible increase in GPR54 expression during that time.

When the YTK GPR54 expression level in the gonad was compared between the immature and pubertal group, the expression level was significantly lower in male pubertal fish group at sampling 1 (which were at peak of spawning season) compared with its expression levels in the group which were undergoing regression or the immature group.

In SBT, the limited sample enabled only the confirmation that GPR54 is expressed in both the brain and gonad, and in both immature and maturing fish. Expression of SBT GPR54 in the gonads was also detected in zebrafish (Biran *et al*., 2008), Senegalese Sole (Mechaly *et al*., 2008) and fathead minnow (Filby *et al*., 2008). The functional significance of GPR54 expression in the gonad has not yet been determined (Biran *et al*., 2008; Mechaly *et al*., 2008; Filby *et al*., 2008), and its role on regulating GnRH expression in the gonad is still to be examined, however the consistent detection of GPR54 transcripts in the gonads and their fluctuation in expression levels argue for a role for GPR54 in the gonad of both males and females. It still remains to be determined if GPR54 activates the GnRH system in the gonad.

In conclusion, GPR54 cDNA sequences from SBT and YTK have been isolated. An RT-qPCR assay has been developed to determine the transcript levels of YTK and SBT GPR54 in the gonad and in the brain during reproductive development. The reference gene Hprt1 was found to be unsuitable in the case of YTK, and other reference genes would need to be tested for this species.

Acknowledgement:
We thank Cleanseas tuna for kindly providing all fish samples and the Seafood CRC for project funding to AE. We are grateful to Kelli Anderson for assistance in GPR54 cloning.

References:

Baldwin, J.M., 1994. Structure and function of receptors coupled to G-proteins. Current Opinion in Cell Biology. 6, 180-190.

Biran, J., Ben-Dor, S., Levavi-Sivan, B., 2008. Molecular identification and functional characterization of the kisspeptins/kisspeptins receptor system in lower vertebrates. Biology of Reproduction. 79, 776-786.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., 2009. The MIQE guilines: Minimum information for publication of quantitative real-time PCR experiments. Clinical Chemistry. 55, 611-622.

Davis, T., Farley, J., Bahar, S., 1998. Size partitioning by depth of southern blufin tuna on the spawning grounds. CCSBT Scientific Committee. Document 9807/5.

de Roux, N., Genin, E., Carel, J.C., Matsuda, F., Chaussain, J.L., Milgrom, E., 2003. Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derive peptide receptor GPR54. Proceedings of the National Academy of Sciences. USA. 100**,** 10972-10976.

Dufour, S., Huang, Y.S., Rousseau, K., Sbaihi, M., Le Belle, N., Vidal, B., Marchelidon, J., Querat, B., Burzawa-Gerard, E., Chang, C.F., Schmitz, M., 2000. Puberty in teleosts: new insights into the role of peripheral signals in the stimulation of pituitary gonadotropins. Proc. The 6th International Symposium on the Reproductive Physiology of Fish. 455–461.

Dungan, H.M., Clifton, D.K., Steiner, R.A., 2006. Kisspeptin neurons as central processor in the regulation of GnRH secretion. Endocrinology. 147, 1154-1158.

Elizur, A., 2009. The KiSS1/GPR54 system in fish. Peptides. 30(1), 164-170.

Evans, B.J., Wang, Z., Mobley, L.T., Khosravi, D., Fujii, N., Navenot, J.-M., Peiper, S.C., 2008. Physical association of GPR54 C-terminal with protein phosphatase 2A. Biochemical and Biophysical Research Communications. 377, 1067-1071.

Filby, A., Tyler, C., 2007. Appropriate 'housekeeping' genes for use in expression profiling the effects of environmental estrogens in fish. BMC Molecular Biology. 8, 10.

Filby, A.L., van Aerle, R., Duitman, J. W., Tyler, C.R., 2008. The kisspeptin/gonadotropin-releasing hormone pathway and molecular signaling of puberty in fish. Biololgy of Reprouction. 78, 278-289.

Funes, S., Hedrick, J. A., Vassileva, G., Markowitz, L., Abbondanzo, S., Golovko, A. 2003. The KiSS-1 receptor GPR54 is essential for the development of the murine reproductive system. Biochemical and Biophysical Research Communications. 312, 1357–63.

Li, S., Zhang, Y., Liu, Y., Huang, X., Huang, W., Lu, D., Zhu, P., Shi, Y., Cheng C.H.K., Liu, X., Lin, H., 2009. Structural and functional multiplicity of the kisspeptin/GPR54 system in goldfish (*Carassius auratus*). Endocrinology. DOI: 10.1677.

Martinez-Chavez, C.C., Minghetti, M., Migaud, H., 2008. GPR54 and rGnRH I gene expression during the onset of puberty in Nile tilapia. General and Comparative Endocrinology. 156**,** 224–233.

Mechaly, A.S., Vinas, J., Piferrer, F., 2008. Identification of two isoforms of the kisspeptin-1 receptor (kiss1r) generated by alternative splicing in a modern teleost, the Senegalese sole (*Solea senegalensis*). Biology of Reproduction. DOI: 10.1095/bioreprod.108.072173.

Messager, S., Chatzidaki, E.E., Ma, D., Hendrick, A.G., Zahn, D., Dixon, J., *et al*., 2005. Kisspeptin directly stimulates gonadotropin-releasing hormone release via G protein-coupled receptor 54. Proceedings of the National Academy of Sciences. USA. 102**,** 1761-1766.

Mohamed, J.S., Benninghoff, A.D., Holt, G.J., Khan, I.A., 2007. Developmental expression of the G protein-coupled receptor 54 and three GnRH mRNAs in the teleost fish cobia. Journal of Molecular Endocrinology 38, 235–244.

Muir, A.I., Chamberlain, L., Elshourbagy, N.A., Michalovich, D., Moore, D.J., Calamari, A., Szekere, P.J., Sarau, H.M., Chambers, J.K., Murdock, P., *et al*., 2001. AXOR12, a novel human G protein-coupled receptor, activated by the peptide KiSS-1. Journalof Biological Chemistry. 276**,** 28969–28975.

Murphy, K.G., 2005. Kisspeptin: regulators of metastasis and the hypothalamic-pituitary-gonadal axis. Journal of Neuroendocrinology. 17, 519-525.

Navarro, V.M., Castellano, J.M., Garcia-Galiano, D., Tena-Sempere, M., 2007. Neuroendocrine factors in the initiation of puverty: The emergent role of kisseptin. Reviews in Endocrine Metabolism and Disorders. 8, 11-20.

Nocillado, J.N., Levavi-Sivan, B., Carrick, F., Elizur, A., 2007.Temporal expression of G-protein-coupled receptor 54 (GPR54), gonadotropin-releasing hormones (GnRH), and dopamine receptor D2 (drd2) in pubertal female grey mullet, *Mugil cephalus*. General and Comparative Endocrinology. 150, 278–287.

Okuzawa K. 2002. Puberty in teleosts. Fish Physiology and Biochemistry 26: 31-41.

Parhar, I.S., Ogawa, S., Sakuma, Y., 2004. Laser-captured single digoxigenin-labeled neurons of gonadotropin-releasing hormone types reveal a novel G protein-coupled receptor (Gpr54) during maturation in cichlid fish. Endocrinology 145, 3613–3618.

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Research. 29**,** 2002-2007.

Plant, T.M., Ramaswamy, S., Dipietro, M.J., 2006. Repetitive activation of hypothalamic G protein-coupled receptor 54 with intravenous pulses of kisspeptin in the juvenile monkey (Macaca mulatta) elicits a sustained train of gonadotropin-releasing hormone discharges. Endocrinology. 147, 1007-1013.

Roa, J., Aguilar, E., Dieguez, C., Pinilla, L. Tena-Sempere, M., 2008. New frontiers in kisspeptin/GPR54 physiology as fundamental gatekeepers of reproductive function. Frontiers in Neuroendocrinology. 29, 48-69.

Schulz, R., Goos, H.J.Th., 1999. Puberty in male fish: concepts and recent developments with special reference to the African catfish (*Clarias gariepinus*). Aquaculture. 177, 5-12.

Seminara, S.B., Messager, S., Chatzidaki, E.E., Thresher, R.R., Acierno, J.S., Shagoury, J.K., Bo-Abbas, Y., Kuohung, W., Schwinof, K.M., Hendrick, A.G., Zahn, D., Dixon, J., Kaiser, U.B., Slaugenhaupt, S.A., Gusella, J.F., O'Rahilly, S., Carlton, M.B., Crowley, W.F., Jr., Aparicio, S.A., Colledge, W.H., 2003. The GPR54 gene as a regulator of puberty. New England Journal of Medicine. 349,1614-1627.

Seminara, S.B., 2005. Metastin and its G protein-coupled receptor, GPR54: critical pathway modulating GnRH secretion. Frontiers in Neuroendocrinology. 26, 131-138.

Sisk, C.L., Foster, D.L., 2004. The neural basis of puberty and adolescence. Nature Neuroscience. 7, 1040-1047.

Teles, M.G., Bianco, S.D.C., Brito, N.V., Trarbach, E.B., Kuohung, W., Xu, S., Seminara, S.B., Mendonca, B.B., Kaiser, U.B., Latronico, A.C., 2008. A GPR54-activating mutation in a patient with central precocious puberty. New England Journal of Medicine 358, 709-715.

Weltzien, F. A., Andersson, E., Andersen, O., Shalchian-Tabrizi, K., Norberg, B., 2004. The brain–pituitary–gonad axis in male teleosts, with special emphasis on XatWsh (*Pleuronectiformes*). Comparative Biochemistry and Physiology A. 137, 447–477.

Weltzien, F., Pasqualini, C., Vernier, P., Dufour, S., 2005. A quantitative real-time RT-PCR assay for European eel tyrosine hydroxylase. General and Comparative Endocrinology. 142, 134-142.

Tables

Table 1

Degenerate and gene-specific primers used to isolate the SBT and YTK GPR54 cDNA by RT-PCR and RACE RT-PCR

Primer name	Direction	5' to 3' sequence	TM (°C)
F6*	Forward	5' GCAYCCBTTCYTSACRGAYGC 3'	57
F7*	Forward	5' CTCATCATGCTRGTSGGRCT 3'	54
F8*	Forward	5' TGGHTGCCACYGACATCATYTT 3'	53
R5*	Reverse	5' TGAABGGSACRCAGCAMASCA 3'	56
R6*	Reverse	5' CACATGAAGTYSCCAAAKATCCA 3'	53
R7*	Reverse	5' GTSGCYTGYACHGTCACCTG 3'	56
3'SF1**	Forward	5' TTCTTCACAGACGCCTGG 3'	50
3'YSF2**	Forward	5' GCAACTCTCTGGTTATTTATGTCAT 3'	53
3'R1*	Reverse	5' CCAGCAGAYGGYGAAGAGGA 3'	56
3'R2*	Reverse	5' TAGGACATGCAGTTGGCCCA 3'	54
5'SR1**	Reverse	5' CCAGGCGTCTGTGAAGAA 3'	50
5'YS R2**	Reverse	5' ATGACATAAATAACCAGAGAGTTGC 3'	53
S54 3F1(b)	Forward	5' CAGGAGCAAGGTCTCCAAGATGGTGGT 3'	63
S54 3F2(b)	Forward	5' CAAGACATGGGCCAACTGCATGTCCTA 3'	61
3'YF1**	Forward	5' TTCTTCACGGATGCCTGG3'	50
3'YS F2**	Forward	5' GCAACTCTCTGGTTATTTATGTCAT 3'	53
NUP	Forward	5' AAGCAGTGGTATCAACGCAGAGT 3'	55
NUP	Reverse	5' ACTCTGCGTTGATACCACTGCTT 3'	55

* Degenerate primers were designed from regions conserved between cobia (*Rachycentron canadum,* DQ790001), grey mullet (*Mugil cephalus,* DQ683737*)*, tilapia (*Oreochromis niloticus,* AB162143*)*, zebrafish (*Danio rerio,* EU047917) and fathead minnow (*Pimephales promelas*) GPR54 sequences available at the GenBank. A: Adenine, G: Guanie, T: Thymine, C: Cytosine. R: A/g; Y: C/T; M: A/C; K: g/T; S: g/C; W: A/T; H: A/C/T; B: g/C/T; V: A/g/C; D: A/g/T; N: A/g/C/T.

** Gene-specific primers designed for 5' and 3' RACE RT-PCR

Table 2

Gene-specific primers used for RT-qPCR assays

Gene	**Primer name (direction)**	**5' to 3' sequence**	**TM(°C)**	**Amplicon size (bp)**
SBT	T54F2 (forward)	5' TGGTGGGCAACTCTCTGGTTATT 3'	55	164
GPR54	T54R2 (reverse)	5' GCCAAATATCCATCCAGGCAGA 3'	55	
SBT	ThprtF1(forward)	5' CTACGCCACCGATTTGGAGA 3'	54	196
Hprt1	ThprtR1(reverse)	5' GGAGCGGTCACTGTTCCTGT 3'	56	
YTK	Y54F2 (forward)	5' CAAACACAGGCAGATGAGGA 3'	52	130
GPR54	Y54R1 (reverse)	5' GCCAAAGATCCATCCAGGGAGA 3'	57	
YTK	YhprtF2(forward)	5' CCCCATGGACTCATCTTGGA 3'	54	144
Hprt1	YhprtR2(reverse)	5' CAGAGCCTTGATGTAGTCCAGCAGG 3'	61	

Table 3: Average body weight of YTK (Kg, mean ± SD).

	Immature Fish Group		**Pubertal Fish Group**	
	Male (n=6)	**Female (n=6)**	**Male (n=6)**	**Female (n=6)**
Sampling 1 (Dec 08)	1.86±0.23 kg	1.93±0.24 kg	5.55±1.69 kg	5.56±0.85 kg
Sampling 2 (Jan 09)	2.30±0.33 kg	2.48±0.26 kg	6.34±2.50 kg	5.74±0.48 kg

Table 4 Average gonad weight of YTK (g, mean ± SD)

	Immature Fish Group		**Pubertal Fish Group**	
	Male	**Female (n=6)**	**Male**	**Female (n=6)**
Sampling 1 (Dec 08)	N/A (n=6)	N/A	199.53±74.32 (n=5)	47.13±45.27
Sampling 2 (Jan 09)	0.90±0.01 (n=2)	3.48±0.54	20.95±16.37 (n=4)	20.92±8.14

Table 5: Gonadosomatic index of YTK (GSI mean ± SD, N/A: Not Available)

	Immature Fish Group		**Pubertal Fish Group**	
	Male	**Female (n=6)**	**Male**	**Female (n=6)**
Sampling 1 (Dec 08)	N/A (n=6)	N/A	3.67±1.60 (n=5)	0.77±0.70
Sampling 2 (Jan 09)	0.04 (n=2)	0.14±0.02	0.36±0.28 (n=4)	0.36±0.12

Table 6: Body weight of maturing SBT broodstock fish (Kg).

	Fish 1 (female)	**Fish 2 (female)**	**Fish 3 (male)**
Body weight (Kg)	126.9	129.0	128.0

Table 7: Gonadosomatic index of maturing SBT broodstock fish (N/A: Not Available)

	Fish 1 (female)	**Fish 2 (female)**	**Fish 3 (male)**
GSI	N/A	0.64	0.11

Table 8

Amplification efficiency, slope and R^2 values obtained from the validation assays of the target and reference genes using serially diluted cDNA.

Gene	**Tissue**	**Amplification efficiency**	**Slope**	**R^2**
SBT GPR54	Brain	1.86	-3.704	0.99
	Gonad	2.00	-3.327	0.99
SBT Hprt1	Brain	1.85	-3.753	0.99
	Gonad	2.00	-3.331	0.99
YTK GPR54	Brain	2.00	-3.329	0.99
	Gonad	1.99	-3.353	0.99
YTK Hprt1	Brain	1.98	-3.375	0.99
	Gonad	1.99	-3.348	0.99

Figure legends

Figure 3.1. Phylogram of GPR54 gene sequences across species. Branch lengths are proportional to the amount of inferred evolutionary change. Accession numbers: cobia (DQ790001), Nile tilapia (AB162143), Senegalese sole (EU136710), Goldfish (EU622877), Grey mullet (DQ683737), Atlantic croaker (DQ347412), Fathead minnow (EF672266), Zebrafish GPR54a (EU047917), Zebrafish GPR54b (EU047918) Bullfrog (ACD44939), Rat (AAD19664), human (AAK83235), SBT (Bankit 1218262) and YTK (Bankit 1218311). The tree was generated by Clustal w2 (http://www.ebi.ac.uk/Tools/clustalw2/), according to amino acid sequences.

Figure 3.2. Representative ovarian sections of female YTK. (A) Immature; oocytes at chromatin nucleolar stage (S1: single large nucleus); sampling 1 of immature fish group. (B) Immature; oocytes at perinucleolar stage (S2: nucleus larger in size than S1, multiple nucleoli); sampling 2 of immature fish group. (C) Developing; few vitellogenic oocytes (S4: appearance of vitellogenic protein); sampling 1 of pubertal fish group. (D) Mature resting; cortical alveoli oocytes (S3: yolk vesicles appearing in the cytoplasm); sampling 2 of pubertal fish group. Yellow scale bar = 150μm, 100X magnification.

Figure 3.3. Representative testicular histology of male YTK. (A) Immature; spermatogonia (spg) predominant; sampling 1 of immature fish group. (B) Immature; mainly spermatogonia (spg) and few spermatids (spd); sampling 2 of immature fish group. (C) Developing; spermatozoa (spz) predominant; sampling 1 of pubertal fish group. (D) Developing; spermatids (spd) predominant; sampling 2 of pubertal fish group. Yellow scale bar = 150μm, 400X magnification.

Figure 3.4. Representative gonadal sections of SBT. Three immature individual female SBT contain oocytes with single large nucleus. Red scale bar = 150μm, 100X magnification.

Figure 3.5. RT-qPCR dilution curves and standard curves. (A) SBT brain GPR54; (B) SBT brain Hprt1; (C) SBT gonad GPR54; (D) SBT gonad Hprt1. In the dilution curve, x-axis: cycle number; y-axis: fluorescent signal; different color: different fold dilution of cDNA template. In the standard curve, x-axis: cDNA template concentration;

y-axis: crossing point; R^2: linear regression; M: slope; B: y-intercept.

Figure 3.6. RT-qPCR dilution curves and standard curves. (A) YTK brain GPR54; (B) YTK brain Hprt1; (C) YTK gonad GPR54; (D) YTK gonad Hprt1. In the dilution curve, x-axis: cycle number; y-axis: fluorescent signal; different color: different fold dilution of cDNA template. In the standard curve, x-axis: cDNA template concentration; y-axis: crossing point; R^2: linear regression; M: slope; B: y-intercept.

Figure 3.7. RT-qPCR melt curve analysis. A single peak was obtained for GPR54 and Hprt1 from each tissue at 85°C while peaks were not obtained from the negative control. (A) SBT brain GPR54; (B) SBT brain Hprt1; (C) SBT gonad GPR54; (D) SBT gonad Hprt1; (E) YTK brain GPR54; (F) YTK brain Hprt1; (G) YTK gonad GPR54; (H) YTK gonad Hprt1. X-axis: temperature; y-axis: fluorescent signal.

Figure 3.8. Brain and gonad Hprt1 Cq from two cohorts of male and female YTK at two sampling points and three maturing and immature SBT. For YTK, each bar represents mean (±SEM) from 6 fish per sampling point using real-time quantitative RT-qPCR assay. Different letters indicate significant difference at $P<0.05$.

Figure 3.9. Relative brain and gonadal expression levels of GPR54 from two cohorts of male and female YTK at two sampling points. Each bar represents mean (±SEM) from 6 fish per sampling point using real-time quantitative RT-qPCR assay. Data were normalized against Hprt1. Different letters indicate significant difference at $P<0.05$.

Figure 3.10. Brain and gonad GPR54 Cq from two cohorts of male and female YTK at two sampling points. Each bar represents mean (±SEM) from 6 fish per sampling point using the real-time quantitative RT-qPCR assay. Different letters indicate significant difference at $P<0.05$.

