Induced reproduction in Pātiki (*Rhombosolea leporina*), a novel endemic aquaculture candidate

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Abstract

The rapid global population growth combined with the decline of wild-capture fisheries has resulted in the global expansion of aquaculture. New Zealand's aquaculture industry remains largely underdeveloped and as such, industry growth would benefit from species diversification. The endemic yellowbelly flounder (*Rhombosolea leporina*) is a promising candidate species for New Zealand aquaculture due to its high market value, fast growth rate and the global demand for flatfishes. At present, very limited knowledge exists regarding the reproductive biology of this species, and notably the control of reproduction in captivity. Successful finfish aquaculture requires a robust understanding of reproduction for effective broodstock management. This thesis investigates induced ovulation in yellowbelly flounder using a gonadotropin releasing hormone analogue (GnRHa). The study pairs physiology and genetics to advance the current knowledge of reproduction in this species.

GnRHa was administrated to induce ovulation in yellowbelly flounder, over a 28-day period. One of three treatments were assigned to 27 female yellowbelly flounder (n=9): 50 µg/kg GnRHa, 100 µg/kg GnRHa, or a sham treatment of 50 µg/kg Ringer's solution. Histological and fresh oocyte analyses were carried out and batch fecundity, fertilization and ovulation rates were assessed. Reproductive dysfunction experienced by yellowbelly flounder in this study was through incomplete germinal vesicle migration and a failure to enter hydration. Ovulation was only observed in GnRHa treated fish and the ovulatory periodicity appeared to be approximately 72 hours in fish that ovulated more than once. This study confirms that yellowbelly flounder are multiple group synchronous batch spawners. Although fewer fish than expected ovulated (four), significantly more GnRHa treated fish completed final oocyte maturation and entered hydration compared to the controls. Furthermore, fecundity, fertilization rates and egg viability were high, with fertilization >80% achieved in some initial ovulations. In contrast, eggs obtained from fish that had ovulated for a second time, were characterised by low fecundity and fertilization rates. Nonetheless, this data indicates GnRHa can successfully induce ovulation at dosages of at least 50 µg/kg without adversely affecting egg quality in at least the first batch. Further refinement is essential to optimize a protocol for the reliable induction of reproduction in this species. Low incidences of ovulation may be a factor of dosage or inappropriate ovarian maturity at the time of hormone administration. This highlights the need to confirm oocyte maturity immediately prior to GnRHa treatment.

To aid future work, a classification index for the identification of ovarian developmental stage based on fresh oocyte morphology was developed for yellowbelly flounder.

To further advance future research into yellowbelly flounder reproduction, brain, pituitary and gonad transcriptomic libraries were generated for female yellowbelly flounder using RNA-seq. Protein sequences of eight reproduction related genes were successfully identified and characterised, which were *StAR, Gpr54, Lhβ, Fshβ, Fshr, Cyp19a1a, Cyp19a1b* and *Hsd17b1.* Conserved functional regions, phylogenetic analysis and homology with sequences from other Pleuronectiformes confirmed the identities of these genes and indicated that their functional roles are likely conserved. Characterisation of these genes provides highly valuable information for future quantitative assessment of gene expression throughout the yellowbelly flounder reproductive axis.

The results presented in this thesis will help to inform future work to control and optimize reproduction in captive yellowbelly flounder. In addition, the genetic data will enable molecular tools to be developed, including quantitative PCR primers as well as the potential for recombinant gonadotropins to develop enzyme-linked immunosorbent assays. These tools will be highly valuable to accurately assess the effects of GnRHa treatment on reproduction and gene expression under differing culture conditions. This study indicates that successful ovulation and good quality eggs may be achieved in yellowbelly flounder using GnRHa treatment.

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Chapter 1

General Introduction

1.1 Introduction

With the global human population projected to reach 9.7 billion people by 2050 (United Nations, 2022), and many capture fisheries experiencing notable declines, aquaculture is quickly becoming the fastest growing primary industry and is essential for ensuring global food security. Further, the expeditious increase in global aquaculture production in recent decades has resulted in seafood becoming an increasingly important global food source (Garlock et al., 2020). Consequently, there is an urgent requirement for the escalation of food production and an ever-increasing demand per capita for seafood. This exerts substantial pressure and focus on the further expansion of aquaculture, globally. To meet these demands, it has been forecast that by 2030 aquaculture will account for 59% of fish produced for human consumption (FAO, 2020). Within only 20 years, from 1997 to 2017, global aquaculture production more than tripled, from 34Mt in annual production to 112Mt (Naylor et al., 2021). Further, in 2018, global fish production was estimated to have reached 179 million tonnes, with aquaculture accounting for 46 percent of total fish production and 52 percent of fish supplied for human consumption (FAO, 2020). This rapid expansion of aquaculture in recent years has predominantly been fuelled by the notable declines in wild fish stocks, coupled with increasing awareness regarding the environmental and ecological impacts of overfishing. Already, wild-caught fisheries production has plateaued and further declines in temperate and tropical wild fish stocks are predicted (Cheung & Frölicher, 2020; Lotze et al., 2019). Evidently, the continued growth of the aquaculture industry is essential for ensuring food security and meeting the protein requirements of the rapidly expanding human population.

Aquaculture has been practiced in some regions of the world for many centuries, and can be traced back around 4000 years to the farming of tilapia in ancient Egypt (Beveridge & Little, 2002). Despite the early origins of aquaculture, the transition from traditional aquaculture approaches to intensive modern aquaculture only occurred within the mid-late nineteenth century (Stickney & Treece, 2012). Consequently, the current global aquaculture industry

remains limited to a few key species, and aquaculture technologies are not yet on par with those developed for the agricultural industry. In particular, the optimization of reproduction methodologies for cultured aquatic species is lacking, and as such, one of the key global bottlenecks associated with finfish culture is the control of reproduction. The successful production of any cultured finfish depends upon having a reliable year-round supply of viable eggs with high survival. Thus, the initial development of a novel aquaculture species requires developing methodologies to optimize reproduction. This remains an area of extensive research in aquaculture and is critical for the sustainable and profitable development of the industry, alongside the successful development of new target species (Zohar & Mylonas, 2001).

In comparison to many terrestrial animal food production systems, aquaculture is oftentimes considered a more environmentally sustainable method for producing protein (Garlock et al., 2020). Farming species that occupy lower trophic levels increases the ecological efficiency of aquaculture. These species ultimately utilize less resources and create less waste than those feeding at higher trophic levels (Neori & Nobre, 2012). Further, feed conversion efficiencies in aquaculture production systems are greater in comparison to terrestrial agriculture systems, and have lower emissions than intensive livestock production (Hall, 2011). Considerable research is being undertaken to develop and improve ecological aquaculture technologies to minimise adverse environmental impacts (Edwards, 2015). Therefore, it may be argued that increasing protein production through aquaculture is a more sustainable goal than through intensive terrestrial agriculture. Yet, growth of the commercial aquaculture industry has been significantly constrained due to the delays in species diversification (Stickney & Treece, 2012). The continued development of novel candidate species and farming practices are critical for further expansion of this industry. If aquaculture is to remain a sustainable source of protein, while also increasing production at the substantial rate required by population growth, the development of both novel species for culture and efficient production processes are essential.