Figure 3.11. Relative brain and gonadal expression levels of GPR54 from three immature and maturing broodstock SBT using real-time quantitative RT-qPCR assay. Data were normalized against Hprt1.

Figure 3.12. Brain and gonad GPR54 Cq from three immature and maturing broodstock SBT using real-time quantitative RT-qPCR assay. Different letters indicate significant difference at $P<0.05$.

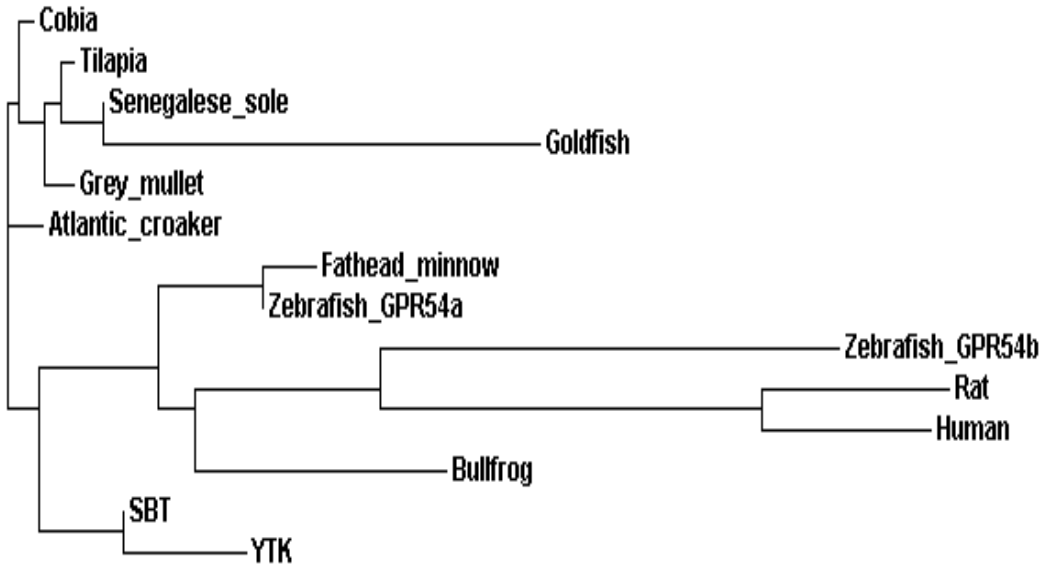

Cobia
Tilapia
Senegalese_sole
Goldfish
Grey_mullet
Atlantic_croaker
Fathead_minnow
Zebrafish_GPR54a
Zebrafish_GPR54b
Rat
Human
Bullfrog
SBT
YTK

Figure 3.1

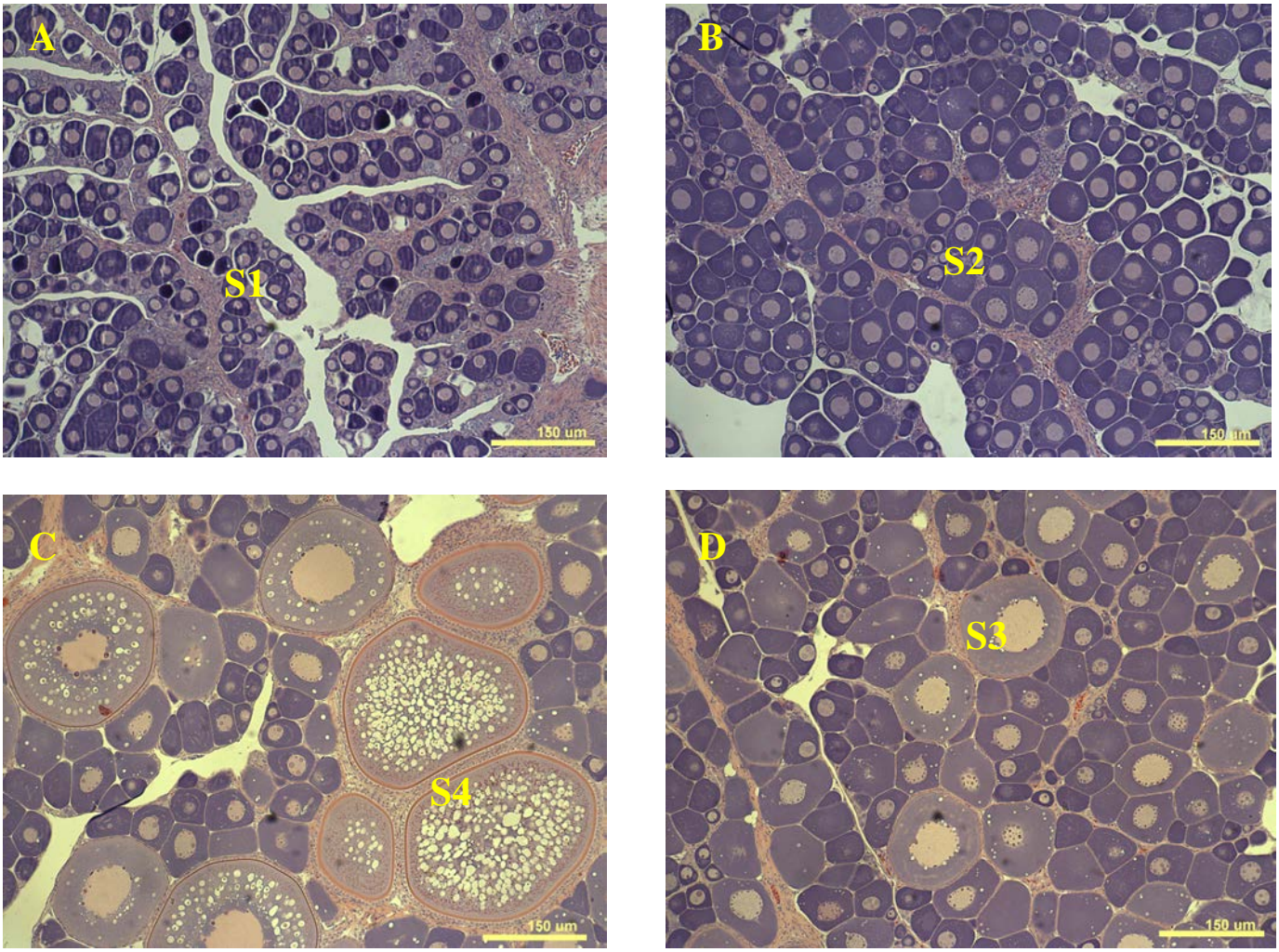


Figure 3.2

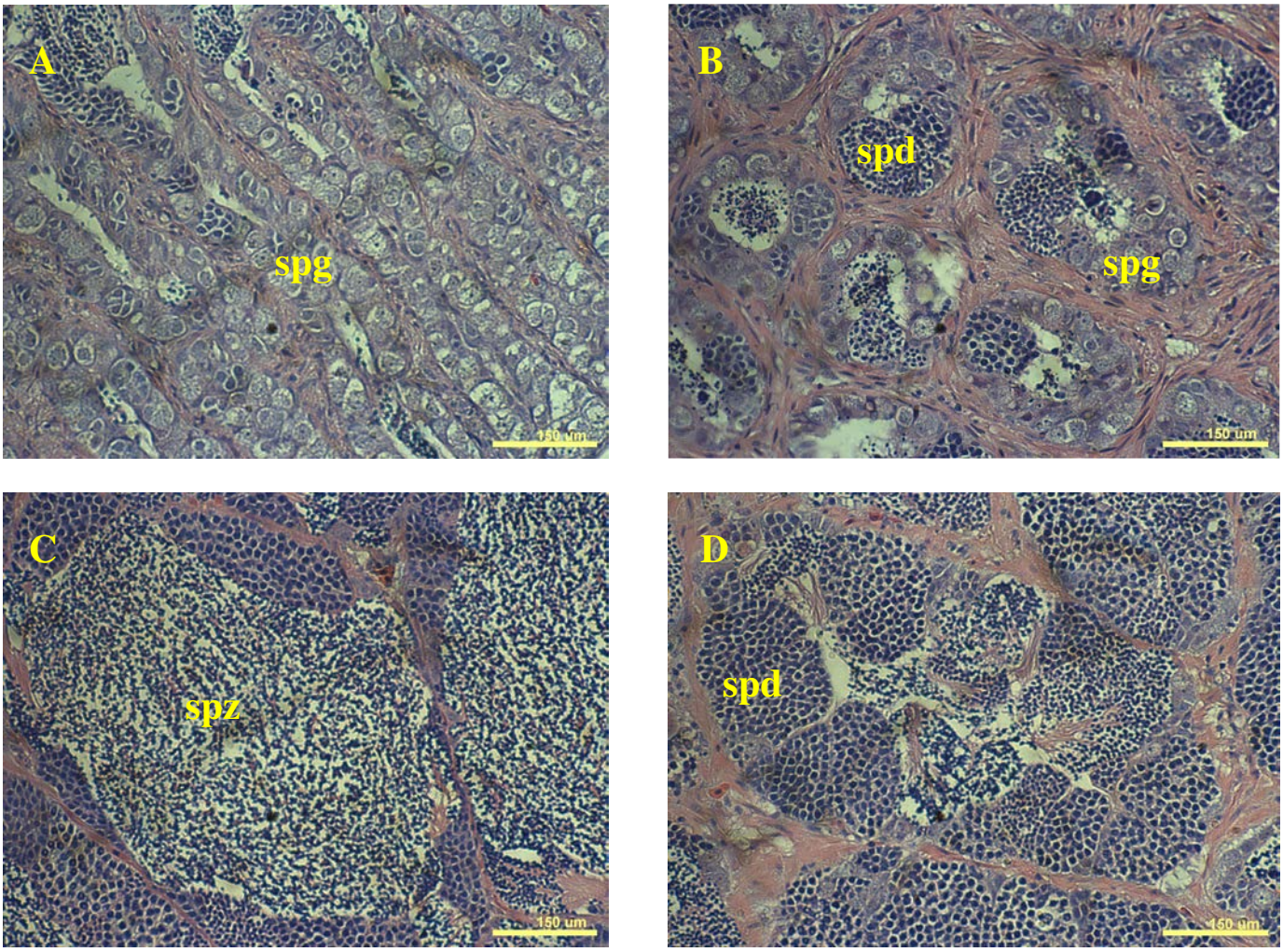
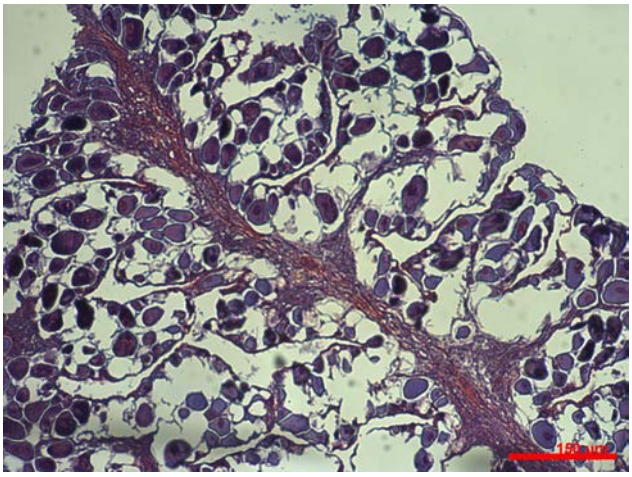
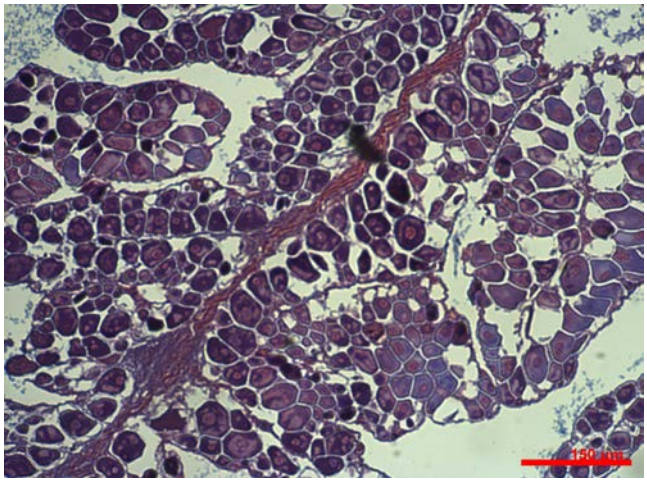


Figure 3.3





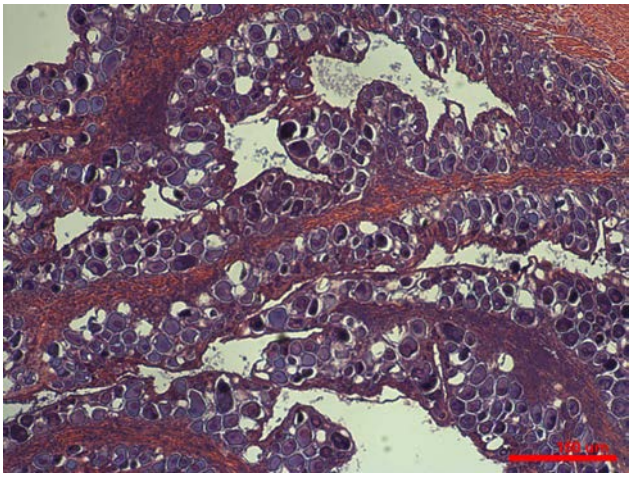


Figure 3.4

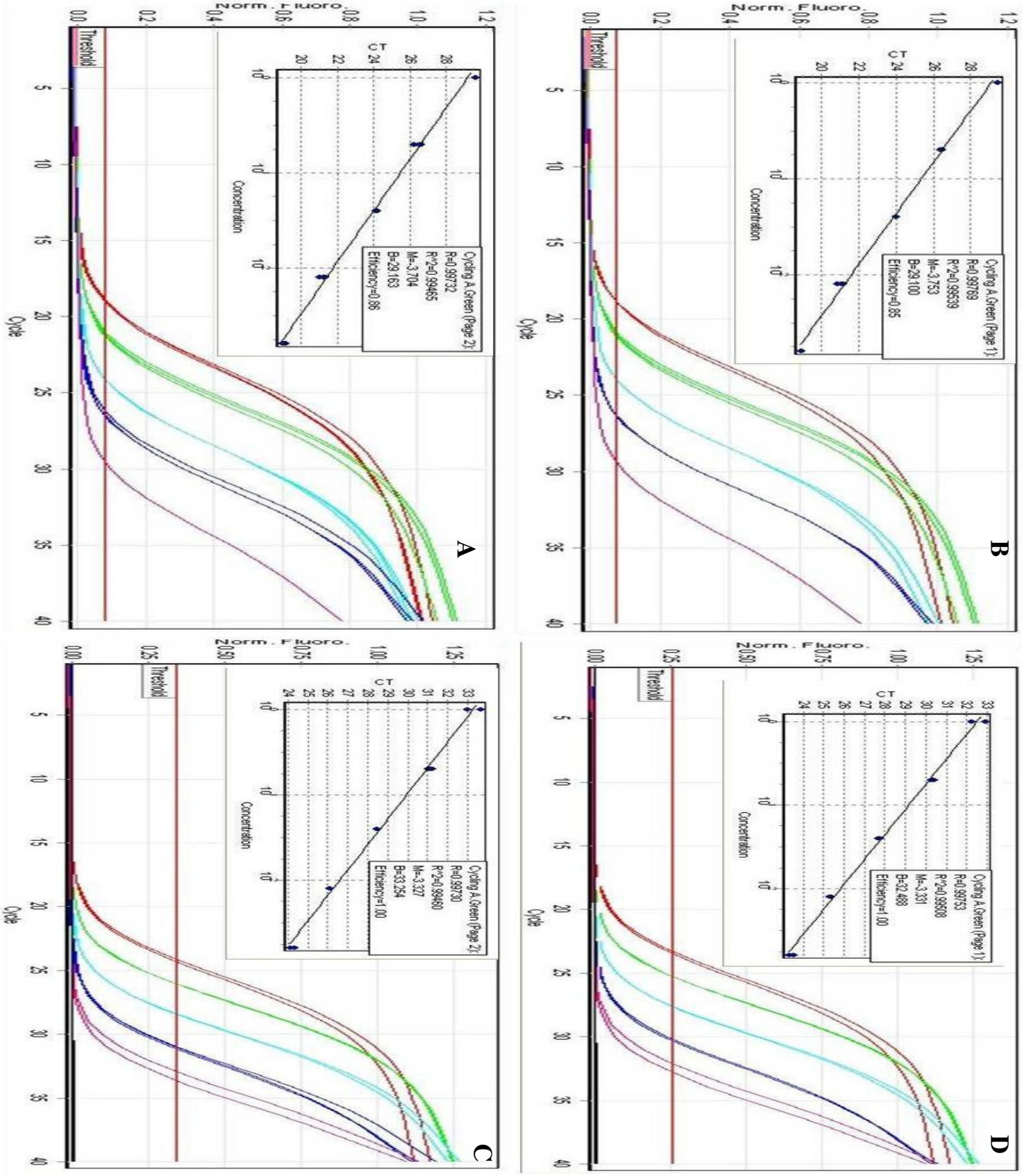

A
B
C
D
Norm. Fluoro.
Cycle
Threshold
Concentration
Cycling A.Green (Page 1):
R=0.99769
R^2=0.99539
M=-3.753
B=29.100
Efficiency=0.85
Cycling A.Green (Page 2):
R=0.99732
R^2=0.99465
M=-3.704
B=29.163
Efficiency=0.86
Cycling A.Green (Page 1):
R=0.99753
R^2=0.99508
M=-3.331
B=32.488
Efficiency=1.00
Cycling A.Green (Page 2):
R=0.99730
R^2=0.99460
M=-3.327
B=33.254
Efficiency=1.00