1.2 Aquaculture in New Zealand

Aquaculture in New Zealand originated during pre-European times, where Māori engaged in traditional practices including the maintenance of fish nurseries for stock replenishment and enhancement, and restoring coastal marine areas with translocated bull kelp (Cram et al., 2010). Following traditional aquaculture, the commercialization of aquaculture in New Zealand began with the farming of the native rock oyster, *Saccostrea glomerata*, in the mid-

1960s. This was followed by the green-lipped mussel, *Perna Canaliculus* (Ministry for the Environment, 2007), which remains a key aquaculture species. Commercial finfish farming in New Zealand began between 1976 and 1983 with the rearing of Chinook salmon (*Oncorhynchus tshawytscha)* in marine cages (Fløysand et al., 2016; Stenton-Dozey et al., 2021). Due to major legislation changes that took place in 1983, the operation of marine farms were first permitted, and by 1993 Stewart Island became the key location for salmon farming (Fløysand et al., 2016). In present day, the New Zealand aquaculture industry achieves $600+ million in annual sales, with the New Zealand Aquaculture Strategy aiming to boost aquaculture to become a $3 billion industry by 2035 (Ministry for Primary Industries (MPI), 2019). It is important to recognise that the development of the industry has been constrained by complex regulatory frameworks (Banta & Gibbs, 2009), in conjunction with the high perceived recreational and amenity values associated with many coastal regions in New Zealand ideal for aquaculture. Thus, the social implications associated with aquaculture in New Zealand present a further barrier hindering its development.

Despite New Zealand's 15,000 km long coastline, the marine areas allocated for aquaculture production only total around 20,000 hectares (Stenton-Dozey et al., 2021). Furthermore, the current aquaculture industry in New Zealand is largely limited to three key species, Greenshell™ mussels, Chinook salmon, and Pacific oysters (*Crassostrea gigas*). The commercial production of several additional species, including the backfoot pāua (*Haliotis iris)* and the Bluff oyster (*Tiostrea chilensis),* has been established, although on much smaller scales (Stenton-Dozey et al., 2021). Further, research on the development of the finfishes, yellowtail kingfish (*Seriola lalandi)* and hāpuku (*Polyprion oxygeneios)* for aquaculture has also been undertaken, although commercial production of these species has not yet been established (Stenton-Dozey et al., 2021). Further growth of New Zealand's aquaculture industry could stem from the expansion of the already established aquaculture species targets. However, it has been highlighted that diversification to include new species that have both significant environmental and market advantages may be critical for the expansion and growth of a sustainable and resilient aquaculture industry in New Zealand (Camara & Symonds, 2014; Valenza-Troubat et al., 2022).

1.3 Teleost reproduction

1.3.1 Seasonality of reproduction

Reproduction in most teleosts is timed to coincide with favourable environmental conditions to enhance larval survival and growth, therefore ensuring the greatest chance of survival for successive generations (Cushing, 1969; Methot, 1983). Higher latitudes are characterized by cooler climates where larval food availability is often limited to spring algal blooms (Platt et al., 2003), thus restricted spawning seasons are prevalent. Exogenous cues in the form of environmental stimuli, such as temperature and daylength (i.e., photoperiod), are the primary factors entraining the seasonal cycles of reproductive development in finfishes. For instance, studies on Salmonids revealed that photoperiod alone can modify the timing of reproduction (Pankhurst & Porter, 2003). Several other external cues can influence the reproductive timing in some finfishes. Lunar and semi-lunar cycles seem to dictate spawning in some coral reef fishes (Taylor, 1984), and spawning behaviour of wild Atlantic cod is tied to a lunar cycle (Grabowski et al., 2015). Ultimately, integration of external environmental stimuli into internal stimuli determines the timing of reproduction and spawning in finfishes.

1.3.2 Regulation of reproduction

In teleosts, sexual reproduction is primarily regulated by a cascade of neuroendocrine and endocrine factors involved in the brain-pituitary-gonad (BPG) axis (Figure 1.1.) (Nagahama, 1994). The physiological activation of this axis occurs during puberty and comprises three key constituents; the brain, pituitary and gonads (Weltzien et al., 2004). Within the natural environment, exogenous cues in the form of environmental stimuli are transduced into endogenous cues. These stimuli trigger the hypothalamic synthesis of the decapeptide neurohormone, gonadotropin-releasing hormone (GnRH), which selectively binds to its receptor in the pituitary (Figure 1.1.). Uniquely, the teleost hypothalamus-pituitary pathway is characterised by the direct release of hypothalamic neurohormones by nerve endings situated near or on their target cells (Weltzien et al., 2004; Zohar et al., 2010). GnRH secretion stimulates the synthesis and release of two distinct pituitary gonadotropin hormones, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Zohar et al., 2010). Both FSH and LH, are recognised for their essential roles in regulating the processes involved in gametogenesis and gonadal steroidogenesis (Planas & Swanson, 2008). They exert their effects via their G protein-coupled receptors located in the gonads, follicle stimulating hormone receptor (Fshr)

and luteinising hormone receptor (Lhr) (Kobayashi et al., 2008; Mittelholzer et al., 2009). The actions of FSH and LH on the gonads of teleosts are indirect, through initiating the gonadal production and secretion of sex steroid hormones (Figure 1.1.), which mediate different aspects of reproductive development (Rocha et al., 2008). Despite the considerable diversity of steroid hormones, specific steroids play a primary role in reproduction. C_{19} steroids (androgens) regulate a multitude of reproductive processes in both females and males (Pankhurst, 2008). This includes regulating gonadal growth in males and behaviour, pituitary and hypothalamic function in both sexes. Similarly, C_{18} steroids (estrogens) are synthesized in both sexes, although are produced in higher quantities in females. Throughout reproduction, estrogens in female teleosts contribute to the regulation of ovarian growth, secondary sexual characteristics and aspects of oogenesis (Pankhurst, 2008).

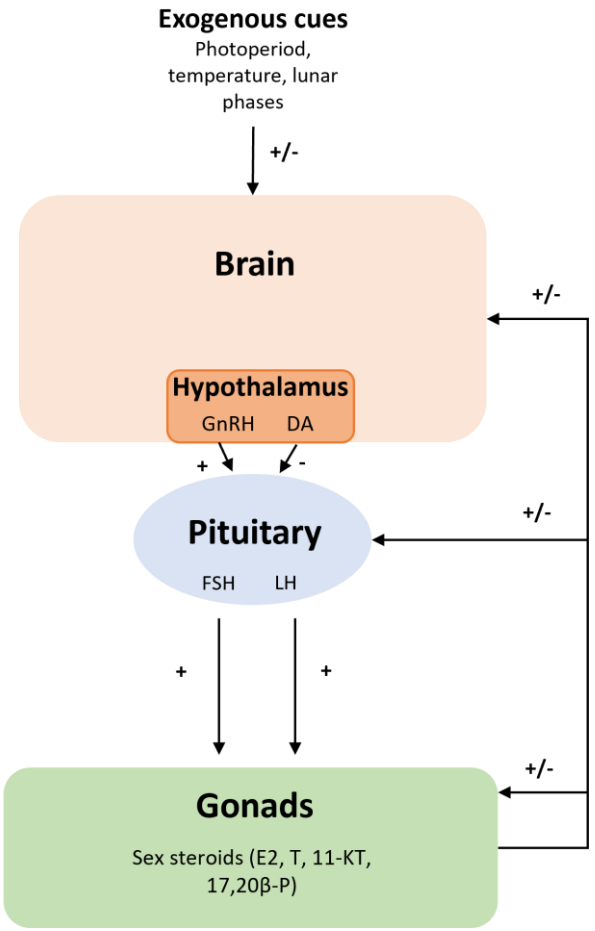

Figure 1.1. Schematic representation of the brain-pituitary-gonad axis of teleosts. This figure is simplified and generalized, although species-specific and sex-specific variations exist. Abbreviations are GnRH, gonadotropin releasing hormone; DA, dopamine; FSH, follicle-stimulating hormone; LH, luteinizing hormone; E2, estradiol; T, testosterone; 11-KT, 11-ketotesosterone; 17,20β-P, 17,20β-dihydroxy-4-pregnen-3-one.