Figure 3.5

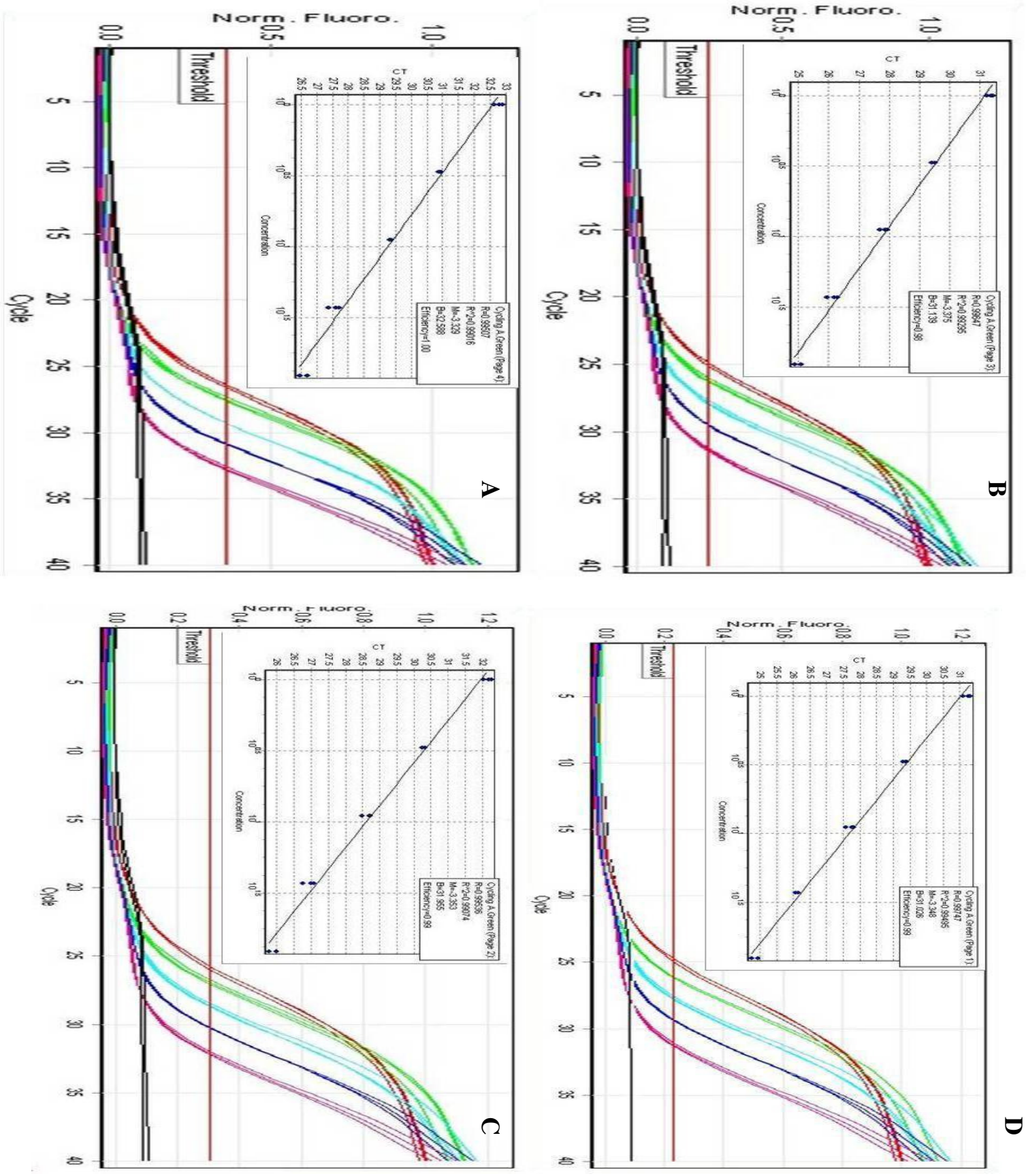


Figure 3.6

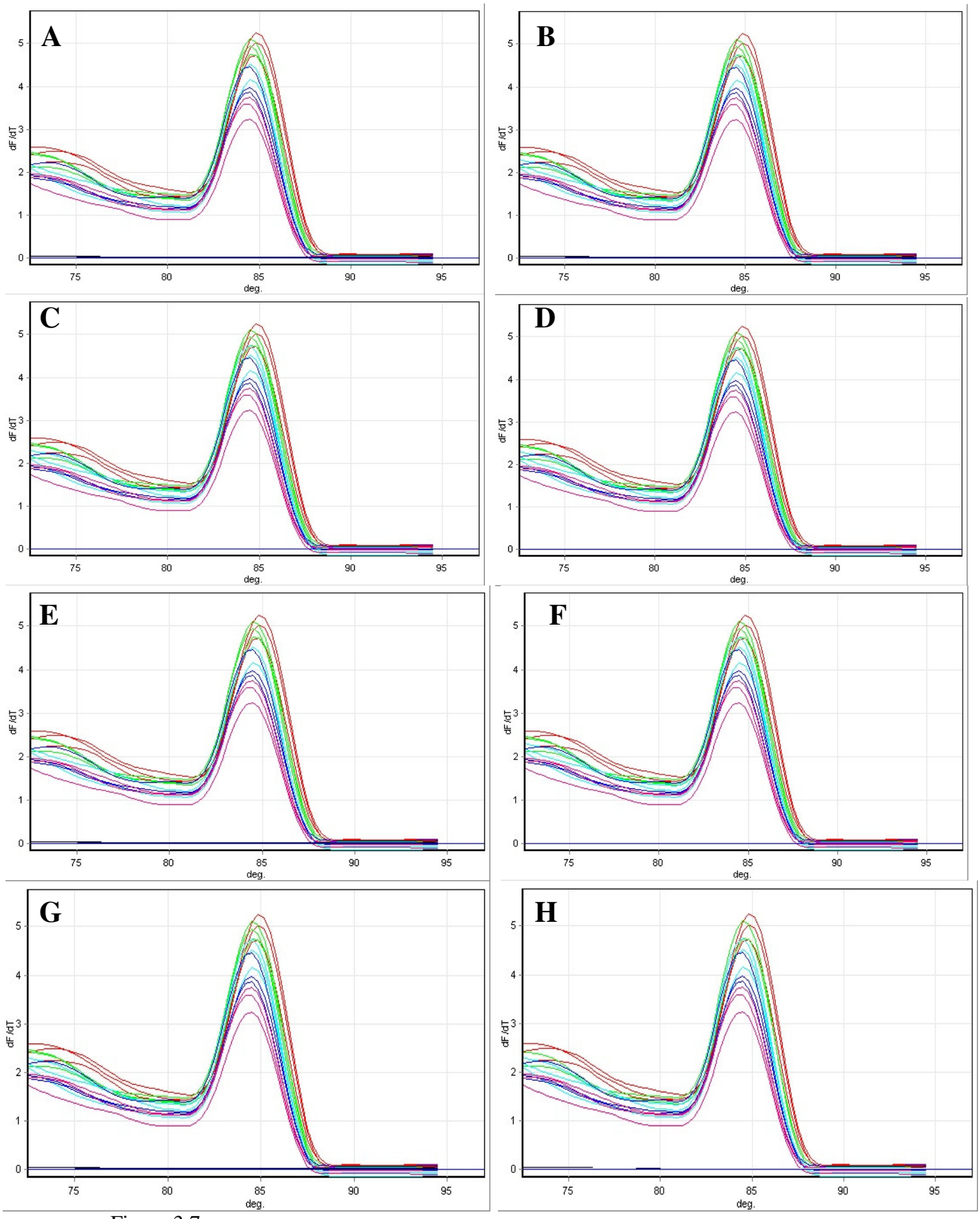
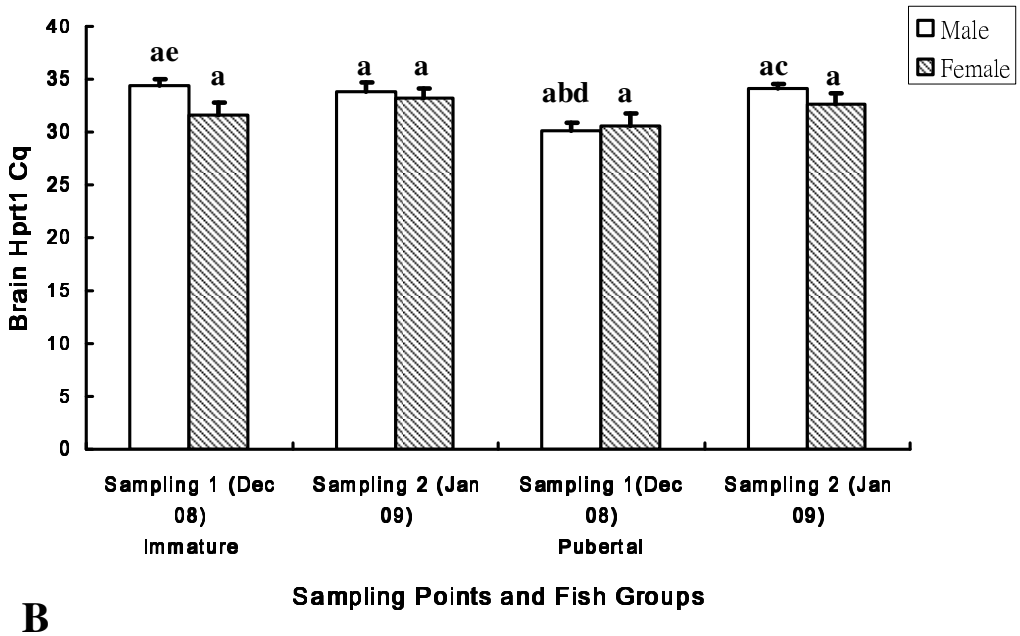
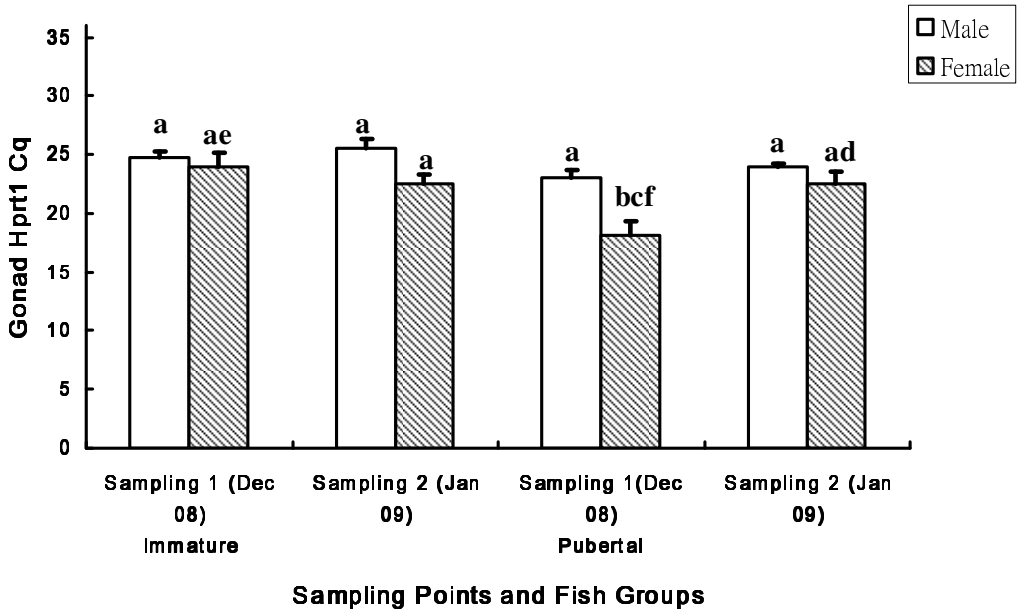


Figure 3.7

A


Male
Female
Brain Hprt1 Cq
40
35
30
25
20
15
10
5
0
ae
a
a
a
abd
a
ac
a
Sampling 1 (Dec 08)
Sampling 2 (Jan 09)
Sampling 1(Dec 08)
Sampling 2 (Jan 09)
Immature
Pubertal
Sampling Points and Fish Groups

B


Male
Female
Gonad Hprt1 Cq
35
30
25
20
15
10
5
0
a
ae
a
a
a
bcf
a
ad
Sampling 1 (Dec 08)
Sampling 2 (Jan 09)
Sampling 1(Dec 08)
Sampling 2 (Jan 09)
Immature
Pubertal
Sampling Points and Fish Groups

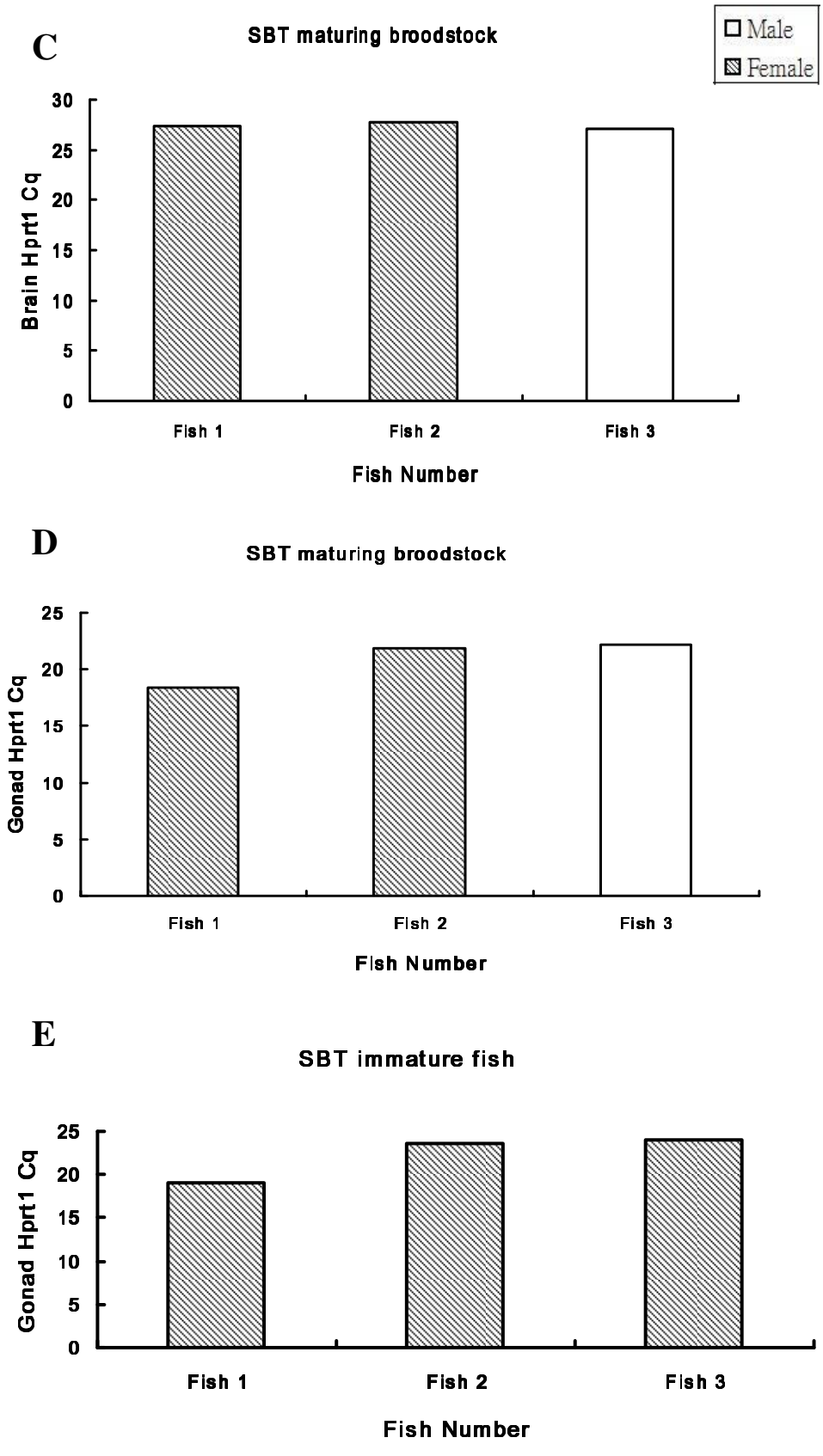

C
SBT maturing broodstock
Male
Female
Brain Hprt1 Cq
30
25
20
15
10
5
0
Fish 1
Fish 2
Fish 3
Fish Number
D
SBT maturing broodstock
Gonad Hprt1 Cq
25
20
15
10
5
0
Fish 1
Fish 2
Fish 3
Fish Number
E
SBT immature fish
Gonad Hprt1 Cq
25
20
15
10
5
0
Fish 1
Fish 2
Fish 3
Fish Number

Figure 3.8

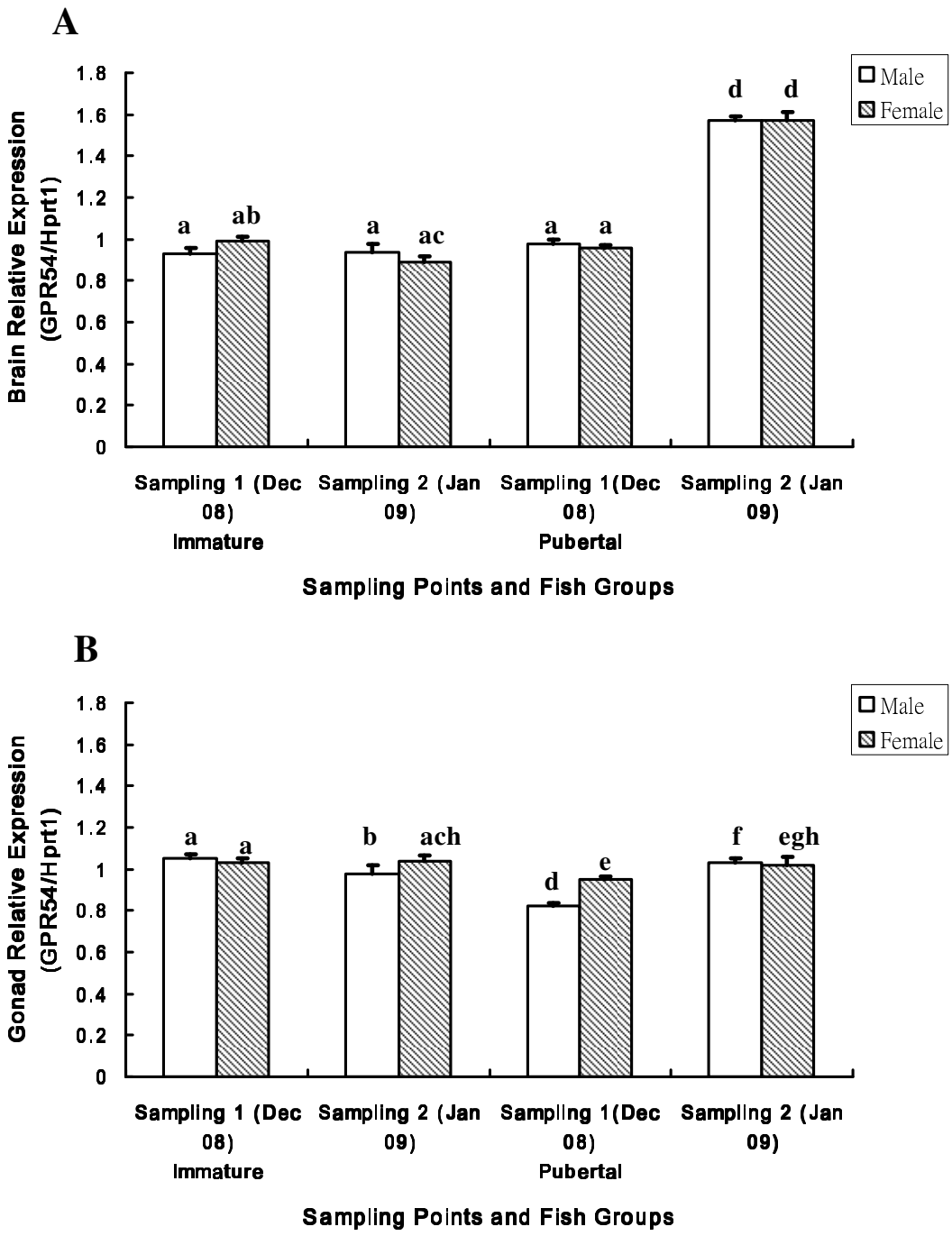

A
Brain Relative Expression (GPR54/Hprt1)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
a
ab
a
ac
a
a
d
d
Male
Female
Sampling 1 (Dec 08)
Sampling 2 (Jan 09)
Sampling 1(Dec 08)
Sampling 2 (Jan 09)
Immature
Pubertal
Sampling Points and Fish Groups
B
Gonad Relative Expression (GPR54/Hprt1)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
a
a
b
ach
d
e
f
egh
Male
Female
Sampling 1 (Dec 08)
Sampling 2 (Jan 09)
Sampling 1(Dec 08)
Sampling 2 (Jan 09)
Immature
Pubertal
Sampling Points and Fish Groups

Figure 3.9

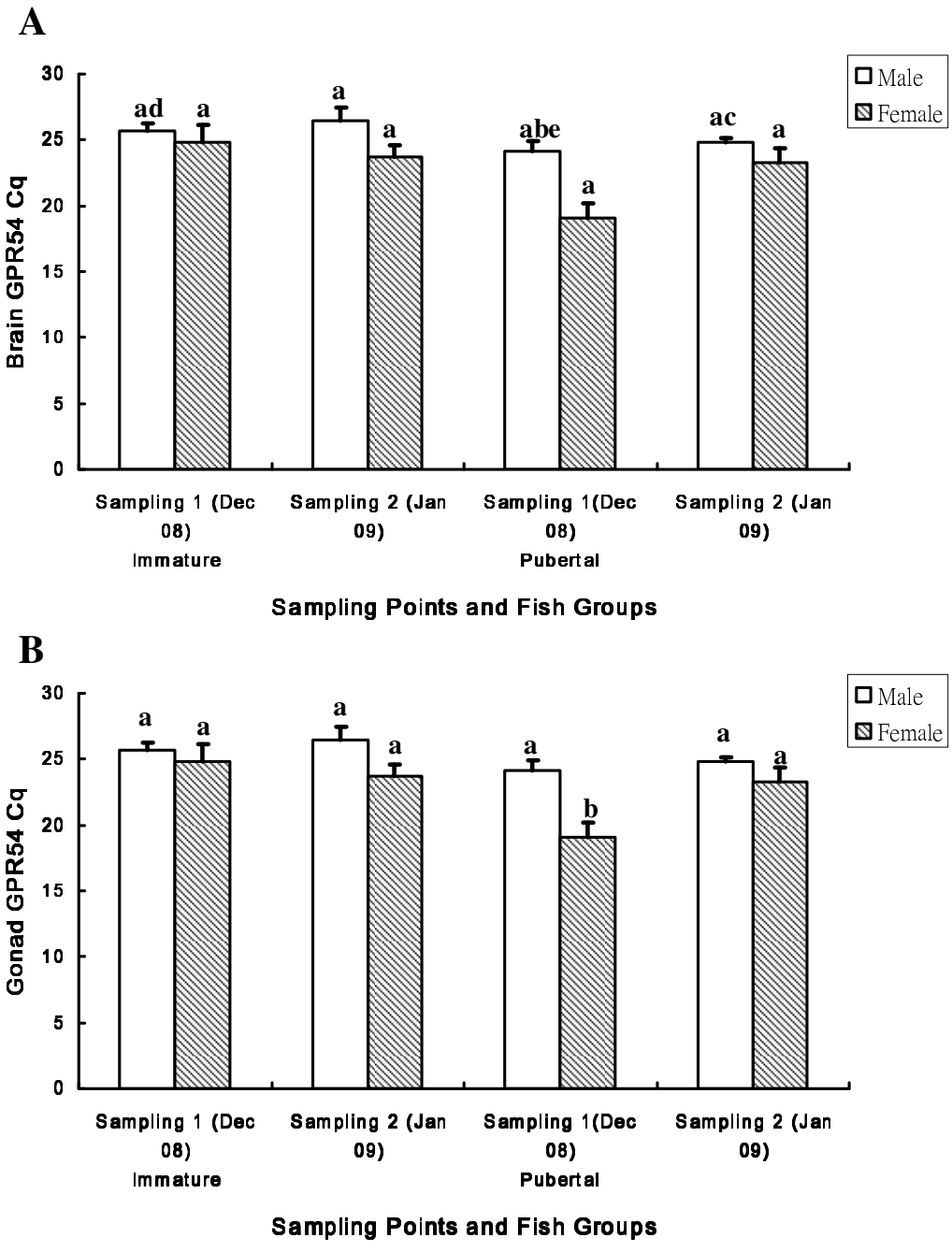
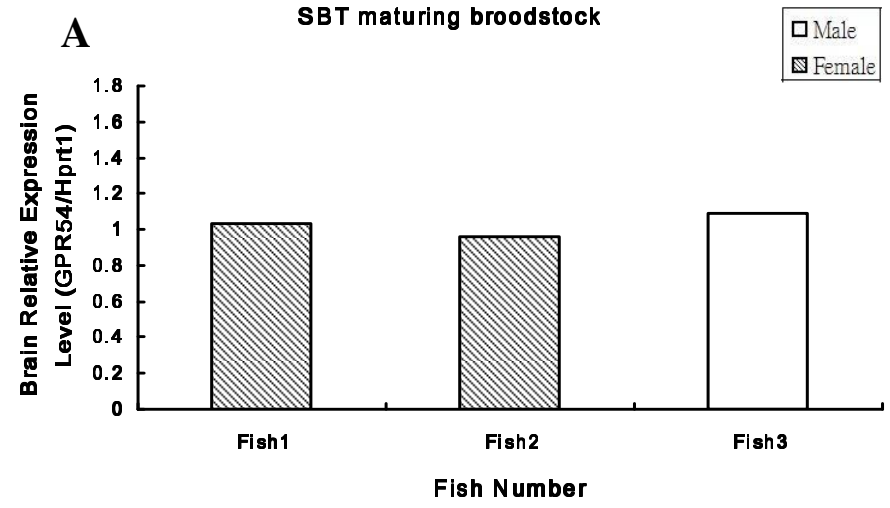
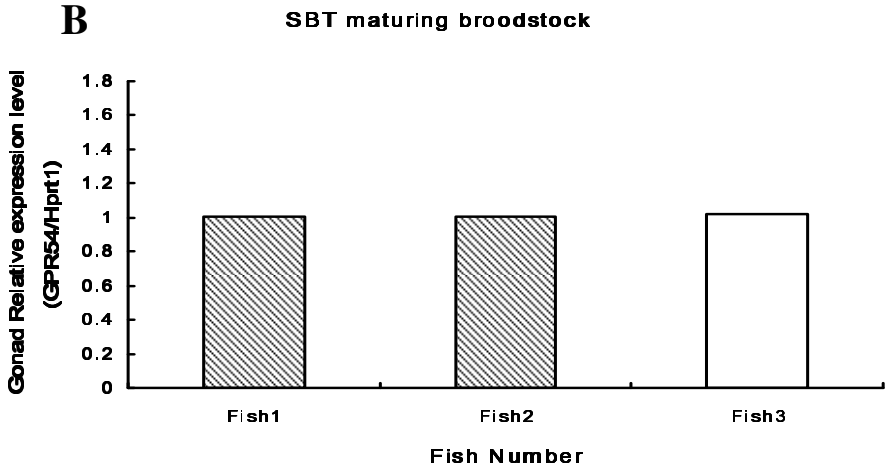

A
Male
Female
Brain GPR54 Cq
30
25
20
15
10
5
0
ad
a
a
a
abe
a
ac
a
Sampling 1 (Dec 08)
Sampling 2 (Jan 09)
Sampling 1(Dec 08)
Sampling 2 (Jan 09)
Immature
Pubertal
Sampling Points and Fish Groups
B
Male
Female
Gonad GPR54 Cq
30
25
20
15
10
5
0
a
a
a
a
a
b
a
a
Sampling 1 (Dec 08)
Sampling 2 (Jan 09)
Sampling 1(Dec 08)
Sampling 2 (Jan 09)
Immature
Pubertal
Sampling Points and Fish Groups

Figure 3.10


A
SBT maturing broodstock
Male
Female
Brain Relative Expression Level (GPR54/Hprt1)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Fish1
Fish2
Fish3
Fish Number


B
SBT maturing broodstock
Gonad Relative expression level (GPR54/Hprt1)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Fish1
Fish2
Fish3
Fish Number

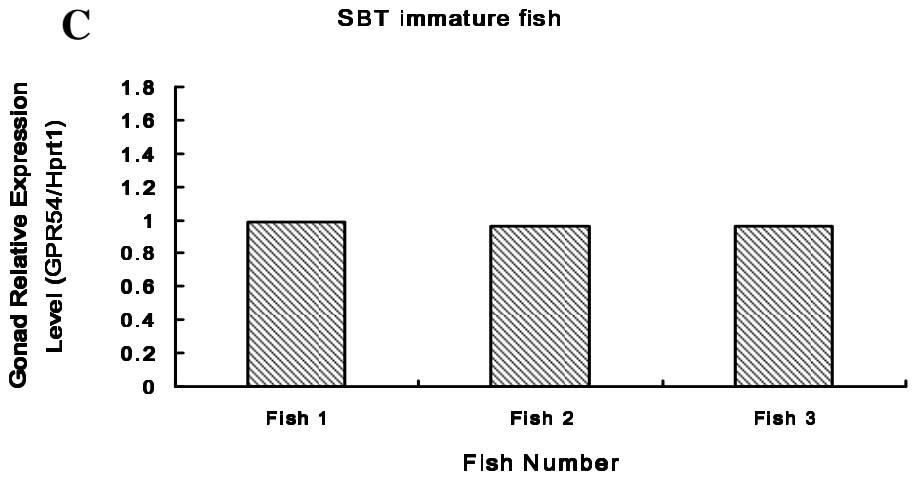

C
SBT immature fish
Gonad Relative Expression Level (GPR54/Hprt1)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Fish 1
Fish 2
Fish 3
Fish Number

Figure 3.11

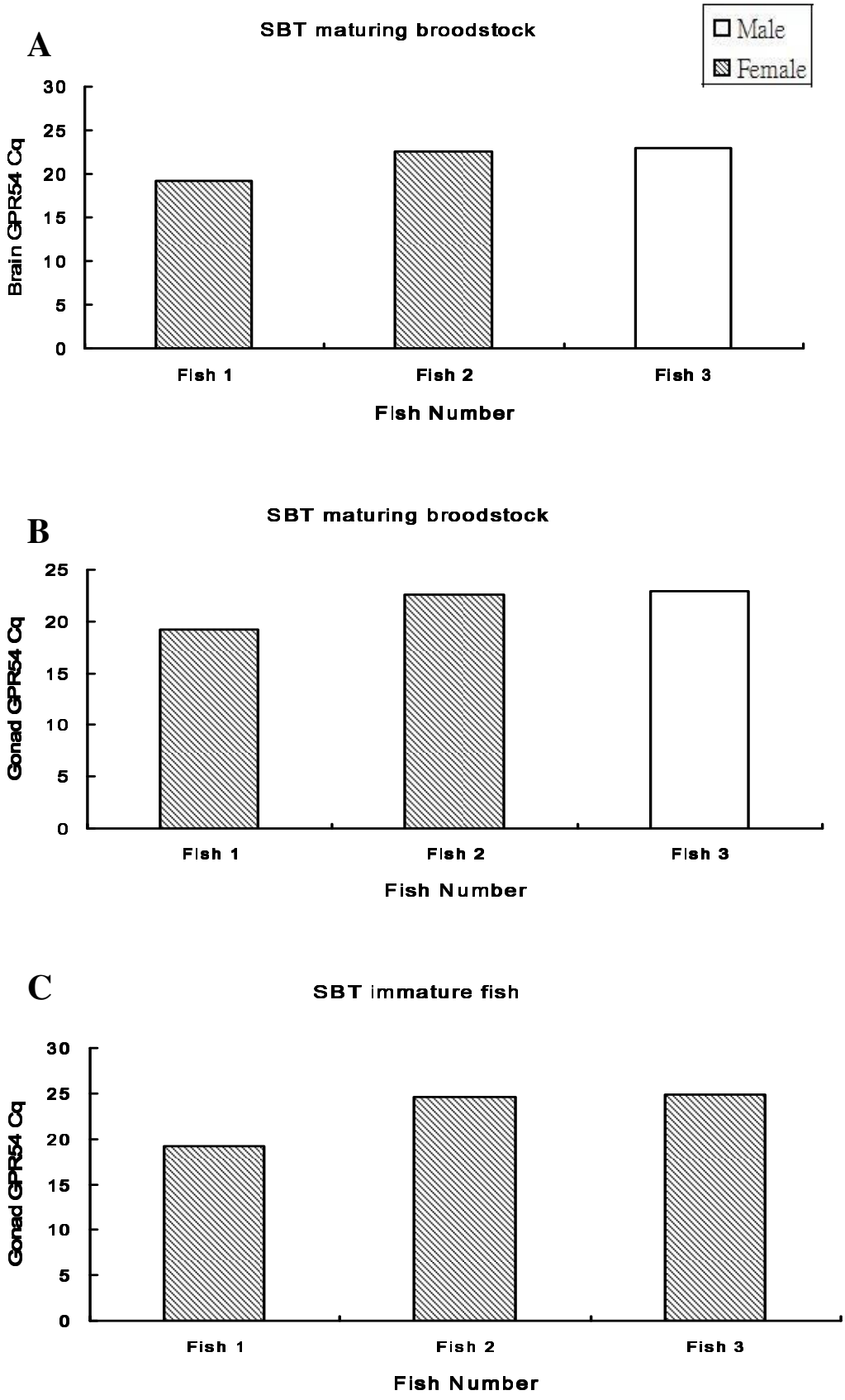

A
SBT maturing broodstock
Male
Female
Brain GPR54 Cq
30
25
20
15
10
5
0
Fish 1
Fish 2
Fish 3
Fish Number
B
SBT maturing broodstock
Gonad GPR54 Cq
25
20
15
10
5
0
Fish 1
Fish 2
Fish 3
Fish Number
C
SBT immature fish
Gonad GPR54 Cq
30
25
20
15
10
5
0
Fish 1
Fish 2
Fish 3
Fish Number

Figure 3.12

5. Appendix 1

The information added in this appendix refers to some of the sections in the manuscript, where further details were warranted in the context of an honours thesis. The numbering refers to the relevant section in the manuscript. i.e. A2.3. is the appendix for section 2.3. in the manuscript. This appendix therefore is not intended to be evaluated as an independent chapter, but to complement for the manuscript.

A2.3. RNA extraction

Total RNA was extracted from SBT and YTK brain with TriZol Reagent (Invitrogen, Carlsbad, CA). Whole brain was homogenized in proportional amount of TriZol Reagent, i.e., 100mg of tissue in 1ml of TriZol reagent. Aliquots (1ml) of the homogenized tissue stock were stored at -80°C. Total RNA was extracted from the 1ml aliquot and the concentration of total RNA was determined using a spectrophotometer (Genequant ProTM, Biochrom, Cambridge, UK). A blank reference was set up with the same nuclease free water which was used to resuspend the RNA after isolation. Spectrophotometry was performed at 260nm and 280nm on isolated RNA, which was loaded into a microcapillary tube. The 260/280 ratio, which in an indicator of RNA purity was automatically calculated (a ratio of 1.8-2.1 is optimal). In the same reading the concentration of RNA was expressed in µg/µl. The RNA was treated with DNAse (Promega) according to the manufacturer's instructions. The concentration of DNase-treated total RNA was determined again using a spectrophotometer (Genequant ProTM, Biochrom, Cambridge, UK). Total RNA from gonadal tissue samples (15-20mg) were extracted in accordance with the illustra RNA spin Mini-prep (GE healthcare, Catalogue # 25-0500-71, UK) total RNA extraction kit. The kit incorporates filtration columns that eliminate contaminating DNA in the RNA extracts. The concentration of RNA was determined by spectrophotometer (Genequant ProTM, Biochrom, Cambridge, UK). The extracted RNA from both brain and gonad were stored in -80°C until use.

A2.3.1. Visualization of RNA by formaldehyde denaturing gel electrophoresis

Integrity of the brain and gonad RNA samples was assessed by visualizing on formaldehyde denaturing gel electrophoresis. A 1.2% formaldehyde gel was prepared by adding 0.6g of agarose (Sigma-Aldrich, Castle Hill, Australia) to 50ml of 1X

formaldehyde (FA) buffer in a conical flask. The mixture was then microwaved on high for approximately 1 minute or until the mixture boiled and the agarose is fully dissolved and the mixture becomes crystal clear. The mixture was then cooled to ~60°C . Ethidium bromide and 0.9ml of 37% (12.3M) formaldehyde were added and the solution was poured into a gel plate and was left to solidify.

One volume of 5X RNA loading buffer was added to 4 volumes of RNA sample and RNA ladder. The RNA mixtures were incubated for 3 to 5 minutes at 65°C in a heat block then chilled on ice before being loaded onto the formaldehyde gel.

The gel was electrophoresed in 1X formaldehyde (FA) buffer at 60V for 1hour. For RNA visualization, the gel was placed onto a UV screen and photographed using GeneSnap image acquisition software (SYNGENE, Cambridge, UK).

A2.4.2.1.Visualization of amplified PCR products by Agarose Gel Electrophoresis

A 2% agarose gel was prepared by adding 2.4g of agarose (Sigma-Aldrich, Castle Hill, Australia) to 120ml of 0.6X TBE buffer (pH8.0, 100mM Tris HCL, 50mM EDTA, 100mM Boric acid) in a conical flask. The mixture was microwaved on high for approximately 1.5 minute or until the mixture was boiling and the agarose was fully dissolved and the solution became crystal clear. The mixture was cooled to ~60°C and ethidium bromide was added. The solution was then poured into gel plate and was left to solidify. 2µl of 1Kb plus DNA size marker (AXYGEN, Union City, CA USA, 1µg/µl) was loaded into the appropriate well(s) and a mixture of 1µl loading dye (bromophenol blue, Amersham Biosciences Australia) and 5µl PCR amplification product was loaded into separate wells. The gel was electrophoresed in 0.6X TBE buffer at 125V for 30 minutes. The gel was then placed onto a UV screen and photographed using GeneSnap image acquisition software (SYNGENE, Cambridge, UK).

A2.4.2.2. Purification of PCR products

Before PCR products can be cloned they must be purified to ensure that only the PCR product of interest is cloned (i.e. primer dimers and other non-specific products are not ligated into the vector). DNA purification also rids the product of other contaminants that may interfere with reactions at a later stage of processing. Gel purification of PCR products from section 2.4.2. was achieved by using the QIAquick PCR purification columns (Qiagen, Victoria, Australia) according to the manufacturer's

instructions
(http://francois.schweisguth.free.fr/protocols/QIAquick_PCR_Purification_Kit.pdf).

A2.4.2.3. Ligation, transformation and cloning of PCR products

The purified PCR product (3µl: 25-50ng) was ligated into the pGEM®-T easy vector (Promega, New South Wales, Australia), according to manufacturer's instructions. An aliquot of 3 µl (20-40ng) of the ligation reaction was used to transform JM109 high efficiency competent cells (Promega, New South Wales, Australia). The cells were then plated onto a nutrient enriched medium which also contained 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-gal) and isopropyl-beta-D-thiogalactopyranoside (IPTG) for color selection of transformants. The plates were incubated at 37°C overnight. White transformed colonies were selected for further analysis. A DNA positive control that is known to ligate efficiently was supplied by Promega (Promega, New South Wales, Australia) and was included in the transformation.

A2.4.2.4. Colony PCR

Colony PCR was performed on randomly selected white colonies using gene-specific primer pairs (Table 1). The template used was a scraping of one fresh white colony per reaction tube. The PCR reaction mix and the cycling conditions were the same as previously described in section 2.4.2. The PCR products were visualized as described in section A2.4.2.1. Colonies that contained the clone corresponding to the correct size PCR product were re-picked and grown overnight at 37°C in a 200µl liquid broth culture (with shaking at 250 rpm) containing 2µl ampicillin (50ug/ml).

A2.4.2.5. Plasmid DNA purification

Plasmid DNA was purified using Gene JET™ Plasmid Minipreps Kit DNA purification system (Fermentas Life Science, Quantum Scientific, Muranie Qld, Australia, Catalogue # K0502) as per manufacturer's instruction. Concentration of purified plasmid was determined by spectrophotometer (Genequant ProTM, Biochrom, Cambridge, UK) as described in section A2.3., except that DNA was used instead of RNA.

A2.4.2.6. Sequencing

The purified plasmids from multiple colonies were prepared for sequencing as specified by the Australian Genome Research Facility (AGRF, Brisbane, QLD, Australia). Plasmids were prepared by combining the following: 500ng plasmid DNA, 1µl of 6.4pmol M13 forward primer, nuclease-free water added up to final volume of 10µl. Sequences returned from AGRF were analysed to first remove vector sequences using Sequencher software (Version 4.8; Gene Codes Corporation, Ann Arbour, ME, USA). Sequence analysis involved visualization of sequence chromatograms through Sequencher^{TM} 4.8, checking that the nucleotides were positioned correctly, and ensuring that the sequences were flanked by forward and reverse primers. Basic Local Alignment Tool (BLAST) searches (http://ncbi.nml.nih.gov/BLAST/) were conducted on the sequences which were flanked by correct forward and reverse primers, to identify homologies with other available sequences. The nucleotide sequences were converted to amino acid sequence using ExPASy Proteomics tools (http://au.expasy.org/tools/). The deduced amino acids sequences were aligned with other existing sequences using Clustal W2 (http://www.ebi.ac.uk/Tools/clustalw2/).