1.3.3 Oogenesis

Oogenesis can be defined as the process whereby primordial germ cells (PGC's) develop into eggs ready for fertilization (Patiño & Sullivan, 2002). Regulation of oocyte development and maturation in female teleosts is primarily regulated by the gonadotropin hormones, FSH and LH. This is through both the subsequent actions of sex steroids, and through actions which are independent of sex steroids (Lubzens et al., 2010; Planas & Swanson, 2008). Sex steroid biosynthesis occurs within the two follicular cell layers and is initiated by the mobilization of cholesterol within thecal cells, which is later bio converted to testosterone (Nagahama, 1997; Tenugu et al., 2021). During oogenesis, estradiol (E2) is produced through the aromatization of testosterone in the inner granulosa layer under FSH stimulation (Nagahama, 1994; Yaron & Levavi-Sivan, 2011). At the onset of final maturation, LH stimulation causes a shift in the steroidogenic pathway, initiating the synthesis of the active maturation-inducing steroid (MIS) within the follicular cell layers, which leads to final oocyte maturation (Nagahama & Yamashita, 2008).

1.4 Control of reproduction in aquaculture

Controlling reproduction is essential for efficient aquaculture, as sourcing wild larvae for grow-out is oftentimes unreliable and unsustainable (De Silva et al., 2008; Naylor et al., 2000). Establishing a captive broodstock for reliable seedstock production is therefore critical. Attaining a detailed understanding of the species reproductive biology, alongside the ability to control reproduction in a culture environment are prerequisites for the sustainable and successful aquaculture of any finfish. Furthermore, production of a reliable year-round supply of hatchery reared fish is highly desirable for consumers and key for ensuring the culture of a species remains sustainable and economically efficient. Achieving control of reproduction in captive finfish can pose the greatest challenge to commercial aquaculture (Howell et al., 2009; Mylonas et al., 2004).

1.4.1 Reproductive dysfunction

Reproduction remains one of the key areas limiting the development of commercial aquaculture. This results from the potential for bottlenecks to occur at numerous stages throughout the process of gonadal recrudescence and maturation.

Reproductive dysfunction refers to the full or partial inhibition of the reproductive axis. Captivity is a common cause of reproductive dysfunction in teleosts and captivity-associated reproductive dysfunction has been reported across many teleost orders, including; Pleuronectiformes (Guzmán et al., 2009), Salmoniformes (Bromage et al., 1992), Anguilliformes (Zohar & Mylonas, 2001), Siluriformes (Viveiros et al., 2002), Carangiformes (Micale et al., 1999) and Perciformes (Bardon-Albaret et al., 2015). Factors such as elevated stress and a lack of appropriate exogenous cues are considered to be major inducers of reproductive dysfunction (Jerez et al., 2018; Mylonas et al., 2010). Captive containment and some husbandry practices are major stressors (Barnett & Pankhurst, 1998) and these effects are often amplified in wild-caught broodstock (Cleary et al., 2000; Jerez et al., 2018). Seasonally timed environmental stimuli are often absent in captivity, which may also disrupt important reproductive behaviours (De Silva et al., 2008). For example, the migration to breeding grounds undertaken by anadromous and diadromous finfishes are characterised by significant temperature, salinity, and depth changes (Colman, 1973; Fleming, 1998). The absence of these natural stimuli may result in inhibition of reproduction (Ben Ammar et al., 2015; Haffray et al., 1995).

Despite some degree of gonadal growth and reproductive maturity often being achieved, reproductive dysfunction may still occur in cultured finfish. In female fish, this occurs during three key aspects of reproductive development. Firstly, vitellogenesis may be inhibited and oocyte development arrested during the early stages, when the oocytes are still previtellogenic (Zohar & Mylonas, 2001). Secondly, vitellogenesis may progress but inhibition of final oocyte maturation occurs, which often results in follicular atresia (Mañanós et al., 2009). The third form of reproductive dysfunction occurs where oocytes mature and are ovulated, but spawning is inhibited. This final form of reproductive dysfunction has been reported in many cultured finfishes, such as Atlantic halibut (Skaalsvik et al., 2015) and some Salmonids (Bromage et al., 1992). The failure to spontaneously release eggs is a significant problem faced during the commercial culture of finfishes, and anecdotal reports from the pilot culture of greenback flounder stated the retention of unspawned eggs caused broodstock mortality (Pankhurst & Fitzgibbon, 2006). This may be remediated by hand-stripping of ovulated eggs, such as in the Atlantic halibut industry (Skaalsvik et al., 2015). Reproductive dysfunction is less common in captive male fishes and is typically associated with reduced volumes of sperm and diminished milt fluidity (Agulleiro et al., 2007; Mylonas et al., 1997).

Regardless of sex, reproductive dysfunction in any form may impact hatchery efficiency and thereby increase the economic risk associated with finfish aquaculture.

1.4.2 Hormonally induced reproduction in aquaculture

Hormonal intervention is an important therapeutic for finfish when the effects of captivity cannot be easily overcome. Subsequent generations raised entirely under cultured conditions typically require less intervention to overcome reproductive dysfunction (Cleary et al., 2000; Soares et al., 2015). A common breeding strategy is to use hormonal intervention to induce reproduction in wild-caught broodstock, and then breed from the first (F1) generation offspring, to minimize the need for further treatment to induce successful reproduction.

Different hormonal treatments exist. Administration of the mammalian gonadotropin human chorionic gonadotropin (hCG) (Guzmán et al., 2011), or carp pituitary extract (CPE) (Berlinsky et al., 1997) have traditionally been used for overcoming captivity induced reproductive dysfunction. Injection of hCG can successfully induce ovulation, stimulate spermiation and increase sperm production in teleost fishes (Guzmán et al., 2011; Żarski et al., 2017). However, due to the low potency of mammalian gonadotropins, large and repeated doses may be required to overcome reproductive dysfunction (Berlinsky et al., 1997; Lim, 2016). Similarly, injection of CPE has successfully induced ovulation in numerous finfishes, including pikeperch (*Sander lucioperca)* (Falahatkar & Poursaeid, 2014), tench (*Tinca tinca*) (Kouril et al., 2007) and summer flounder (Berlinsky et al., 1997). However, the usefulness of CPE is limited as it is difficult to standardize; additional hormones may present which can cause adverse effects, and there is an elevated risk of disease transmission (Chatakondi et al., 2011).