A2.4.3. Isolation of GPR54 full length cDNA sequence using RACE (Rapid Amplification of cDNA Ends) PCR

To facilitate the PCR amplification of the full SBT and YTK cDNA sequences, gene-specific primers were designed from the partial cDNA sequence obtained from SBT and YTK, using Primer3 software (http://wi.mit.edu) (Table 1). The 5' end of the transcript was isolated with two rounds of 5'RACE-PCRs following the SMART^{TM} RACE PCR protocol (Clontech, Palo Alto, CA). The reverse primer, 5YSR2 (Table 1), was used for the first round of 5'RACE-PCR with the adapter primer, NUP. Components included in a 25µl reaction and thermal cycling conditions were as described in section 2.4.2. Semi-nested 5'RACE-PCR was carried out using 1µl of the primary PCR product as template. The second round of 5'RACE-PCR was performed using internal reverse primers 5SR1 and 5YR1 (Table1) for SBT and YTK respectively, and NUP. The reaction components and thermal cycling parameters are similar to those for the first round of 5'RACE-PCR.

Two rounds of 3'RACE-PCR were conducted to obtain the 3'-end of the cDNA. The primers for the first round of 3'RACE-PCR were forward primers, S54 3F1(b) and Y54 3F1(b) (Table 1) for SBT and YTK, respectively, for the primary reaction. The corresponding primers for the second round of 3'RACE-PCR were forward primers, S54 3F2(b) and Y543F2(b) (Table 1) for SBT and YTK, respectively. Products obtained from the 5' and 3' RACE-PCRs were purified, sub-cloned and sequenced following the same protocol described in sections A2.4.2.2. to A2.4.2.6.

A2.5. Degenerate primer design for isolation of reference gene, Hypoxanthine phosphoribosyltransferase 1, Hprt1, from SBT and YTK

Degenerate Hprt1 PCR primers were designed from the Hprt1 mRNA sequences of salmon *(Salmo salar* BT043501) and zebrafish (*Danio rerio,* EU047917). The Hprt1 DNA sequences for these species were aligned using Clustal W2 (http://www.ebi.ac.uk/Tools/clustalw2/) which enabled primer (F1-F2 R1-R2) (Table 9) designed from homologous sections of DNA.

In order to isolate the Hprt1 gene from both SBT and YTK, PCR was performed with all combinations of the primers F1, F2, and R1, R2, (Table 9) using cDNA from SBT and YTK gonadal tissues. The PCR cycling parameters and visualization were the same as described in sections A2.4 and A2.4.1. The isolated Hprt1 sequence was purified, cloned and sequenced following the same procedure described in sections A2.4.2.2. to A2.4.2.6. Hprt1 gene specific primers (Table 2) for RT-qPCR were designed using Primer3 software (http://wi.mit.edu).

Table 9
Degenerate primers used to isolate the SBT and YTK Hprt1 cDNA by RT-PCR.

Primer name	Direction	5' to 3' sequence	TM (°C)
F1	Forward	5'GAKGAGCARGGTTWTGACCTGG 3'	57
F2	Forward	5' ATGAAGGACATGGGKGGRCACC 3'	57
R1	Reverse	5' AAGTCTGGTMTGTAYCCAACYCTCCT 3'	60
R2	Reverse	5' TCGTTGTAGTCWAGYGCATATC 3'	53

Degenerate primers were designed from the conserved regions of salmon (*salmon salar,* BT043501*)* and zebrafish (*Danio rerio,* NM 212986) Hprt1 sequences available at the GenBank. A: Adenine, G: Guanie, T: Thymine, C: Cytosine. R: A/g Y: C/T M: A/C K: g/T S: g/C W: A/T H: A/C/T B: g/C/T V: A/g/C D: A/g/T N: A/g/C/T

A3.1. Isolation of GPR54 cDNA

A3.1.1. Isolation of SBT brain GPR54 cDNA

Initial PCR amplification using all the degenerate primer combinations (Table 1 F6 to R7) resulted in smeary products only. Semi-nested PCR using F6-R5 (lane 1) and F6-R6 (lane 2) were performed, using first round PCR products as template. From these reactions, single products were obtained (Fig. 5.1). The primer positions are shown in Fig. 5.10

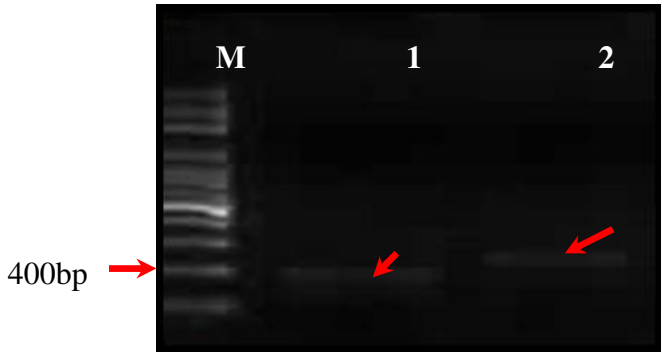


Figure 5.1: Semi-nested PCR amplification products using degenerate primer combinations F6-R5 and F6-R6, SBT GPR54. Arrows indicate products that were purified and sequenced. (M-1Kb DNA ladder).

The sequences of the two isolated fragments overlapped when aligned. The larger product was a 240 bp sequence encoding for 80 amino acid residues. BLAST analysis indicated that the sequences were GPR54.

Gradient PCR using gene-specific primers 3'SF1-3'R2 (Table 1; positions shown in Fig. 5.10), amplified the most intense single product at 56.7°C (Fig. 5.2, lane 6). Cloning and subsequent sequencing of this product yielded 824 bp partial SBT GPR54 cDNA, corresponding to 294 amino acid residues.

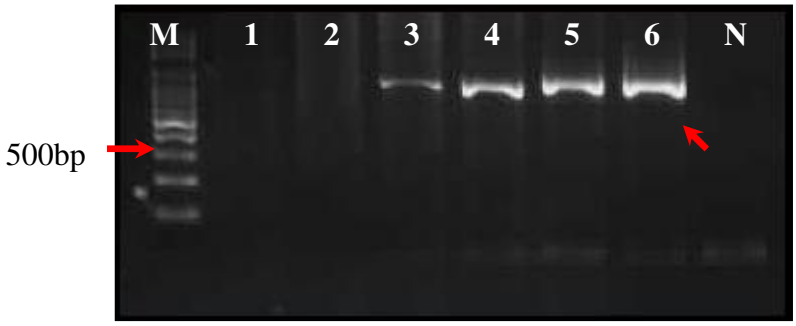


Figure 5.2: Gradient PCR (temperature range from 47°C to 57°C. Lane 1: 47°C, lane 2: 48.6°C, lane 3: 50.9°C, lane 4: 53.0°C, lane 5: 55.4°C, and lane 6: 56.7°C) amplification products using gene-specific primer combination 3'SF1-3'R2, SBT GPR54. Arrow indicates product purified and sequenced. (M-1Kb DNA ladder; N-Negative control).

For the isolation of the 5'end sequences of GPR54, primers 5'YSR2- NUP (Table 1; positions shown in Fig. 22) amplified a single product at 48.6°C (Fig. 5.3, lane2), using 5'RACE SBT brain cDNA as template.

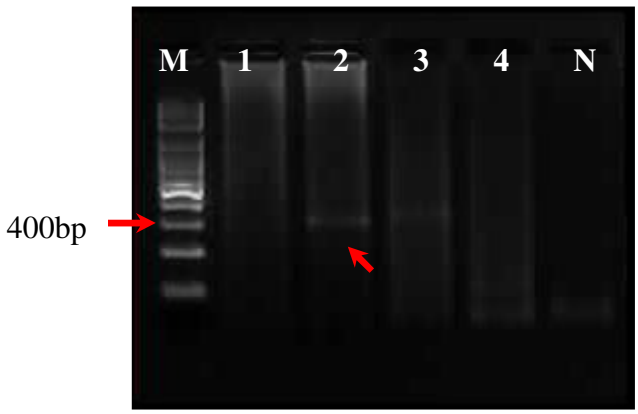


Figure 5.3: Gradient PCR (temperature range from 47°C to 57°C. Lane 1: 47.3°C, lane 2: 49.2°C, lane 3 at 52.5°C, and lane 4 at 56.2°C) amplification products using gene-specific and universal primers, 5'YSR2- NUP, SBT GPR54. Arrow indicates product used for semi-nested PCR reaction. (M-1Kb DNA ladder; N-Negative control).

Primers 5'SR1-NUP (Table 1; positions shown in Fig. 5.10) further amplified a single product at 48.6°C in a semi-nested PCR reaction (Fig. 5.4). Cloning and sequencing of this product revealed 276 bp sequence encoding the deduced 24 amino acid residues of the SBT GPR54 N-terminus plus the 5' untranslated region of the transcript.

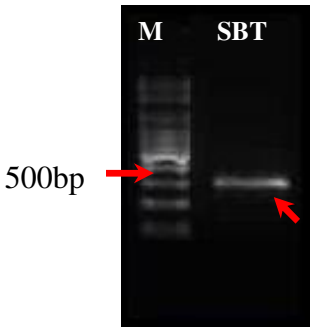


Figure 5.4: Semi-nested PCR amplification products amplified using gene-specific and universal primers, 5'SR1 and NUP, SBT GPR54. Arrow indicates sequenced product. (M-1Kb DNA ladder).

For the 3' end isolation, primers S54 3F1(b)-NUP (Table 1; positions are shown in Fig. 5.10) amplified two products using a 3'RACE brain cDNA as template (Fig. 5.5).

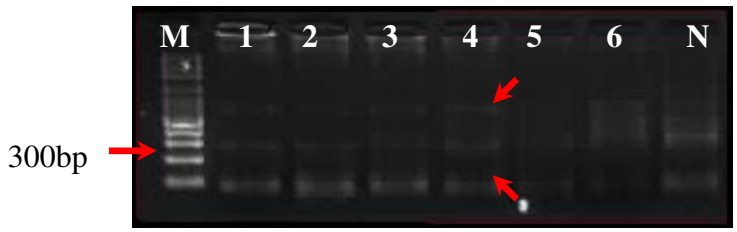


Figure 5.5: Gradient PCR (temperature range from 58°C to 68°C. Lane 1: 58°C, lane 2: 59.1°C, lane 3: 61.9°C, lane 4: 64.0°C, lane 5: 66.4°C, lane 6: 67.7°C, and lane 7: 68°C) amplification products using gene-specific and universal primers, S54 3F1(b)-NUP, SBT GPR54 Arrows indicate products used for semi-nested PCR reaction. (M-1Kb DNA ladder; N-Negative control).

Primers S54 3F2(b)-NUP (Table 1; positions are shown in Fig. 5.10) further amplified two products in a semi-nested PCR reaction (Fig. 5.6).

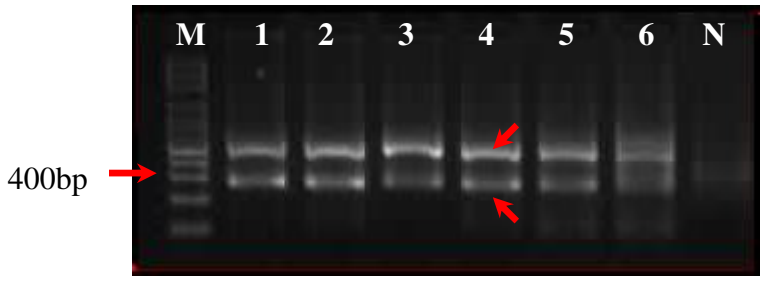


Figure 5.6: Gradient PCR (temperature range from 58°C to 68°C. Lane 1: 58°C, lane 2: 59.1°C, lane 3: 61.9°C, lane 4: 64.0°C, lane 5: 66.4°C, lane 6: 67.7°C, and lane 7: 68°C) amplification products using gene-specific and universal primers, S54 3F2(b)-NUP, SBT GPR54, in a semi-nested PCR reaction. Arrows indicate products purified and sequenced. (M-1Kb DNA ladder; N-Negative control).

A 455 bp SBT GPR54 3'end sequence was obtained from the larger isolated product. This longer product had a 238 bp 3'UTR. The shorter 244 bp SBT GPR54 3'end sequence, which was obtained from the shorter isolated product, had a 30 bp sequence after the stop codon that included a 20 bp poly A tail. The sequences of the two products, starting from the forward primer, matched.

A3.1.3. Isolation of YTK brain GPR54 cDNA.

The primers F6-R6 (Table 1) amplified a single product (Fig. 5.7; lane 5) while the primer combination F7 and R7 (Table 1) also yielded a single product in (Fig. 5.7; lane 6) using YTK brain cDNA as template. The primers positions are shown in Fig. 5.11.

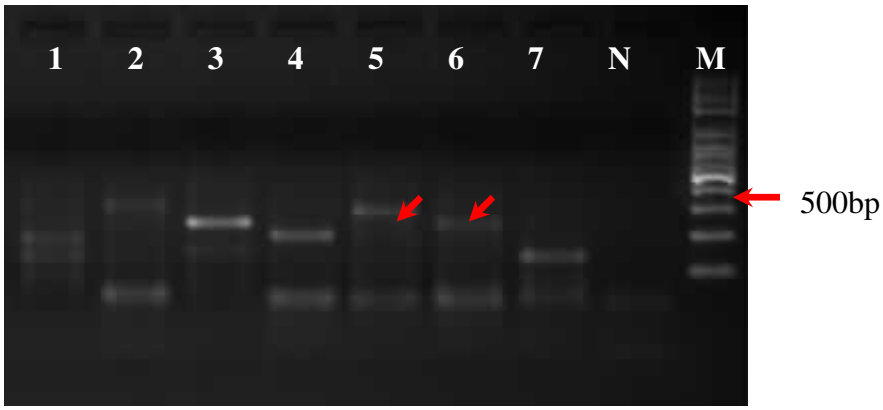


Figure 5.7: PCR amplification products using degenerate primer combinations F6-R6 and F7-R7, YTK GPR54. Arrows indicate products purified and sequenced. (M-1Kb DNA ladder; N-Negative control).

The sequences of the two isolated fragments overlapped when aligned. A 282 bp nucleotide sequences, corresponding to 94 amino acid residues, were obtained from the PCR products.

Primers 3'YF1-3'R2 (Table 1; positions are shown in Fig. 5.11) did not yield any visible product (Fig. 5.8). However, when a nested PCR reaction was performed, using internal primers 3'YS F2-3'R1 (Table 1; positions are shown in Fig. 5.11), a single product was amplified at 56.7°C (Fig. 5.9). Cloning and sequencing of this product revealed 537 bp, corresponding to179 amino acid residues, of the YTK GPR54.

Figure 5.8: Gradient PCR (temperature range: 47°C to 57°C. Lane 1: 47°C, lane 2: 48.6°C, lane 3: 50.9°C, lane 4: 53.0°C, lane 5: 55.4°C, and lane 6: 56.7°C) amplification products using primers 3'YF1-3'R2, YTK GPR54. (M-1Kb DNA ladder; N-Negative control). Arrow indicates product used in nested PCR reaction.

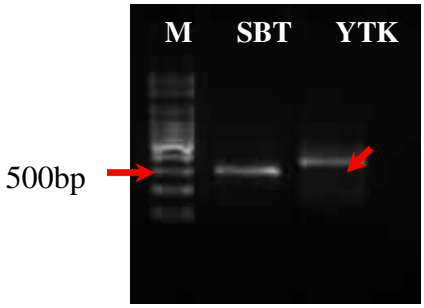


Figure 5.9: Nested PCR amplification products using primers 3'YS F2-3'R1, YTK GPR54. Arrow indicates product purified and sequenced. (M-1Kb DNA ladder).

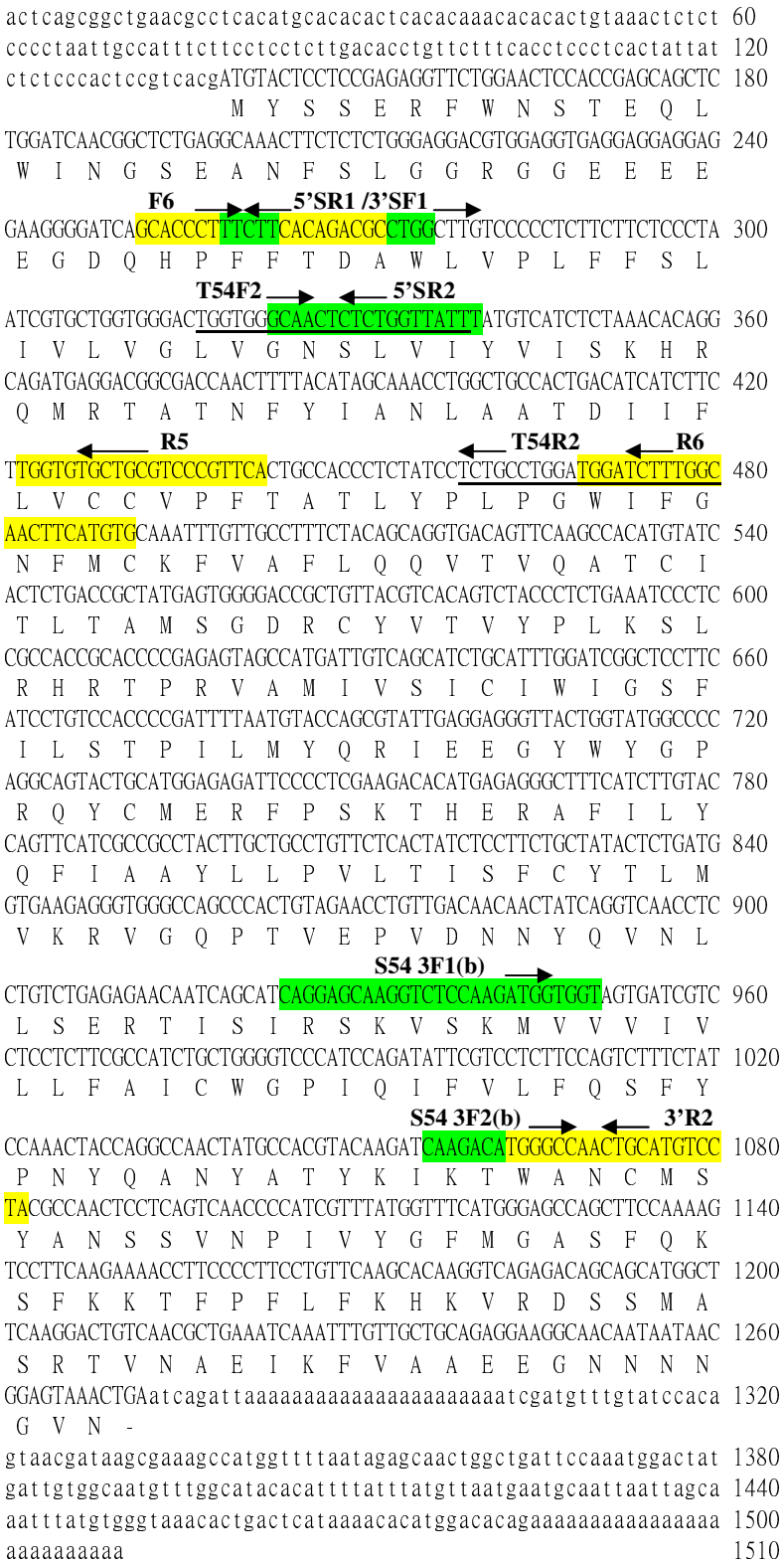


Figure 5.10: Nucleotide and deduced amino acid sequence of SBT brain GPR54 cDNA. The sequence contains an open reading frame of 1134bp encoding a predicted 378 amino acid peptide, a 138bp 5'UTR and 238 3'UTR. Numbers indicate the nucleotide position. The 5' and 3' UTR nucleotide sequences are in

lower case while the predicted coding nucleotide sequence from the start codon to the stop codon is in upper case. Positions of degenerate and gene-specific primers used to isolate the SBT GPR54 cDNA by RT-PCR and RACE RT-PCR are marked yellow and green, respectively. Gene specific-primers designed for RT-qPCR are underlined. → denotes a forward direction primer and ← denotes a reverse direction primer.

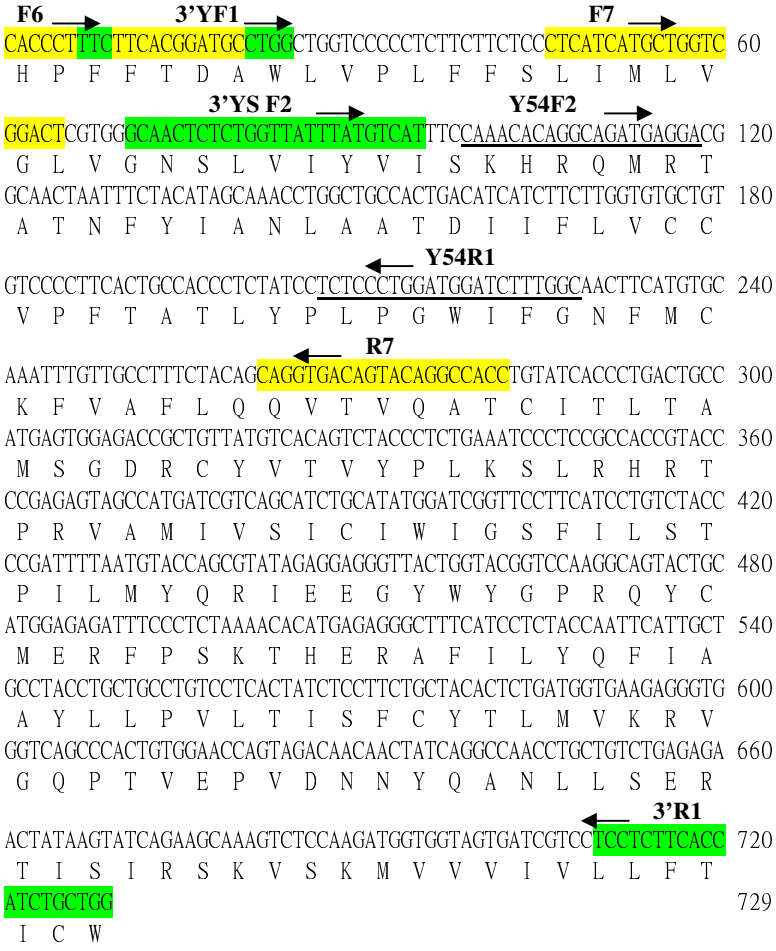


Figure 5.11: Partial nucleotide (729 bp) and deduced amino acid (243a.a) sequences of YTK brain GPR54 cDNA. Numbers indicate the nucleotide position. Positions of degenerate and gene-specific primers used to isolate the YTK GPR54 cDNA by RT-qPCR are marked yellow and green, respectively. Gene-specific primers designed for RT-qPCR are underlined. → denotes a forward direction primer and ← denotes a reverse direction primer.

```
SBT                  METYSSERFWNSTEQL---WINGSEANFSLGGR---GGEEEEEGDQHPFFTDAWLVPLFF 54
YTK                  ---------------------------------------------HPFFTDAWLVPLFF 14
Grey_Mullet          --MHSSEEPWNSSEHV---WVNGSEANFSLGRRRVDEEKEEEEGGQHPFLTDAWLVPLFF 55
Zebrafish_GPR54a     --MFSGED-WNSSE-----LLNGSFRNSS---------MEDSEDGEHPFLTDAWLVPLFF 43
Bullfrog             --MALGEGMLDEMLNLSWEYVNGSFLGNASRIN--ESLVNRTETGPPPFLTDAWLVPLFF 56
Zebrafish_GPR54b     --MAETNSTGDAAEHIMCNYDANIYQCNQ---------TDLMRFQSPVPLTDTWLVPLFF 49
Human                --MHTVATSGPNASWGAPANASGCPGCGAN---------ASDGPVPSPRAVDAWLVPLFF 49
Rat                  --MAAEATLGPNVSWWAPSNASGCPGCGVN---------ASDGPGSAPRPLDAWLVPLFF 49
                                                                   *:*******

                       TM I                            TM II
SBT                  SLIM--LVGLVGNSLVIYVISKHRQMETRTATNFYIANLAATDIIFLVCCVPFTATLYPL 112
YTK                  SLIMETLVGLVGNSLVIYVISKHRQMETRTATNFYIANLAATDIIFLVCCVPFTATLYPL 74
Grey_Mullet          SLIM--LVGLVGNSLVIYVISKHRQMRT--ATNFYIANLAATDIIFLVCCVPFTATLYPL 111
Zebrafish_GPR54a     SLIM--LVGLIGNSLVIYVISKHRQMRT--ATNFYIANLAATDIIFLLCCVPFTATLYPL 99
Bullfrog             ALIM--LVGLIGNSLVIYVISKHRQMRT--ATNFYIANLASTDIMFLVCCVPFTATLYPL 112
Zebrafish_GPR54b     TLIM--FVGLVGNLIVIYVVIKNQQMKT--VTNLYIVNLATTDILFLVCCVPFTATVYVL 105
Human                AALM--LLGLVGNSLVIYVICRHKPMRT--VTNFYIANLAATDVTFLLCCVPFTALLYPL 105
Rat                  AALM--LLGLVGNSLVIFVICRHKHMQT--VTNFYIANLAATDVTFLLCCVPFTALLYPL 105
                     : :*   ::**:** :**:*: ::: *.*  .**:**.***:**: **:******* :* *

                                  TM III
SBT                  PGWIFGNFMETCKFVAFLQQVTVQATCITLTAMETSGDRCYVTVYPLKSLRHRTPRVAME 172
YTK                  PGWIFGNFMETCKFVAFLQQVTVQATCITLTAMETSGDRCYVTVYPLKSLRHRTPRVAME 134
Grey_Mullet          PGWIFGNFM--CKFVAFLQQVTVQATCITLTAMS--GDRCYVTVYPLKSLRHRTPKVAM- 166
Zebrafish_GPR54a     PGWIFGDFM--CKFVAFLQQVTVQATCITLTAMS--GDRCYVTVYPLKSLHHRTPRVAM- 154
Bullfrog             PSWVFGDFM--CKFVAYLQQVTVQATCITLTAMS--ADRCYATLYPLRSLRHRTPRVAM- 167
Zebrafish_GPR54b     PSWIFGDFM--CRLVNYLQQVTAQATCITLSAMS--VDRFYVTVYPLQSLHHRTPQMALS 161
Human                PGWVLGDFM--CKFVNYIQQVSVQATCATLTAMS--VDRWYVTVFPLRALHRRTPRLALA 161
Rat                  PTWVLGDFM--CKFVNYIQQVSVQATCATLTAMS--VDRWYVTVFPLRALHRRTPRLALT 161
                     * *::*:**  *::* ::***:.**** **:**.    ** *.*::**::*::***::*:

                        TM IV
SBT                  TIVSICIWIGSFILSTPILMETYQRIEEGYWYGPRQYCMETERFPSKTHERAFILYQFIA 232
YTK                  TIVSICIWIGSFILSTPILMETYQRIEEGYWYGPRQYCMETERFPSKTHERAFILYQFIA 194
Grey_Mullet          -IVSVCIWIGSFILSTPILM--YQRIEEGYWYGPRQYCME--RFPSKTHERAFILYQFIA 221
Zebrafish_GPR54a     -IVSICIWIGSFILSIPIFL--YQRLEDGYWYGPRKYCME--RFPSKTHEKAFILYQFIA 209
Bullfrog             -IVSMCIWIGSLLLSTPIIP--YQKIQKGYWYGPRTYCIE--QFPTDTMKKACILYQFLA 222
Zebrafish_GPR54b     --VCTTIWICSSLLSVPIAL--YQHTESSYWFGPQTYCTE--TFPSVIHKRVYLLYSFLA 215
Human                --VSLSIWVGSAAVSAPVLA--LHRLSP----GPRAYCSE--AFPSRALERAFALYNLLA 211
Rat                  --VSLSIWVGSAAVSAPVLA--LHRLSP----GPHTYCSE--AFPSRALERAFALYNLLA 211
                       *.  **: *  :* *:      ::  .      **: ** *   **:    ::.  **.::*

                      TM V
SBT                  AYLLPVLTISFCYTLMETVKRVGQPTVEPVDNNYQVNLLS--ERTISIRSKVSKMETVVV 290
YTK                  AYLLPVLTISFCYTLMETVKRVGQPTVEPVDNNYQANLLS--ERTISIRSKVSKMETVVV 252
Grey_Mullet          AYLLPVLTITFCYTLM--VKRVGQPTVEPVDNNYQVNLLS--ERTISIRSKVSKM--VVV 275
Zebrafish_GPR54a     VYLLPVITISFCYSFM--LKRVGQASVEPVDNNHQVHLLS--ERTISIRSKISKM--VVV 263
Bullfrog             VYLLPLLTICLCYSLM--LKRVGRPVVEPMDNNYQVQLLS--ERTIAMRSKISKM--VIV 276
Zebrafish_GPR54b     VYLLPLITICMCYTFM--LKRMAQATVQPVQGCNQISLQTSSERAEAVRSRVSRM--VVV 271
Human                LYLLPLLATCACYAAM--LRHLGRVAVRPAPADSALQGQVLAERAGAVRAKVSRL--VAA 267
Rat                  LYLLPLLATCACYGAM--LRHLGRAAVRPAPTDGALQGQLLAQRAGAVRTKVSRL--VAA 267
                      ****:::   **  *  :::::.:  *.*                :*: ::*:::*::  * .

                      TM VI                                     TM VII
SBT                  IVLLFAICWGPIQIFVLFQSFYPN---YQANYATYKIKTWANCMETSYANSSVNPIVYGF 347
YTK                  IVLLFTICW--------------------------------------------------- 261
Grey_Mullet          IVLLFAVCWGPIQIFVLFQSFYPN---YQPNYATYKIKTWANCMS--YANSSVNPIVYGF 330
Zebrafish_GPR54a     IVVLFTICWGPIQIFVLFQSFYPN---FKANYATYKIKTWANCMS--YANSSINPIVYGF 318
Bullfrog             IVLLFTICWGPIQLFSLFQGFYPG---FQANYATYKIKTWANCMS--YANSSINPIVYGF 331
Zebrafish_GPR54b     MVLLFLLCWGPIQILILLQAFCAED--VSRSYTLYKLKIWAHGMS--YSNSSINPVIYAF 327
Human                VVLLFAACWGPIQLFLVLQALGPAGSWHPRSYAAYALKTWAHCMS--YSNSALNPLLYAF 325
Rat                  VVLLFAACWGPIQLFLVLQAL-PLGGLAPSKLCAYALKIWAHCMS--YSNSALNPLLYAF 324
                     :*:**   **
```

```
SBT                 METGASFQKSFKKTFPFLFKH-KVRDSSMETASRTVNAEIKFVAAEEGNNNNGVN----- 401
YTK                 ------------------------------------------------------------
Grey_Mullet         MG--ASFQKSFRKTFPFLFKH-KVRDSSMAS--RTANAEIKFVAAEEGNNNNAVN----- 380
Zebrafish_GPR54a    MG--ASFRKSFRKTFPFLFRH-KVRDSSVAS--RTANAEIKL----------------- 355
Bullfrog            MG--ASFRKSFKKAFPFMFRN-KVRDGSITS--GTVNNEMKFVAMESTNNELK------- 379
Zebrafish_GPR54b    MG--ANFRKAFRSVCPLIFKR-RSTEP-----LATYNREMNFLSS--------------- 364
Human               LG--SHFRQAFRRVCPCAPRRPRRPRRPGPSDPAAPHAELLRLGSHPAPARAQKPGSSGL 383
Rat                 LG--SHFRQAFCRVCPCGPQRQRRPHASAHSDRAAPHSVPHSRAAHPVRVRTPEPGNP-- 380

SBT                 ---------------
YTK                 ---------------
Grey_Mullet         ---------------
Zebrafish_GPR54a    ---------------
Bullfrog            ---------------
Zebrafish_GPR54b    ---------------
Human               AARGLCVLGEDNAPL 398
Rat                 VVHSPSVQDEHTAPL 395
```

Figure 5.12: Alignment of the deduced amino acid sequences of the SBT and YTK GPR54 cDNA sequence with those of mullet (GenBank Accession No. DQ683737), zebrafish-GPR54 1 (EU047917), zebrafish-GPR54 2 (EU047918), bullfrog (EU681171), human (NM 032551), and rat (NM 023992). The predicted TMDs are marked green. The number on the right represents the number of amino acid; "*" denote conserved amino acid residues; ":" denote conserved substitutions between related amino acid family; "." denote semi-conserved substitution. Sequences were aligned by Clustal W2 (http://www.ebi.ac.uk/Tools/clustalw2/).

Fig. 5.12 shows the homology of the deduced amino acid residues of SBT and YTK GPR54 to other vertebrate GPR54 cDNAs. The SBT GPR54 shared more than 90% homology with YTK, 79-89% homology to zebrafish GPR54-1 and mullet GPR54, 37% homology to zebrafish GPR54-2, 76% homology to bullfrog GPR54 and 70-71% homology to its rat and human homologues. Analysis of the predicted amino acid sequences of SBT GPR54 revealed seven putative transmembrane (TM) domains. TM1-7 showed 82-100% identity to other fish species. Comparisons with the TM domain of amphibian, GPR54 revealed 71-93% identity in TM1-7. SBT GPR54 TM1-7 shared only 43-86% homology with mammalian GPR54.

A3.4. RT-qPCR validation assays

A3.4.1. Isolation of SBT and YTK partial Hprt1 cDNA sequences

In order to develop a real-time quantitative PCR (RT-qPCR) for the determination of relative expression levels of GPR54 in SBT and YTK, partial SBT and YTK Hprt1 cDNA sequences of the reference gene, Hprt1, were isolated.

For SBT, primers F1-R1 and F1-R2 (Table 9) amplified a single product, using SBT gonad cDNA as template (lane 1 and 3, respectively; Fig. 5.13). The primer positions are shown in Fig. 5.21.

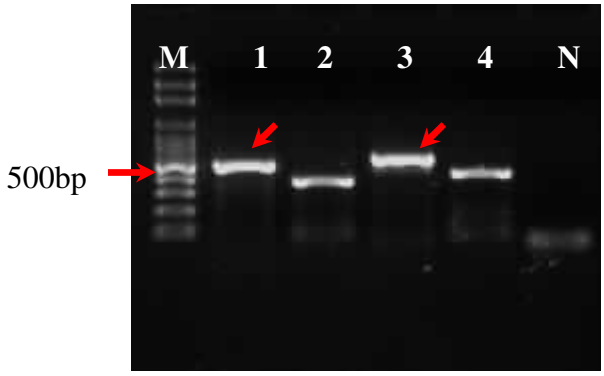


Figure 5.13: PCR amplification products using degenerate primers F1-R1 and F2-R1, SBT Hprt1. Arrows indicate products purified and sequenced. (M-1Kb DNA marker; N-Negative control).

The sequences of the two isolated fragments overlapped when aligned. A 555 bp of partial SBT Hprt1 cDNA sequences were obtained from these PCR products.

Partial YTK Hprt1 cDNA sequence (555 bp) was amplified with primers F1-R2 (Table 9; positions are shown in Fig. 5.22), using YTK gonad cDNA as template (Fig. 5.14, lane 3).

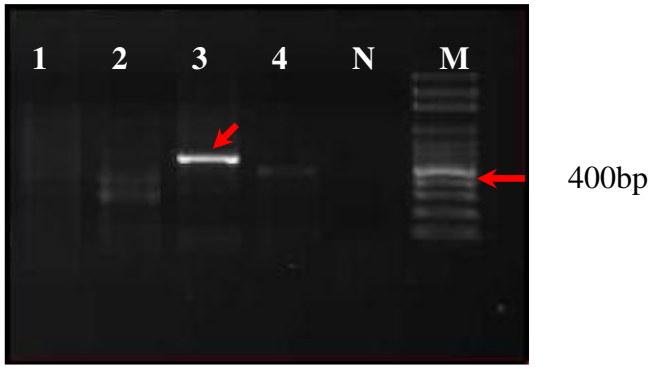


Figure 5.14: PCR amplification products using degenerate primers F1-R2, YTK Hprt1. Arrow indicates product purified and sequenced. (M-1Kb DNA marker; N-Negative control).

A3.4.2. SBT and YTK GPR54 and Hprt1 RT-qPCR standard curves

The gene-specific primers used for the RT-qPCR amplification of target and reference genes are shown in Table 3. The primers, tested on plasmid clones of the genes, showed product specificity (Figs 5.15-5.18).

For SBT target gene, GPR54, primers T54F2-T54R2 yielded a single product with expected size of 164 bp (Fig. 5.15). For the reference gene, Hprt1, primers ThprtF1-ThprtR1 amplified a single product of 196 bp (Fig. 5.16).

For YTK GPR54 gene, primers Y54F2-Y54R1 yielded a single product with expected size of 130 bp (Fig. 5.17). Primers YhprtF2-YhprtR2 amplified a single product with 144 bp in length for the Hprt1 cDNA (Fig. 5.18).

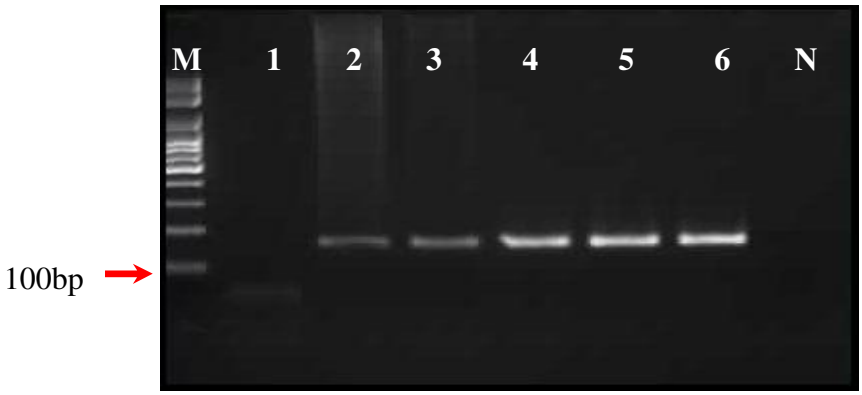


Figure 5.15: Gradient PCR (temperature range from 47°C to 63.5°C. Lane 1: 47°C, lane 2: 48.9°C , lane 3: 53.6°C , lane 4: 57.2°C, lane 5: 61.3°C, and lane 6: 63.5°C). SBT GPR54 target amplification products (164 bp) using RT-qPCR gene-specific primers T54F2-T54R2 are shown in lanes 1-6. (M-1Kb DNA marker; N-Negative control).

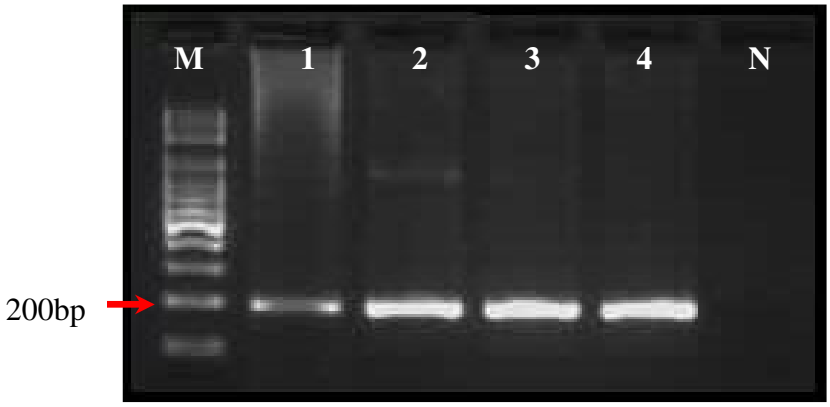


Figure 5.16: Gradient PCR (temperature range from 49.8°C to 64°C. Lane 1: 49.8° C, lane 2: 55.5°C , lane 3: 60.8°C , and lane 4: 64°C). SBT Hprt1 target amplification products (196 bp) using RT-qPCR gene-specific primers ThprtF1-ThprtR1 in lanes 1-4. (M-1Kb DNA marker; N-Negative control).