In recent years, the use of gonadotropin-releasing analogues (GnRHa) and luteinizing hormone releasing hormone analogue (LHRHa), have superseded the use of CPE and mammalian gonadotropins. These GnRH analogues are based on the natural hormonal cascade in the BPG axis (Zohar & Mylonas, 2001). Ovulation and increased milt production are achieved through the stimulatory effect of GnRHa on the endogenous synthesis of gonadotropins, particularly LH, and consequently elevated MIS production to complete gametogenesis (Mateos et al., 2002; Nyuji et al., 2019; Prat et al., 2001). Successful GnRHa treatment has been achieved in numerous cultured finfish, including longfin yellowtail (*Seriola rivoliana*) (Fernández-Palacios et al., 2015), Senegalese sole (Guzmán et al., 2009) and greater

amberjack (Nyuji et al., 2019). The success of GnRHa has been attributed to the direct stimulatory effect of GnRHa on the endocrine system, as it acts directly on the pituitary at a 'high' level of the reproductive axis. Therefore, GnRHa has the distinct advantage over alternative exogenous hormones in that it acts on the pituitary rather than the gonads (Zohar & Mylonas, 2001). Resultantly, GnRHa can more completely stimulate the endocrinological events that occur naturally throughout reproduction. Due to the synthetic nature of GnRHa, these hormones are highly potent agonists compared to native GnRH (Zohar & Mylonas, 2001), requiring only small doses. GnRHa yields effective results across different species with a low risk of triggering an immune response due to its highly conserved small decapeptide structure (Chen & Fernald, 2008; Zohar & Mylonas, 2001).

Other factors, such as dose and delivery method, also need to be considered when investigating the efficacy of GnRHa treatment. The optimal dose for successful reproduction varies between species. For example, 4 µg/kg is optimal for African catfish (Shokr, 2020), while 20 µg/kg is effective in longfin yellowtail (Fernández-Palacios et al., 2015). Thus, determining the optimal dosage often requires considerable research. Delivery methods vary from intramuscular and intraperitoneal injections, to sustained release forms such as pellets and microspheres (Guzmán et al., 2009; Mugnier et al., 2000; Zohar & Mylonas, 2001). Further, the possibility of dopamine inhibition negating the effect of GnRHa treatment is an additional key concern. This results largely from the actions of dopamine to down regulate the expression of the GnRH-receptor in the pituitary, and therefore limit the production and secretion of the pituitary gonadotropins (Levavi-Sivan et al., 2004). This may be remediated through the administration of a dopamine antagonist in conjunction with GnRHa to successfully induce reproduction in broodstock (Kucharczyk et al., 2020). The efficacy of GnRHa treatment on ovarian development may be assessed using simple measures such as oocyte diameter size frequency and histological analysis (Guzmán et al., 2009; Jerez et al., 2018; Nyuji et al., 2019), alongside fertilization rates and fecundity. Further, oil globule diameter has been utilized in several species, such as kingfish (*Seriola lalandi*) (Setiawan et al., 2016). Analysis of plasma sex steroid levels (Guzmán et al., 2009; Mylonas et al., 1997) and gonadotropin levels have also been useful (Nyuji et al., 2019).

1.5 Flatfish aquaculture

Globally, marine flatfishes (order Pleuronectiformes) have been important in human diets, and consequently are considered valuable targets for aquaculture. They host considerable commercial interest due to their highly sought-after white flesh and nutritional profile, making them a desirable source of protein for consumers (Puvanendran et al., 2003). Flatfishes are considered low-fat, and much like many other marine species their flesh contains high quantities of poly-unsaturated fatty acids, such as DHA and EPA (Hibbeln et al., 2006), which can be increased through diet (Xu et al., 2018). Due to overexploitation, many flatfish stocks have declined and global wild capture fisheries catch has dropped considerably since its peak in the 1970's (Cheung & Oyinlola, 2018). Resultantly, flatfish aquaculture has experienced steady growth, particularly in Asia and Europe. A variety of flatfishes are commercially cultured at present, with six key species dominating production: European turbot (*Scophthalamus maximus*), olive flounder (*Paralichthys olivaceus*), tongue sole (*Cynoglossus semilaevis)*, Atlantic halibut, Senegalese sole and common sole (*Solea solea*).

Controlling reproduction remains one of the chief limitations in flatfish aquaculture. For instance, in both Senegalese sole and common sole, captivity-associated reproductive dysfunction and the closure of the lifecycle remain the primary bottlenecks limiting commercial production (Martín et al., 2019; Ofelio et al., 2020). However, highly successful induction of reproduction in flatfishes has been achieved through various forms of hormonal intervention (Guzmán et al., 2009; Mugnier et al., 2000; Sampaio et al., 2008). Treatment using GnRH analogues are now perhaps the most implemented technique for overcoming reproductive dysfunction. For instance, the implantation of sustained-release GnRHa pellets in captive female European turbot induced ovulation in all sexually mature females, and increased the incidence of spawning (Mugnier et al., 2000). Similarly, oocyte maturation and daily spawning can be successfully induced in Senegalese sole using GnRHa (Guzmán et al., 2009).

Commercial production of flatfishes in New Zealand currently remains undeveloped. The aquaculture potential for several species of New Zealand flatfish, such as brill (*Colistium guntheri)* and New Zealand turbot (*Colistium nudipinnus)* (Diggles et al., 2000; Tait & Hickman, 2001) has been investigated previously, although is yet to reach commercialisation. This previous interest represents an opportunity in the New Zealand aquaculture industry to

expand to include the culture of a flatfish species, which would allow for expansion in both the domestic and export markets.

1.6 Yellowbelly flounder

1.6.1 General biology

Pātiki tōtara, or yellowbelly flounder (*Rhombosolea leporina),* is a right-eyed flatfish species belonging to the order Pleuronectiformes and family Pleuronectidae. This species is endemic to New Zealand, having a wide distribution around the coastline. Yellowbelly flounder inhabit inshore coastal habitats such as estuaries and embayments, although migration to deeper waters (30m) has been observed during spawning (Colman, 1973). Early accounts of this species reported annual spawning occurring from June to November, with fecundity described to vary from approximately 250,000 eggs spawned from a 30cm fish to 1.25 million eggs spawned by a 45cm fish (Colman, 1973). Recent research has confirmed the extended spawning season, observing that under natural conditions yellowbelly flounder spawning takes place annually across a prolonged seven month period, from June to December (Koverman, 2018). It is likely that the process of reproduction begins well in advance of this spawning season. Further, this species are thought to undergo sexual differentiation into males and females at 57mm and 47mm tail length, respectively (Koverman, 2018). The precise age at which reproductive maturity occurs has not yet been determined.

1.6.2 Significance of yellowbelly flounder

Due to being endemic to New Zealand, yellowbelly flounder have significant cultural, recreational, and commercial importance. This is reflected in the 2021 annual flatfish (inclusive of eight flatfish species) capture statistics, with a customary capture allowance of 55,000 kg, a recreation allowance of 177,000 kg and a commercial catch of 1,814,188 kg (Ministry for Primary Industries, *n.d.*). Yellowbelly flounder have considerable cultural significance to Māori, pātiki being a traditional food in coastal areas (Wham, 2020), and are well recognised as a tāonga species to many iwi around Aotearoa/New Zealand. In particular, the historical abundance of pātiki in the Tauranga Harbour meant that they are a treasured tāonga to Ngāti Ranginui, Ngāi Te Rangi and Ngāti Pūkenga, the three iwi of Tauranga Moana. Many traditional stories and songs speak of pātiki, and the diamond shapes of traditional designs represent the flounder morphology. The tendency of pātiki to inhabit inshore coastal

areas likely contributed to this cultural significance, due to ease of harvest compared to offshore species. Historical references such as the quote "Ko au te Pātiki, ko te Pātiki ko au" - "I am the flounder, and the flounder is me" once said by Hōri Tūpaea, former leading chief of Ngāi Te Rangi captures the deep connection held between iwi and this tāonga species.