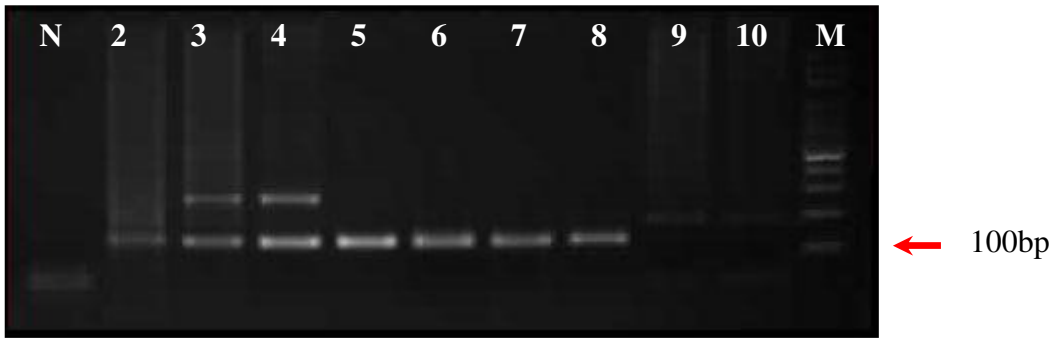


Figure 5.17: Gradient PCR (temperature range from 47°C to 64°C. Lane 1: 47°C, lane 2: 48.9°C , lane 3: 53.6°C , lane 4: 57.2°C, lane 5: 61.3°C, and lane 6: 63.5°C). YTK GPR54 target amplification products (130 bp) using RT-qPCR gene-specific primers Y54F2-Y54R1 are shown in lanes 2-8. (M-1Kb DNA marker; N-Negative control).

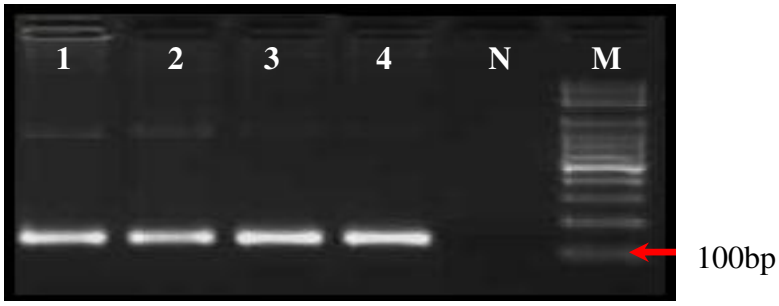


Figure 5.18: Gradient PCR (temperature range from 49.8°C to 64°C, Lane 1: 49.8° C, lane 2: 55.5°C , lane 3: 60.8°C , and lane 4: 64°C). YTK Hprt1 target amplification products (144 bp) using RT-qPCR gene-specific primers YhprtF2-YhprtR2 are shown in lanes 1-4. (M-1Kb DNA marker; N-Negative control).

A3.4.3. Agarose gel electrophoresis of RT-qPCR products

RT-qPCR amplification products from brain and gonad templates from both SBT and YTK were visualized on ethidium bromide stained agarose gel (Figs 5.19-5.20). Within each fish species, the target gene, GPR54 and the reference gene, Hprt1 were amplified from brain and gonad templates using the species-specific primer pairs (Table 2) during qRT-PCR.

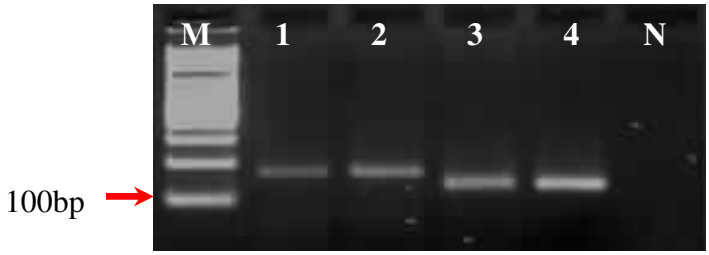


Figure 5.19: RT-qPCR amplification of target gene, GPR54. Lane 1: SBT brain (164 bp); lane 2: SBT gonad (164 bp); lane 3: YTK brain (130 bp); lane 4: YTK gonad (130 bp). (M-1Kb DNA ladder; N-Negative control).

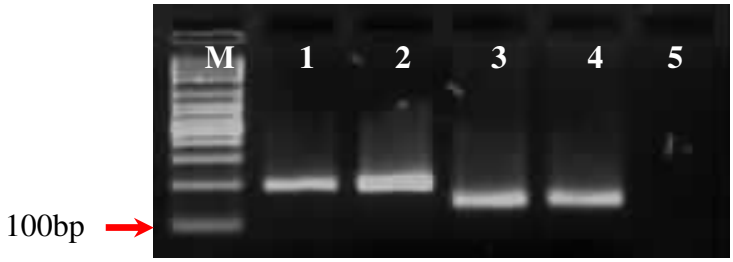


Figure 5.20: RT-qPCR amplification of reference gene, Hprt1, Lane 1: SBT brain (196 bp); lane 2: SBT gonad (196 bp); lane 3: YTK brain (144 bp); lane 4: YTK gonad (144 bp). (M-1Kb DNA ladder; N-Negative control).

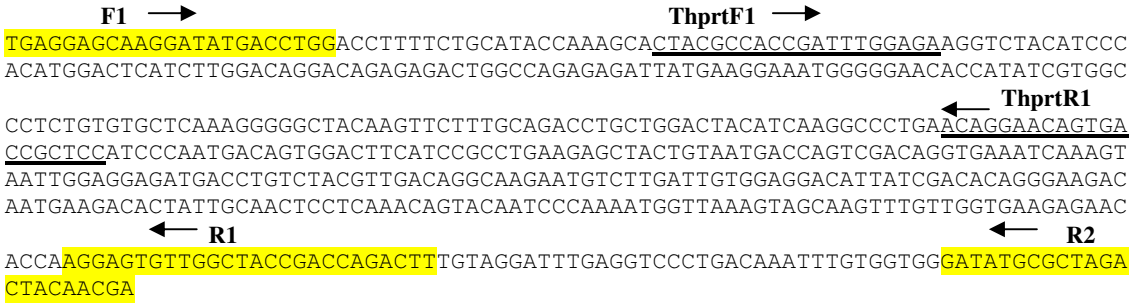


Figure 5.21: Isolated partial SBT Hprt1 cDNA sequence (555 bp) and primers positions. Positions of degenerate primers are marked in yellow. Gene-specific primers designed for RT-qPCR are underlined. → denotes a forward direction primer and ← denotes a reverse direction primer.

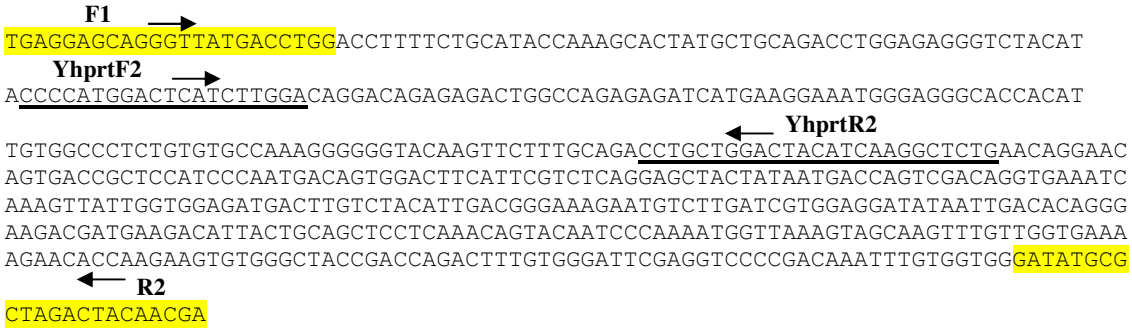


Figure 5.22: Isolated partial YTK Hprt1 cDNA sequence (555 bp) and primers positions. Positions of degenerate primers are marked in yellow. Gene-specific primers designed for RT-qPCR are underlined. → denotes a forward direction primer and ← denotes a reverse direction primer.

A 3.7. Statistical analysis of YTK brain and gonad expression relative to the experimental variables

A frequency plot using descriptive statistical analysis (Fig. 5.23) including tissue types, genders, sampling points and fish groups suggested that the brain and gonad GPR54 expression levels data were not normally distributed.

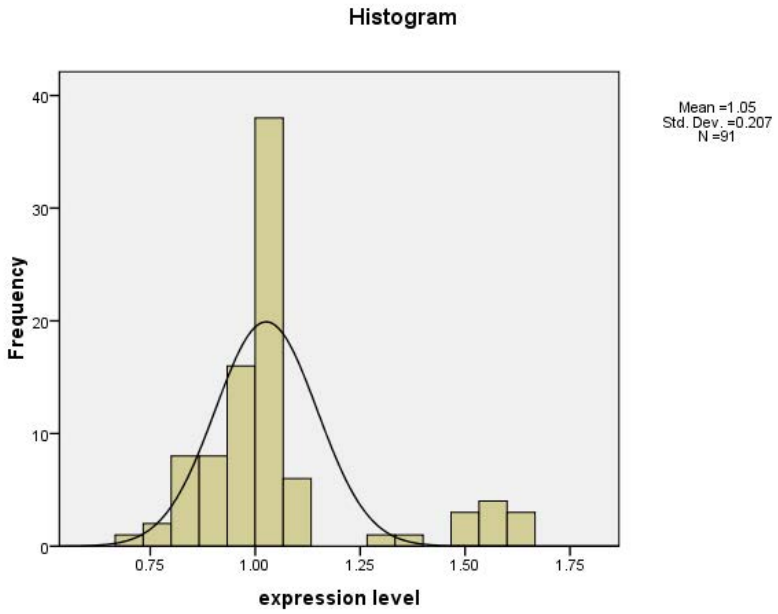


Figure 5.23: Histogram of GPR54 expression levels from all YTK collected samples.

The distribution of data was tested for normality by Kolmogorov-Smirnov test, which confirmed that the distribution was not normal (K-S statistic= 0.292 [91] P<0.001). The distribution of GPR54 expression level was statistically significantly skewed (skewness = 1.648 ± 0.253, P<0.001) and significantly kurtotic (kurtosis = 2.317 ± 0.500, P<0.001). Natural log and $\log_{10}$ transformations of GPR54 expression level failed to normalize the data. Therefore Kruskal-Wallis, K-independent non-parametric test was applied. The YTK brain and gonad relative GPR54 expression levels, raw GPR54 Cq and Hprt1 Cq, between sampling points, developmental stage (immature vs. pubertal) and sex were tested. The results are presented in Tables 10. the table shows that some of the results obtained by non-parametric analysis of the raw data (not normalised against the reference gene) are the same as the relative expression levels, while others differ significantly (P<0.05).

Table 10. Summary of statistical analysis of sampling data for RT-qPCR

	P *V.S.* I		P *V.S.* I		P *V.S.* I		P *V.S.* I		P *V.S.* I		P *V.S.* I	
	Brain Hprt1 Cq		Gonad Hprt1 Cq		Brain GPR54 Cq		Gonad GPR54 Cq		Brain GPR54 relative expression		Gonad GPR54 relative expression	
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
S1	P=0.006*	P=0.715	P=0.150	P=0.004*	P=0.006*	P=0.715	P=0.262	P=0.004*	P=0.770	P=0.222	P=0.004*	P=0.042*
S2	P=0.810	P=0.715	P=0.144	P=1.000	P=0.749	P=0.465	P=0.144	P=0.873	P=0.004*	P=0.006*	P=0.006*	P=0.285

	S1 *V.S.* S2		S1 *V.S.* S2		S1 *V.S.* S2		S1 *V.S.* S2		S1 *V.S.* S2		S1 *V.S.* S2	
	Brain Hprt1 Cq		Gonad Hprt1 Cq		Brain GPR54 Cq		Gonad GPR54 Cq		Brain GPR54 relative expression		Gonad GPR54 relative expression	
	P	I	P	I	P	I	P	I	P	I	P	I
♂	P=0.004*	P=0.631	P=0.262	P=0.361	P=0.006*	P=0.631	P=0.337	P=0.465	P=0.004*	P=0.686	P=0.004*	P=0.006*
♀	P=0.273	P=0.361	P=0.016*	P=0.873	P=0.273	P=0.361	P=0.016*	P=0.873	P=0.006*	P=0.018*	P=0.053	P=0.746

	♂ *V.S.* ♀		♂ *V.S.* ♀		♂ *V.S.* ♀		♂ *V.S.* ♀		♂ *V.S.* ♀		♂ *V.S.* ♀	
	Brain Hprt1 Cq		Gonad Hprt1 Cq		Brain GPR54 Cq		Gonad GPR54 Cq		Brain GPR54 relative expression		Gonad GPR54 relative expression	
	P	I	P	I	P	I	P	I	P	I	P	I
S1	P=0.337	P=0.100	P=0.004*	P=0.150	P=0.337	P=0.100	P=0.004*	P=0.150	P=0.145	P=0.081	P=0.037*	P=0.157
S2	P=0.361	P=0.522	P=0.262	P=0.068	P=0.361	P=0.631	P=0.200	P=0.144	P=1.000	P=0.920	P=0.003*	P=0.044*

* Statistically significant. Differences were accepted as significant at P<0.05.
P: Pubertal; I: Immature
S1: Sampling 1 (Dec 08); S2: Sampling 2 (Jan 09)
♂: Male; ♀: Female

Table 11. Average crossing point (Cq) of reference gene, Hprt1, in the YTK brain (mean ± SD).

	Pubertal Fish Group		**Immature Fish Group**	
	Male (n=6)	**Female (n=6)**	**Male (n=6)**	**Female (n=6)**
Sampling 1 (Dec 08)	30.13±0.77	30.66±1.07	34.45±0.52	31.62±1.39
Sampling 2 (Jan 09)	34.19±0.30	32.62±1.12	33.79±0.88	33.26±0.79

Table 12. Average crossing point (Cq) of reference gene, Hprt1, in the YTK gonad (mean ± SD).

	Pubertal Fish Group		**Immature Fish Group**	
	Male (n=6)	**Female (n=6)**	**Male (n=6)**	**Female (n=6)**
Sampling 1 (Dec 08)	22.97±0.70	18.19±0.59	24.81±1.07	23.92±2.07
Sampling 2 (Jan 09)	23.97±0.56	22.44±1.25	25.48±0.87	22.48±1.41

Tables 11 and 12 present the raw Cq data for YTK brain and gonad. These data are also presented and discussed in the manuscript (Fig. 3.8).

Future work:

Further work is required in order to obtain a more comprehensive understanding of the role of GPR 54 in the puberty onset of YTK and SBT. The following are some of the subsequent research that need to be carried out.

1. A number of alternative reference genes should be tested and the samples reanalysed using an appropriate reference gene, which does not change significantly in response to the experimental conditions and developmental stage. Likely candidates are RPS4, UBQ, and eEF1A1.
2. Cloning the GPR54 transcript from the gonads of YTK and SBT. During this study only the brain GPR54 cDNA was isolated. There is a precedent in Senegalese Sole (Mechaly *et al*., 2008) that gonad contains two types of GPR54, one of which displays intron retention that resulted from alternative splicing. Other species, such as zebrafish (Biran *et al*., 2008) displays two different GPR54 transcripts, which are encoded by two genes. The gonadal GPR54 in YTK and SBT should be examined in order to establish whether GPR54 isoforms or multiple genes are present in these species.
3. Examining the expression profile of GPR54 across more time points, especially

covering the time when the fish enter puberty. This would contribute to elucidating the role of GPR54 in puberty onset in YTK and SBT.

5.1 Appendix 2

Instruction to authors:

Aquaculture

Guide for Authors

An International Journal

Statistics
Impact Factor: 1.735
Issues per year: 48

INTRODUCTION
Types of paper - Contact details for submission - Page charges
BEFORE YOU BEGIN
Ethics in Publishing - Policy and ethics - Conflict of interest - Submission declaration - Contributors - Copyright - Retained author rights - Role of the funding source - Funding body agreements and policies - Language and language services - Submission - - Referees

PREPARATION
Use of wordprocessing software - LaTeX - Article structure - Subdivision - numbered sections - Introduction - Material and methods - Theory/calculation - Results - Discussion - Conclusions - Appendices - Vitae - Essential title page information - Abstract - - Keywords - Classification codes - Abbreviations - Acknowledgements - Nomenclature and units - - Accession numbers - - Math formulae - - Footnotes - Electronic artwork - Color artwork - Figure captions - Text graphics - Tables - References - Citation in text - Web references - References in a special issue - Reference style - Journal Abbreviations Source - Supplementary material - Submission checklist

AFTER ACCEPTANCE
Use of the Digital Object Identifier - Proofs - Offprints
AUTHOR INQUIRIES

INTRODUCTION

Types of paper

Original Research Papers should report the results of original research. The material should not have been previously published elsewhere, except in a preliminary form. *Review Articles* can cover either narrow disciplinary subjects or broad issues requiring interdisciplinary discussion. They should provide objective critical evaluation of a defined subject. Reviews should not consist solely of a summary of published data. Evaluation of the quality of existing data, the status of knowledge, and the research required to advance knowledge of the subject are essential.
Short Communications are used to communicate results which represent a major

breakthrough or startling new discovery and which should therefore be published quickly. They should not be used for preliminary results. Papers must contain sufficient data to establish that the research has achieved reliable and significant results.
Technical Papers should present new methods and procedures for either research methodology or culture-related techniques.
The *Letters to the Editor* section is intended to provide a forum for discussion of aquacultural science emanating from material published in the journal.

Contact details for submission

Papers for consideration should be submitted via the electronic submission system mentioned below to the appropriate Section Editor:

Nutrition:
D.M. Gatlin

Husbandry and Management:
B.Costa-Pierce

Physiology and Endocrinology:
E.M. Donaldson

Diseases:
P.R. Smith

Genetics:
G. Hulata

Page charges

This journal has no page charges.

BEFORE YOU BEGIN

Ethics in Publishing

For information on Ethics in Publishing and Ethical guidelines for journal publication see http://www.elsevier.com/publishingethics and http://www.elsevier.com/ethicalguidelines.

Policy and ethics

The work described in your article must have been carried out in accordance with *The Code of Ethics of the World Medical Association* (Declaration of Helsinki) for animal experiments http://europa.eu.int/scadplus/leg/en/s23000.htm; *Uniform Requirements for manuscripts submitted to Biomedical journals*

http://www.nejm.org/general/text/requirements/1.htm. This must be stated at an appropriate point in the article.

Conflict of interest

All authors are requested to disclose any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the submitted work that could inappropriately influence, or be perceived to influence, their work. See also http://www.elsevier.com/conflictsofinterest.

Submission declaration

Submission of an article implies that the work described has not been published previously (except in the form of an abstract or as part of a published lecture or academic thesis), that it is not under consideration for publication elsewhere, that its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere in the same form, in English or in any other language, without the written consent of the copyright-holder.

Contributors

Each author is required to declare his or her individual contribution to the article: all authors must have materially participated in the research and/or article preparation, so roles for all authors should be described. The statement that all authors have approved the final article should be true and included in the disclosure.

Copyright

Upon acceptance of an article, authors will be asked to complete a 'Journal Publishing Agreement' (for more information on this and copyright see http://www.elsevier.com/copyright). Acceptance of the agreement will ensure the widest possible dissemination of information. An e-mail will be sent to the corresponding author confirming receipt of the manuscript together with a 'Journal Publishing Agreement' form or a link to the online version of this agreement.
Subscribers may reproduce tables of contents or prepare lists of articles including abstracts for internal circulation within their institutions. Permission of the Publisher is required for resale or distribution outside the institution and for all other derivative works, including compilations and translations (please consult http://www.elsevier.com/permissions). If excerpts from other copyrighted works are included, the author(s) must obtain written permission from the copyright owners and credit the source(s) in the article. Elsevier has preprinted forms for use by authors in these cases: please consult http://www.elsevier.com/permissions.

Retained author rights

As an author you (or your employer or institution) retain certain rights; for details you are referred to: http://www.elsevier.com/authorsrights.

Role of the funding source

You are requested to identify who provided financial support for the conduct of the research and/or preparation of the article and to briefly describe the role of the sponsor(s), if any, in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication. If the funding source(s) had no such involvement then this should be stated. Please see http://www.elsevier.com/funding.

Funding body agreements and policies

Elsevier has established agreements and developed policies to allow authors whose articles appear in journals published by Elsevier, to comply with potential manuscript archiving requirements as specified as conditions of their grant awards. To learn more about existing agreements and policies please visit http://www.elsevier.com/fundingbodies.

Language and language services

Please write your text in good English (American or British usage is accepted, but not a mixture of these). Authors who require information about language editing and copyediting services pre- and post-submission please visit http://www.elsevier.com/languagepolishing or our customer support site at http://epsupport.elsevier.com for more information. Please note Elsevier neither endorses nor takes responsibility for any products, goods or services offered by outside vendors through our services or in any advertising. For more information please refer to our Terms & Conditions: http://www.elsevier.com/termsandconditions.

Submission

Submission to this journal proceeds totally online. Use the following guidelines to prepare your article. Via the homepage of this journal (http://www.elsevier.com/journals) you will be guided stepwise through the creation and uploading of the various files. The system automatically converts source files to a single Adobe Acrobat PDF version of the article, which is used in the peer-review process. Please note that even though manuscript source files are converted to PDF at submission for the review process, these source files are needed for further processing after acceptance. All correspondence, including notification of the Editor's decision and requests for revision, takes place by e-mail and via the author's homepage, removing the need for a hard-copy paper trail. Authors should avoid responding by messages received from the system using the 'Reply' button on their e-mail message; this will send the message to the system support and not to the editorial office, and will create unnecessary load of sorting out and forwarding

Referees

Please submit, with the manuscript, the names, addresses and e-mail addresses of 3 potential referees. Note that the editor retains the sole right to decide whether or not the suggested reviewers are used.

PREPARATION

Use of wordprocessing software

It is important that the file be saved in the native format of the wordprocessor used. The text should be in single-column format. Keep the layout of the text as simple as possible. Most formatting codes will be removed and replaced on processing the article. In particular, do not use the wordprocessor's options to justify text or to hyphenate words. However, do use bold face, italics, subscripts, superscripts etc. Do not embed "graphically designed" equations or tables, but prepare these using the wordprocessor's facility. When preparing tables, if you are using a table grid, use only one grid for each individual table and not a grid for each row. If no grid is used, use tabs, not spaces, to align columns. The electronic text should be prepared in a way very similar to that of conventional manuscripts (see also the Guide to Publishing with Elsevier: http://www.elsevier.com/guidepublication). Do not import the figures into the text file but, instead, indicate their approximate locations directly in the electronic text and on the manuscript. See also the section on Electronic illustrations.
To avoid unnecessary errors you are strongly advised to use the "spell-check" and "grammar-check" functions of your wordprocessor.

LaTeX

If the LaTeX file is suitable, proofs will be produced without rekeying the text. The article should preferably be written using Elsevier's document class "elsarticle", or alternatively the standard document class "article".
The Elsevier LaTeX style file package (including detailed instructions for LaTeX preparation) can be obtained from the Quickguide: http://www.elsevier.com/latex. It consists of the file: elsarticle.cls, complete user documentation for the class file, bibliographic style files in various styles, and template files for a quick start.

Article structure

Subdivision - numbered sections

Divide your article into clearly defined and numbered sections. Subsections should be numbered 1.1 (then 1.1.1, 1.1.2, ...), 1.2, etc. (the abstract is not included in section numbering). Use this numbering also for internal cross-referencing: do not just refer to "the text". Any subsection may be given a brief heading. Each heading should appear on its own separate line.

Introduction

State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

Material and methods

Provide sufficient detail to allow the work to be reproduced. Methods already published should be indicated by a reference: only relevant modifications should be described.

Theory/calculation

A Theory section should extend, not repeat, the background to the article already dealt with in the Introduction and lay the foundation for further work. In contrast, a Calculation section represents a practical development from a theoretical basis.

Results

Results should be clear and concise.

Discussion

This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is often appropriate. Avoid extensive citations and discussion of published literature.

Conclusions

The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

Appendices

If there is more than one appendix, they should be identified as A, B, etc. Formulae and equations in appendices should be given separate numbering: Eq. (A.1), Eq. (A.2), etc.; in a subsequent appendix, Eq. (B.1) and so on.

Vitae

Include in the manuscript a short (maximum 100 words) biography of each author, along with a passport-type photograph accompanying the other figures.

Essential title page information

- *Title.* Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.
- *Author names and affiliations.* Where the family name may be ambiguous (e.g., a double name), please indicate this clearly. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-case superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name, and, if available, the e-mail address of each author.
- *Corresponding author.* Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication. **Ensure that telephone and fax numbers (with country and area code) are provided in addition to the e-mail address and the complete postal address.**
- *Present/permanent address.* If an author has moved since the work described in the article was done, or was visiting at the time, a "Present address" (or

"Permanent address") may be indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

Abstract

A concise and factual abstract is required. The abstract should state briefly the purpose of the research, the principal results and major conclusions. An abstract is often presented separately from the article, so it must be able to stand alone. For this reason, References should be avoided, but if essential, then cite the author(s) and year(s). Also, non-standard or uncommon abbreviations should be avoided, but if essential they must be defined at their first mention in the abstract itself.
The abstract should be not longer than 400 words.

Keywords

Immediately after the abstract, provide a maximum of 4-6 keywords, using American spelling and avoiding general and plural terms and multiple concepts (avoid, for example, "and", "of"). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

Classification codes

Please provide up to 6 standard PACS codes. The available codes may be accessed at PACS: ⇨http://www.elsevier.com/locate/pacs.

Abbreviations

Define abbreviations that are not standard in this field in a footnote to be placed on the first page of the article. Such abbreviations that are unavoidable in the abstract must be defined at their first mention there, as well as in the footnote. Ensure consistency of abbreviations throughout the article.

Acknowledgements

Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).

Nomenclature and units

Follow internationally accepted rules and conventions: use the international system of units (SI). If other quantities are mentioned, give their equivalent in SI. You are urged to consult IUPAC: Nomenclature of Organic Chemistry: ⇨http://www.iupac.org/ for further information.
1. Authors and editors are, by general agreement, obliged to accept the rules governing

biological nomenclature, as laid down in the International Code of Botanical Nomenclature, the International Code of Nomenclature of Bacteria, and the International Code of Zoological Nomenclature.
2. All biota (crops, plants, insects, birds, mammals, etc.) should be identified by their scientific names when the English term is first used, with the exception of common domestic animals.
3. All biocides and other organic compounds must be identified by their Geneva names when first used in the text. Active ingredients of all formulations should be likewise identified.
4. For chemical nomenclature, the conventions of the International Union of Pure and Applied Chemistry and the official recommendations of the IUPAC IUB Combined Commission on Biochemical Nomenclature should be followed.

Accession numbers

Accession numbers are unique identifiers in bioinformatics allocated to nucleotide and protein sequences to allow tracking of different versions of that sequence record and the associated sequence in a data repository [e.g., databases at the National Center for Biotechnical Information (NCBI) at the National Library of Medicine ('GenBank') and the Worldwide Protein Data Bank]. There are different types of accession numbers in use based on the type of sequence cited, each of which uses a different coding. Authors should explicitly mention the *type of accession number together with the actual number*, bearing in mind that an error in a letter or number can result in a dead link in the online version of the article. Please use the following format: accession number type ID: xxxx (e.g., MMDB ID: 12345; PDB ID: 1TUP). Note that in the final version of the *electronic copy*, accession numbers will be linked to the appropriate database, enabling readers to go directly to that source from the article.
DNA sequences and GenBank Accession numbers. Many Elsevier journals cite "gene accession numbers" in their running text and footnotes. Gene accession numbers refer to genes or DNA sequences about which further information can be found in the databases at the National Center for Biotechnical Information (NCBI) at the National Library of Medicine. Authors are encouraged to check accession numbers used very carefully. **An error in a letter or number can result in a dead link.** Note that in the final version of the electronic copy, the accession number text will be linked to the appropriate source in the NCBI databases enabling readers to go directly to that source from the article.

Example 1: "GenBank accession nos. **AI631510, AI631511, AI632198,** and **BF223228**, a B-cell tumor from a chronic lymphatic leukemia (GenBank accession no. BE675048), and a T-cell lymphoma (GenBank accession no. **AA361117**)".

Authors are encouraged to check accession numbers used very carefully. An error in a letter or number can result in a dead link.

In the final version of the printed article, the accession number text will not appear bold or underlined (see Example 2 below).

Example 2: "GenBank accession nos. AI631510, AI631511, AI632198, and BF223228), a B-cell tumor from a chronic lymphatic leukemia (GenBank accession no. BE675048), and a T-cell lymphoma (GenBank accession no. AA361117)".

In the final version of the electronic copy, the accession number text will be linked to the appropriate source in the NCBI databases enabling readers to go directly to that source from the article (see Example 3 below).

Example 3: "GenBank accession nos. AI631510, AI631511, AI632198, and BF223228), a B-cell tumor from a chronic lymphatic leukemia (GenBank accession no. BE675048), and a T-cell lymphoma (GenBank accession no. AA361117)".

Math formulae

Present simple formulae in the line of normal text where possible and use the solidus (/) instead of a horizontal line for small fractional terms, e.g., X/Y. In principle, variables are to be presented in italics. Powers of e are often more conveniently denoted by exp. Number consecutively any equations that have to be displayed separately from the text (if referred to explicitly in the text).
Give the meaning of all symbols immediately after the equation in which they are first used. In chemical formulae, valence of ions should be given as, e.g. Ca^{2+} and not Ca^{++}. Isotope numbers should precede the symbols, e.g., ^{18}O. The repeated writing of chemical formulae in the text is to be avoided where reasonably possible; instead, the name of the compound should be given in full. Exceptions may be made in the case of a very long name occurring very frequently or in the case of a compound being described as the end product of a gravimetric determination (e.g., phosphate as P_2O_5).

Footnotes

Footnotes should be used sparingly. Number them consecutively throughout the article, using superscript Arabic numbers. Many wordprocessors build footnotes into the text, and this feature may be used. Should this not be the case, indicate the position of footnotes in the text and present the footnotes themselves separately at the end of the article. Do not include footnotes in the Reference list.
Table footnotes
Indicate each footnote in a table with a superscript lowercase letter.

Electronic artwork

General points
• Make sure you use uniform lettering and sizing of your original artwork.
• Save text in illustrations as "graphics" or enclose the font.
• Only use the following fonts in your illustrations: Arial, Courier, Times, Symbol.
• Number the illustrations according to their sequence in the text.
• Use a logical naming convention for your artwork files.
• Provide captions to illustrations separately.
• Produce images near to the desired size of the printed version.
• Submit each figure as a separate file.

A detailed guide on electronic artwork is available on our website:
http://www.elsevier.com/artworkinstructions

You are urged to visit this site; some excerpts from the detailed information are given here.

Formats
Regardless of the application used, when your electronic artwork is finalised, please "save as" or convert the images to one of the following formats (note the resolution requirements for line drawings, halftones, and line/halftone combinations given below):
EPS: Vector drawings. Embed the font or save the text as "graphics".
TIFF: color or grayscale photographs (halftones): always use a minimum of 300 dpi.
TIFF: Bitmapped line drawings: use a minimum of 1000 dpi.
TIFF: Combinations bitmapped line/half-tone (color or grayscale): a minimum of 500 dpi is required.
DOC, XLS or PPT: If your electronic artwork is created in any of these Microsoft Office applications please supply "as is".

Please do not:
• Supply embedded graphics in your wordprocessor (spreadsheet, presentation) document;
• Supply files that are optimised for screen use (like GIF, BMP, PICT, WPG); the resolution is too low;
• Supply files that are too low in resolution;
• Submit graphics that are disproportionately large for the content.

Color artwork

Please make sure that artwork files are in an acceptable format (TIFF, EPS or MS Office files) and with the correct resolution. If, together with your accepted article, you submit usable color figures then Elsevier will ensure, at no additional charge, that these figures will appear in color on the Web (e.g., ScienceDirect and other sites) regardless of whether or not these illustrations are reproduced in color in the printed version. **For color reproduction in print, you will receive information regarding the costs from Elsevier after receipt of your accepted article.** Please indicate your preference for color in print or on the Web only. For further information on the preparation of electronic artwork, please see http://www.elsevier.com/artworkinstructions.
Please note: Because of technical complications which can arise by converting color figures to "gray scale" (for the printed version should you not opt for color in print) please submit in addition usable black and white versions of all the color illustrations.

Figure captions

Ensure that each illustration has a caption. Supply captions separately, not attached to the figure. A caption should comprise a brief title (**not** on the figure itself) and a description of the illustration. Keep text in the illustrations themselves to a minimum but explain all symbols and abbreviations used.

Text graphics

Present incidental graphics not suitable for mention as figures, plates or schemes at the end of the article and number them "Graphic 1", etc. Their precise position in the text can then be indicated. See further under Electronic artwork. Ensure that high-resolution graphics files are provided, even if the graphic appears as part of your normal wordprocessed text file.

Tables

Number tables consecutively in accordance with their appearance in the text. Place footnotes to tables below the table body and indicate them with superscript lowercase letters. Avoid vertical rules. Be sparing in the use of tables and ensure that the data presented in tables do not duplicate results described elsewhere in the article.

References

Citation in text

Please ensure that every reference cited in the text is also present in the reference list (and vice versa). Any references cited in the abstract must be given in full. Unpublished results and personal communications are not recommended in the reference list, but may be mentioned in the text. If these references are included in the reference list they should follow the standard reference style of the journal and should include a substitution of the publication date with either "Unpublished results" or "Personal communication" Citation of a reference as "in press" implies that the item has been accepted for publication.

Web references

As a minimum, the full URL should be given. Any further information, if known (DOI, author names, dates, reference to a source publication, etc.), should also be given. Web references can be listed separately (e.g., after the reference list) under a different heading if desired, or can be included in the reference list.

References in a special issue

Please ensure that the words 'this issue' are added to any references in the list (and any citations in the text) to other articles in the same Special Issue.

Reference style

Text: All citations in the text should refer to:
1. *Single author:* the author's name (without initials, unless there is ambiguity) and the year of publication;
2. *Two authors:* both authors' names and the year of publication;
3. *Three or more authors:* first author's name followed by "et al." and the year of publication.
Citations may be made directly (or parenthetically). Groups of references should be listed first alphabetically, then chronologically.
Examples: "as demonstrated (Allan, 1996a, 1996b, 1999; Allan and Jones, 1995). Kramer et al. (2000) have recently shown ...."
List: References should be arranged first alphabetically and then further sorted chronologically if necessary. More than one reference from the same author(s) in the same year must be identified by the letters "a", "b", "c", etc., placed after the year of publication.
Examples:
Reference to a journal publication:

Van der Geer, J., Hanraads, J.A.J., Lupton, R.A., 2000. The art of writing a scientific article. J. Sci. Commun. 163, 51-59.
Reference to a book:
Strunk Jr., W., White, E.B., 1979. The Elements of Style, third ed. Macmillan, New York.
Reference to a chapter in an edited book:
Mettam, G.R., Adams, L.B., 1999. How to prepare an electronic version of your article, in: Jones, B.S., Smith , R.Z. (Eds.), Introduction to the Electronic Age. E-Publishing Inc., New York, pp. 281-304.

Journal Abbreviations Source

Define abbreviations that are not standard in this field at their first occurrence in the article: in the abstract but also in the main text after it. Ensure consistency of abbreviations throughout the article.

Supplementary material

Elsevier accepts electronic supplementary material to support and enhance your scientific research. Supplementary files offer the author additional possibilities to publish supporting applications, movies, animation sequences, high-resolution images, background datasets, sound clips and more. Supplementary files supplied will be published online alongside the electronic version of your article in Elsevier Web products, including ScienceDirect: http://www.sciencedirect.com. In order to ensure that your submitted material is directly usable, please ensure that data are provided in one of our recommended file formats. Authors should submit the material in electronic format together with the article and supply a concise and descriptive caption for each file. Video files: please supply 'stills' with your files: you can choose any frame from the video or make a separate image. These will be used instead of standard icons and will personalize the link to your supplementary information. For more detailed instructions please visit our artwork instruction pages at http://www.elsevier.com/artworkinstructions.

Submission checklist

It is hoped that this list will be useful during the final checking of an article prior to sending it to the journal's Editor for review. Please consult this Guide for Authors for further details of any item.

Ensure that the following items are present:
One Author designated as corresponding Author:
• E-mail address
• Full postal address
• Telephone and fax numbers
All necessary files have been uploaded
• Keywords
• All figure captions
• All tables (including title, description, footnotes)
Further considerations
• Manuscript has been "spellchecked" and "grammar-checked"

• References are in the correct format for this journal
• All references mentioned in the Reference list are cited in the text, and vice versa
• Permission has been obtained for use of copyrighted material from other sources (including the Web)
• color figures are clearly marked as being intended for color reproduction on the Web (free of charge) and in print or to be reproduced in color on the Web (free of charge) and in black-and-white in print
• If only color on the Web is required, black and white versions of the figures are also supplied for printing purposes
For any further information please visit our customer support site at http://epsupport.elsevier.com.

AFTER ACCEPTANCE

Use of the Digital Object Identifier

The Digital Object Identifier (DOI) may be used to cite and link to electronic documents. The DOI consists of a unique alpha-numeric character string which is assigned to a document by the publisher upon the initial electronic publication. The assigned DOI never changes. Therefore, it is an ideal medium for citing a document, particularly 'Articles in press' because they have not yet received their full bibliographic information. The correct format for citing a DOI is shown as follows (example taken from a document in the journal *Physics Letters B*):
doi:10.1016/j.physletb.2003.10.071
When you use the DOI to create URL hyperlinks to documents on the web, they are guaranteed never to change.

Proofs

One set of page proofs (as PDF files) will be sent by e-mail to the corresponding author (if we do not have an e-mail address then paper proofs will be sent by post) or, a link will be provided in the e-mail so that authors can download the files themselves. Elsevier now provides authors with PDF proofs which can be annotated; for this you will need to download Adobe Reader version 7 (or higher) available free from http://www.adobe.com/products/acrobat/readstep2.html. Instructions on how to annotate PDF files will accompany the proofs (also given online). The exact system requirements are given at the Adobe site: http://www.adobe.com/products/acrobat/acrrsystemreqs.html#70win.
If you do not wish to use the PDF annotations function, you may list the corrections (including replies to the Query Form) and return them to Elsevier in an e-mail. Please list your corrections quoting line number. If, for any reason, this is not possible, then mark the corrections and any other comments (including replies to the Query Form) on a printout of your proof and return by fax, or scan the pages and e-mail, or by post. Please use this proof only for checking the typesetting, editing, completeness and correctness of the text, tables and figures. Significant changes to the article as accepted for publication will only be considered at this stage with permission from the Editor. We will do everything possible to get your article published quickly and accurately. Therefore, it is important to ensure that all of your corrections are sent back to us in one communication: please check carefully before replying, as inclusion of any subsequent corrections cannot be guaranteed. Proofreading is solely your responsibility. Note that

Elsevier may proceed with the publication of your article if no response is received.

Offprints

The corresponding author, at no cost, will be provided with a PDF file of the article via e-mail. The PDF file is a watermarked version of the published article and includes a cover sheet with the journal cover image and a disclaimer outlining the terms and conditions of use. Additional paper offprints can be ordered by the authors. An order form with prices will be sent to the corresponding author.

AUTHOR INQUIRIES

For inquiries relating to the submission of articles (including electronic submission where available) please visit this journal's homepage. You can track accepted articles at http://www.elsevier.com/trackarticle and set up e-mail alerts to inform you of when an article's status has changed. Also accessible from here is information on copyright, frequently asked questions and more. Contact details for questions arising after acceptance of an article, especially those relating to proofs, will be provided by the publisher.