1.6.3 Aquaculture potential of yellowbelly flounder

Yellowbelly flounder have several traits that make them an interesting aquaculture target. Although they do not grow to a large overall size, they exhibit relatively fast growth to a marketable size, with females reaching approximately 30 cm TL within two years and 40 cm TL in three years (Colman, 1974). Further, the extended 7-month spawning season reported for this species (Koverman, 2018) indicates that a good potential to develop year-round fingering supply exists using additional 'out of season' photothermally manipulated broodstock. This would overcome seasonal supply limitations with the current wild-capture industry. This is perhaps one of the most critical factors determining the success and desirability of a novel aquaculture species.

Following metamorphosis, flatfishes are negatively buoyant and predominantly live within benthic environments (Gibson et al., 2014). Due to this primarily sedentary and benthic lifestyle, cultured flatfish have been reported to perform well in shallow, land-based raceway tank systems (Merino et al., 2007; Øiestad, 1999). Shallow raceways have been identified as cost effective and environmentally friendly systems for flatfish culture, allowing for higher stocking densities without limiting growth rates compared to conventional land-based tanks (Labatut & Olivares, 2004). The amenability of yellowbelly flounder for land-based aquaculture eliminates many of the barriers and risks associated with ocean-based aquaculture. Particularly, as the ability to use recirculating aquaculture systems (RAS) can enable 90-99% of water in an aquaculture system to be recycled (Badiola et al., 2012). This enables higher levels of control over environmental and water quality parameters. Due to the tolerance of flatfishes to overcrowding, stocking densities of flatfishes are generally higher than those reported for non-flatfish. For example, stocking densities ranging between 30 to 80 kg m^{-2} have been successful in cultured European turbot (Person-Le Ruyet, 2002). Moreover, there is a range of flatfish species already commercially cultured around the world, therefore existing technologies and research regarding the successful culture of flatfishes is potentially applicable to the culture of yellowbelly flounder. The evidence for successful

GnRHa treatment in other cultured flatfishes (Guzmán et al., 2009; Lim, 2016; Lim et al., 2004) suggests that this could be an effective method to overcome potential reproductive dysfunction in yellowbelly flounder.

Domestic and international demand for flatfishes is strong, and commercial production of flatfishes in European and Asian countries has been occurring for several decades. Yellowbelly flounder supports a commercial fishery throughout New Zealand (Ministry for Primary Industries, *n.d.*). From January to December 2021, commercially caught New Zealand flounder (inclusive of eight species of NZ flatfish) were exported to a total of 11 countries, to a value of approximately $2.78M (Seafood NZ, 2021). Additionally, in the current New Zealand domestic market, the retail value of yellowbelly flounder is approximately $20-28/kg gutted whole weight, which exceeds the market value of whole snapper (*Chrysophrys auratus*). Resultantly, the market value for yellowbelly flounder should be competitive with one of the most popular market fish consumed in New Zealand. Further, the international demand for flatfish indicates good market potential for cultured yellowbelly flounder.

1.7 Research statement and objectives

Despite the intention for the rapid expansion of the New Zealand aquaculture industry, diversification into the culture of novel species is lacking. Therefore, researching new targets is essential if both the sustainability and efficiency of the industry are to be improved, while growing to achieve the government's target of $3 billion by 2035. Yellowbelly flounder present themselves as a promising candidate for the New Zealand aquaculture industry. The development of this species for aquaculture remains largely understudied and little is known regarding their amenability to reproduction under captive conditions. For yellowbelly flounder to become a commercially viable aquaculture candidate in New Zealand, developing a methodology for induced reproduction is essential alongside increasing the current understanding of their reproduction. Previous research on yellowbelly flounder has suggested that this species experiences capture associated stress, alongside a failure of females to undergo oocyte maturation and ovulation (Jeffries, 2019). Consequently, induction of ovulation in females using exogenous hormones will likely be critical in the early stages of aquaculture development for this species. There are currently no published reports of induced ovulation in yellowbelly flounder.

This thesis reports a pilot study within a broader project to develop a protocol for induced ovulation in female yellowbelly flounder. The specific objectives of this study were to investigate the effect of a gonadotropin releasing hormone analogue treatment on reproduction in wild-caught yellowbelly flounder (Chapter 2) and develop a platform of knowledge to support the development of genetic tools (Chapter 3). The response of yellowbelly flounder to two different GnRHa treatments were assessed using measurements of fecundity, egg quality and quantity, alongside histological and fresh morphology of oocytes. In addition, a transcriptomic analysis of the female yellowbelly flounder reproductive axis was undertaken to support the future understanding of reproduction in this species. Collectively, this whole body of work advances the potential to develop yellowbelly flounder as an aquaculture candidate.

1.8 Ethics statement

The work undertaken in this thesis was approved by the Toi Ohomai Institute of Technology Animal Ethics Committee. The project ethics number for this research was AEC 2021.002. Wild capture of yellowbelly flounder was approved under a Ministry for Primary Industries fisheries special permit. Standard approved procedures were following during the capture, handling, husbandry, anaesthesia and euthanasia of fish in this study.

Chapter 2

Oocyte development in relation to gonadotropin releasing hormone analogue treatment

2.1 Introduction

In all finfishes, oocyte development and maturation are endocrinologically controlled and influenced by numerous environmental variables. In captive finfishes however, oocyte development is often controlled through administration of exogenous hormones (Bambill et al., 2006; Guzmán et al., 2009). The successful induction of oocyte development and maturation using exogenous hormones differs greatly among species. This tends to be dependent on a variety of factors, such as the hormone administered, dose and method of administration (Guzmán et al., 2009; Poortenaar & Pankhurst, 2000). Of the available exogenous hormones, gonadotropin releasing hormone analogues (GnRHa) have become extensively used and found to be effective and convenient for inducing oocyte development and maturation in captive finfishes (Mylonas & Zohar, 2000).

2.1.1 Oogenesis in fish

Most finfish are oviparous, although ovarian development in finfishes has been described to fall under three main types: (a) synchronous, (b) group synchronous and (c) asynchronous development (Lubzens et al., 2010). Moreover, ovulation and spawning can occur either once during a spawning season, or multiple times. For instance, Poortenaar and Pankhurst (2000) reported that greenback flounder (*Rhombosolea tapirina*) are multiple batch spawners with multiple group synchronous ovarian development. New Zealand turbot (*Colistium nudipinnis*) and brill (*Colistium guntheri*) also follow a similar pattern of ovarian development and spawning (Poortenaar et al., 2001). The spawning frequency of yellowbelly flounder is currently unknown.

Although the strategies of ovarian development and spawning may differ among species, the key steps involved in the process of oogenesis tend to be highly conserved among finfishes. During oogenesis, oocyte development is characterized by different morphological features

and significant oocyte growth (Lubzens et al., 2010). The ovarian follicle is comprised of the inner granulosa layer and outer thecal layer, which remain unchanged throughout growth (Patiño & Sullivan, 2002). Oogenesis begins with primordial germ cell (PGC) formation, which takes place in early development during embryogenesis (Yoshizaki et al., 2002), followed by sex differentiation which occurs prior to the onset of puberty. This represents the acquisition of the capacity for sexual reproduction, and the functional competence of the reproductive axis (Dufour & Rousseau, 2007).

Oocytes are produced in the ovaries when oogonia, which are proliferating mitotically, enter meiosis (Figure 2.1.) (Suwa & Yamashita, 2007). This is stimulated by estradiol (E2), and the first meiotic division is initiated by progestogens secreted in response to gonadal follicle stimulating hormone (FSH) regulation (Yaron & Levavi-Sivan, 2011). Formation of the ovarian follicle occurs following the initial onset of meiosis (Patiño & Sullivan, 2002). Previtellogenic growth encompasses the first two meiotic arrests, comprising two sub-stages often grouped holistically as previtellogenesis. The first stage, the 'chromatin nucleolus stage', is where oocytes characterized by large nucleoli are clustered in nests. Chromosome condensation proceeds and the oocytes become enveloped and separated by pre-follicle cells (Lubzens et al., 2017). Thereafter, the 'peri-nucleolus stage' is characterized by the envelopment of oocytes in a single layer of follicle cells followed by intracellular organelle proliferation. Early on during the peri-nucleolus stage, meiosis is arrested at prophase I for the remainder of primary and secondary growth (Lubzens et al., 2017). During the late primary growth phase, the oocyte progresses to the cortical alveolus stage, where glycoproteins are synthesized, forming cortical alveoli. These fill the oocyte periphery and increase in size and number throughout the remainder of previtellogenesis (Lubzens et al., 2010; Patiño & Sullivan, 2002). These later play an essential role in the prevention of polyspermy upon fertilization, through the hardening of the chorion by way of the cortical reaction (Lubzens et al., 2017; Wessel et al., 2001).

During the secondary growth stage, the oocyte undergoes vitellogenesis, where FSH regulation of E2 causes estrogenic stimulation of the liver, initiating the production of vitellogenins (yolk-precursor proteins) and choriogenins (Davail et al., 1998). These are transported back to the ovary and subsequently incorporated into the oocyte by receptor mediated endocytosis (Davail et al., 1998; Jun et al., 2018). Vitellogenesis encompasses the

incorporation of vitellogenin proteins, lipids, vitamins and other molecules by the oocyte and the resultant processing of these molecules into yolk proteins (Arukwe & Goksøyr, 2003; Lubzens et al., 2010).

At the completion of vitellogenesis, ovarian maturation and ovulation are initiated, and the oocyte becomes capable of undergoing fertilization (Lubzens et al., 2010; Patiño & Sullivan, 2002). This is initiated by a maturation inducing steroid (MIS), which stimulates the resumption of meiosis I following vitellogenesis (Pankhurst, 2008; Yaron & Levavi-Sivan, 2011)(Figure 2.1.). Luteinizing hormone (LH) is known to initiate final maturation, through the production of the MIS by the follicular cell layers (Nagahama, 1997; Nagahama & Yamashita, 2008). In some finfishes, an LH-driven shift from the production of E2 to the production of the dominant MIS, 17,20β-dihydroxy-4-pregnen-3-one (DHP), takes place within the ovary during the transition from vitellogenesis to final maturation (Nagahama et al., 1995; Ohta et al., 2002). Final maturation begins with the migration of the germinal vesicle to the oocyte periphery, followed by the breakdown of the germinal vesicle (Planas & Swanson, 2008). This corresponds to the first meiotic division and is essential for the expulsion of the first polar body from the oocyte (Figure 2.1.).

Following germinal vesicle migration, osmotic mechanisms result in a considerable influx of water into the oocyte, corresponding to oocyte hydration and a rapid size increase (Fabra et al., 2006; Fyhn et al., 1999). Ovulation requires the separation of the oocyte from the granulosa, followed by the rupturing of the follicular layer and expulsion of the oocyte into the ovarian cavity, where it is ready to be spawned (Bobe et al., 2008). The resumption of meiosis II and the expulsion of the second polar body is activated upon fertilization (Lubzens et al., 2017). Throughout oogenesis, the key stages of ovarian development are regulated through a complex cascade of hormonal signalling, and thus the process of oogenesis is hormone-dependent (Patiño & Sullivan, 2002).

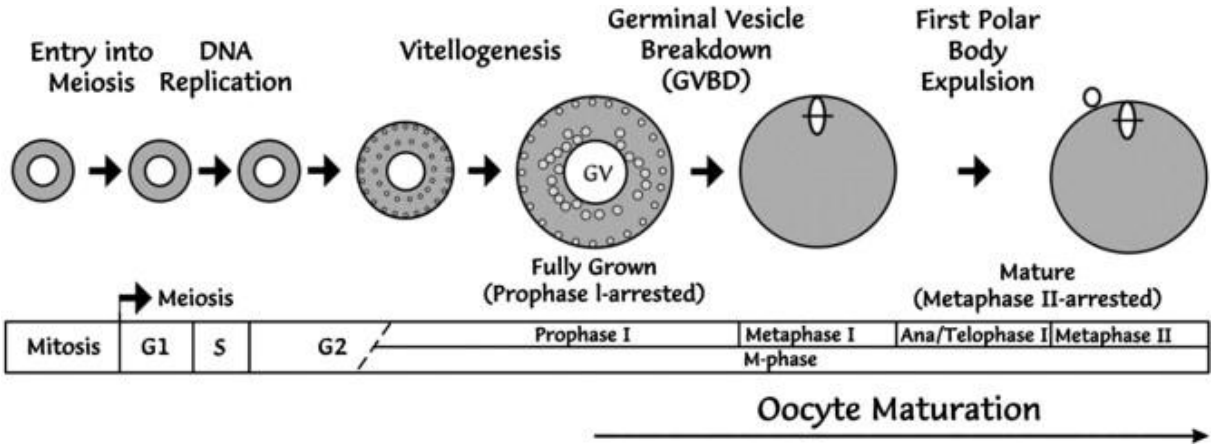

Figure 2.1. A simplified schematic diagram of oogenesis in teleost fishes in relation to meiosis. Sourced from Lubzens et al. (2010).

2.1.2 Reproductive dysfunction in cultured finfish

Although some species of cultured finfish successfully undergo oogenesis and spawn naturally within a captive environment, in many cases hormonal intervention is required to induce oocyte maturation and ovulation. Where captive female fishes are concerned, several forms of reproductive dysfunction are commonly experienced. This ranges from the inhibition of development prior to vitellogenesis, e.g. *Anguilla sp*. (Zohar & Mylonas, 2001), to the inhibition of final oocyte maturation following vitellogenesis (Mañanós et al., 2009), and the failure to undergo natural, spontaneous spawning, as seen in some Salmonids (Bromage et al., 1992) and Atlantic halibut (*Hippoglossus hippoglossus*) (Skaalsvik et al., 2015). The occurrence of reproductive dysfunction in broodstock is often attributed to a combination of the heightened stress associated with captivity, particularly in wild-caught broodstock (Jerez et al., 2018), alongside the absence of natural environmental stimuli associated with reproductive development (Mylonas et al., 2010). In almost all cultured flatfishes, some form of reproductive dysfunction has been observed and has limited the reproductive success in early culture as is reported in cultured Senegalese sole (*Solea senegalensis*) (Howell et al., 2009) and starry flounder (*Platichthys stellatus*) (Lim, 2016). Previous work with yellowbelly flounder at the Toi Ohomai Institute of Technology has indicated that wild-caught broodstock are prone to both acute and chronic capture stress and has identified that hormonal intervention is likely to be an important therapy to achieve successful and reliable reproduction in captivity (Jeffries, 2019).

2.1.3 Exogenous hormone induction of reproduction

In recent years, exogenous hormone treatment with GnRHa has proven effective at inducing reproduction in finfish. GnRHa treatments are associated with several advantages, one of which is that GnRH is highly conserved across species and is synthesized naturally within the hypothalamus. Therefore, GnRHa targets the upper tier of the BPG axis and triggers the natural synthesis and release of the pituitary gonadotropins, FSH and LH. This provides a more natural sequence of reproduction than is stimulated through the use of human chorionic gonadotropin (hCG) and carp pituitary extract (CPE) (Zohar & Mylonas, 2001). Several delivery methodologies exist for the administration of GnRHa, including: 1) intra-muscular injection (Guzmán et al., 2011; Lim, 2016), 2) intraperitoneal injection (Prat et al., 2001), and 3) implantation with sustained release GnRHa-containing pellets (Guzmán et al., 2011). These methods have had considerable success in several species, and notably GnRHa has been administered successfully in a variety of commercially important flatfish species. For example, the administration of GnRHa through both intra-muscular injection and sustained-release pellets in starry flounder reportedly increased egg quantity with no deleterious effect on egg quality (Lim, 2016). Furthermore, GnRHa has induced ovulation in European turbot (*Scophthalmus maximus)* (Mugnier et al., 2000) and Senegalese sole (Guzmán et al., 2009). Induction success using GnRHa in female broodstock may vary depending upon the oocyte developmental stage at the time of hormone administration e.g., vitellogenesis (Berlinsky et al., 1997), or the dose administered. The reported success of GnRHa at overcoming reproductive dysfunction in commercially cultured flatfishes suggests that this method may be viable for inducing reproduction in cultured yellowbelly flounder. This is essential for the development of yellowbelly flounder as a novel aquaculture species in New Zealand, due to the probability that this species will exhibit reproductive dysfunction under culture conditions.

2.1.4 Background

To understand the effect of GnRHa administration on the ovarian development of captive female yellowbelly flounder, it is critical to study the physical effects of the exogenous hormone on gonadal development. Although there is a vast array of research regarding the use of GnRHa for overcoming reproductive dysfunction in cultured flatfishes, the knowledge regarding the use of hormonal manipulations in yellowbelly flounder is limited. Further, there has not yet been a study that has assessed the effects of this hormone on ovarian

development over an extended period. Therefore, this study will extend the current knowledge regarding the suitability of GnRHa treatments to induce reproduction in female yellowbelly flounder for aquaculture.

2.1.5 Aims and objectives

The main aim of this research is to determine the effects of GnRHa treatment on the ovarian development in female yellowbelly flounder, *Rhombosolea leporina,* over a 28-day period using two different doses of GnRHa. In addition, the study will also be used to assess the efficacy of GnRHa to induce ovulation and overcome reproductive dysfunction in captive yellowbelly flounder. This will be assessed through the analysis of oocyte size and stage of development, as well as the occurrence of ovulation, batch fecundity and fertilization rate.

2.2 Methods

The experimental portion of this study was conducted between 10 June 2021 (first administration of hormone injections) and 10 July 2021 (date of final sampling) at Toi Ohomai Institute of Technologies' aquaculture facility in Tauranga, New Zealand. This period coincided with the spawning season of wild yellowbelly flounder in New Zealand and was selected to ensure that the gonadal development of most fish would be nearing maturity.

2.2.1 Fish capture and husbandry

Experiments were conducted on 39 wild adult (>25cm TL) yellowbelly flounder caught using drag netting and set netting in the Waikareao Estuary (-37.68523806117681°, 176.15727847198363°), Tauranga Harbour between 14 May and 4 June 2021. Following capture, fish were transported immediately in large bins full of oxygenated seawater to Toi Ohomai Institute of Technologies' indoor aquaculture facility and housed in six circular 1600L (depth 0.6m) recirculating seawater tanks. Fish were acclimatized to the holding tank water, by adding water from the holding tank to the transportation bins to increase the water temperature by 1°C. This was repeated every 20 minutes until the margin of difference between water temperatures was <1°C.

Fish were maintained at the aquaculture facility and were not fed prior to the commencement of the experiment. Photoperiod was maintained using LED tube lights (Osram Lumilux 58W -

840 Cool White) on a timer set to match the ambient photoperiod. Throughout the experiment, tanks and filters were cleaned when required and water quality parameters were maintained. Partial exchanges with fresh seawater were conducted routinely to keep ammonia, nitrite, and nitrate concentrations below the recommended 0ppm, 0ppm and 40ppm, respectively. Following commencement of the experiment, fish were fed live crabs (*Helice crassa* and *Macrophthalmus hirtipes)* as required and waste was siphoned out to retain water quality. Fish were not fed on sampling days due to the requirement for anesthetization.

2.2.2 Experimental set-up

One week prior to the commencement of the experiment, fish were placed in a 5L aerated seawater bath and anesthetized using 0.6 ml/L 2-phenoxyethanol (Sigma-Aldrich). Fish were retained in the anaesthetic bath until gill ventilation slowed and they were observed to be unresponsive to touch and unable to self-right when turned upside down. Individual weight (g), total length (cm) and sex were recorded. The sex was confirmed visually by using a flashlight underneath the fish to backlight the shape of the gonad. Koverman (2018) determined backlighting to be a reliable technique for determining the sex of yellowbelly flounder. The testes appear as a shorter and wider triangular shape, compared to the much longer, cylindrical shaped ovaries of maturing females. Fish were then placed in a recovery tank of clean aerated seawater and monitored to ensure proper gill ventilation and proprioception had returned before returning to the holding tanks.

One day prior to the experiment commencement, fish were assigned to one of three 1600L recirculating aquaculture system (RAS) tanks. This yielded; nine females and four males per tank. On the experiment start date, fish were treated with a single intraperitoneal injection of either one of two GnRHa treatments (Ovaplant – L, Syndel USA) of 50 µg/kg body weight or 100 µg/kg body weight, or with a sham (50 µg/kg body weight), which consisted of Ringer's solution for marine teleosts (Setiawan et al., 2016). Each fish was randomly assigned a treatment, with each tank assigned three females from each treatment (n=9). Two males in each tank (n=4) were assigned one of either the 50 µg/kg GnRHa or sham treatments. Broodstock were held in mixed tanks of males in females as this is often reported to increase reproductive maturation (Emata, 2003; Morretti, 1999). The weight of the female fish used in this study ranged from 254.2g to 829.24g.

The experiment start date was staggered by one day for each tank, due to the logistical time constraints of experimental sampling and analysing fresh tissue. On day 0, fish were anesthetized using an aerated seawater bath with 2-phenoxyethanol (0.6 ml/L). Fish were then weighed (nearest g), and ovarian biopsies were taken using gentle aspiration with a catheter (external diameter 20 mm) inserted through the genital pore into the upper ovarian lobe. Males were checked for milt using gentle abdominal pressure. Blood samples (0.5 ml) were also taken for future steroid analysis using a 25 gauge needle and 1 ml heparinized syringe. This was then spun at 1300 G for 3 minutes and the plasma collected and frozen at -80 °C for later use. Lastly, gonads were located through placement of a flashlight underneath the fish and each fish was injected intraperitoneally alongside the gonads with their allocated treatment (GnRHa or sham), using an 18 gauge needle. Medical grade tissue glue was used to seal the needle puncture and prevent seepage of the GnRHa or Ringer's solution following injection. Fish were returned to a recovery tank and monitored until they resumed normal gill ventilation and proprioception and transferred back to their tank.

The above procedure was repeated on days 3, 7, 14, 21 and 28, however treatment injections were only administered on day 0.

2.2.3 **Ovulation**

During the first two weeks of the experiment, fish were checked daily for ovulation by visual assessment of external swelling over the ovarian region of the abdomen. Fish were assessed to have reached maximal ovarian distention when there was a distinct and marked longitudinal swelling across the entire length of the abdominal cavity (Figure 2.2.). At this point, they were anesthetized using 0.6 ml/L 2-phenoxyethanol, weighed (g) and checked for signs of ovulation by applying gentle abdominal pressure across the ovary. Fish were considered to have ovulated when eggs were freely expressed from the genital pore with this treatment. If ovulation was evident, the fish were manually stripped through gentle pressure applied from the anterior end of the ovary towards the genital pore, into a clean, dry plastic bowl. Contamination of blood, urine, seawater, and mucus was avoided. The fish was placed into a recovery tank before being transported back to their holding tank. Following egg collection, eggs were transferred to a sterile 100 ml measuring cylinder and egg volume was measured and converted into egg quantity. The number of eggs/ml were based on counts (n=10) of

subsamples of 1 ml of eggs which determined that 1 ml corresponds to approximately 3,500 eggs.

2.2.4 Fertilization

Males were anesthetized and the genital papilla was dried to remove any seawater or urine present to prevent contamination of sperm. Milt was stripped from 2-3 males using gentle abdominal pressure from the anterior end of the testes towards the genital papilla and collected in a pipette. To assess sperm motility, one drop of milt was activated using seawater on a glass slide under 400x magnification on a compound microscope. If high motility was observed, then artificial fertilization was initiated. For artificial fertilization, 10 µl of milt per 1 ml of eggs was gently mixed until milt and eggs became homogenous (dry fertilization). Immediately following, 500 ml of seawater was added for sperm activation. The eggs and sperm were stirred and left for approximately 3 minutes. Eggs were transferred to a sterile 1 L beaker and egg viability was assessed by recording the volume of buoyant (viable) and sinking (unviable) eggs after 15 minutes. Fertilization was assessed 2-3 hours post-fertilization at the 4-8 cell stage by examination of eggs under a compound microscope (Figure 2.3.). Fertilization percentage was determined by the average percentage of developing eggs in a sub-sample of 100 (n=3).

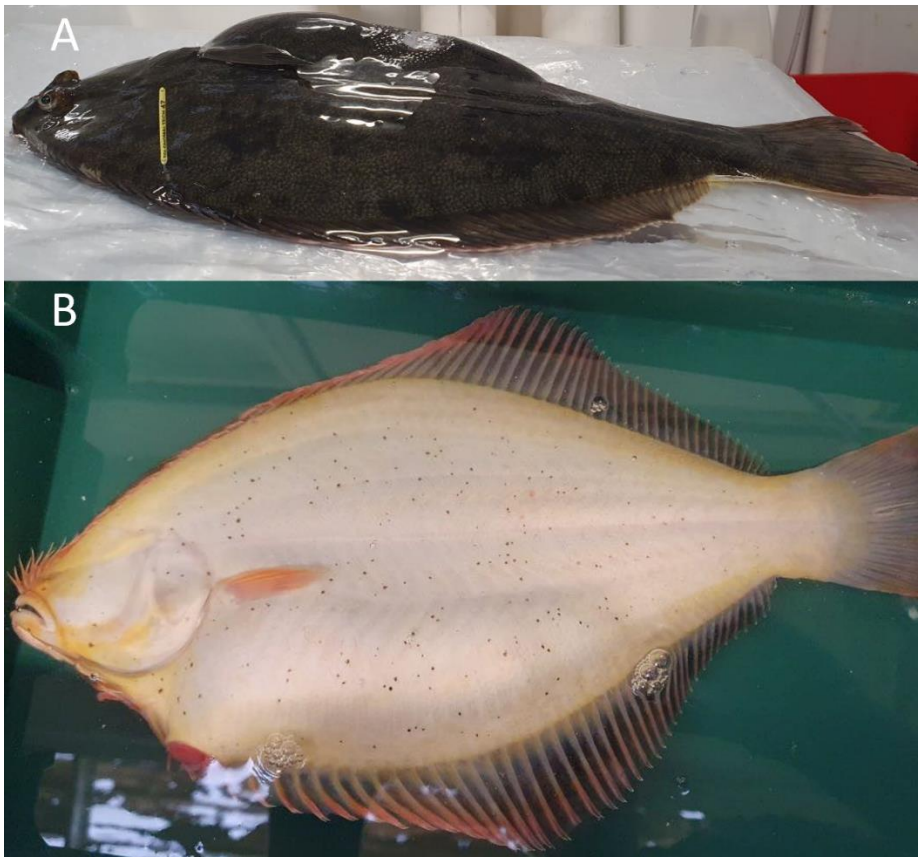

Figure 2.2. The left lateral (A) and ventral (B) view of an adult female yellowbelly flounder with maximal abdominal swelling.

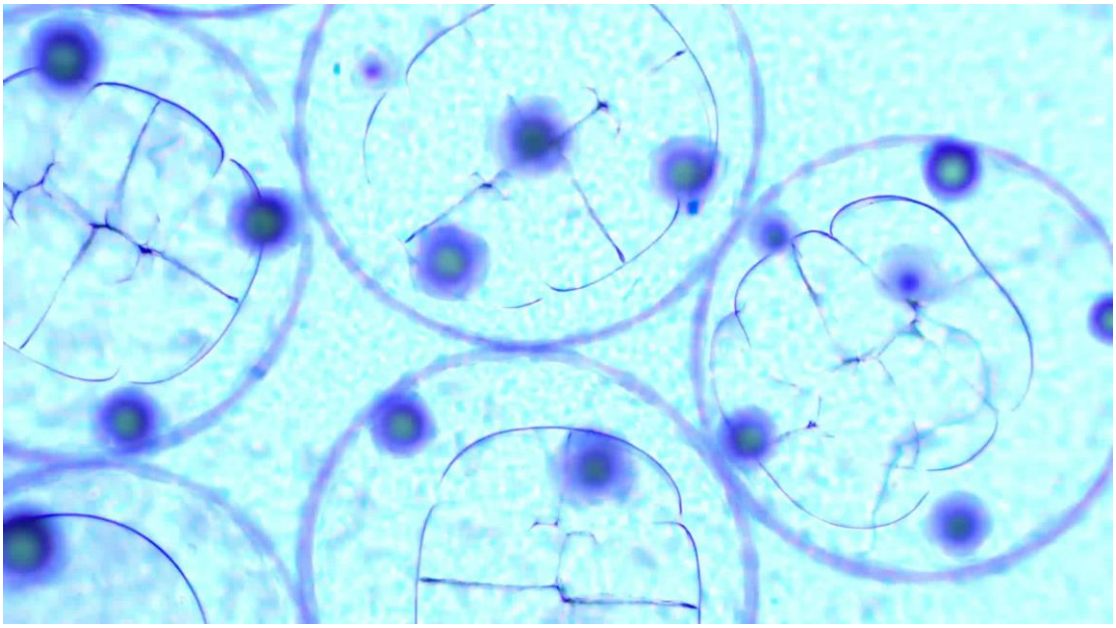

Figure 2.3. The morphology of yellowbelly flounder blastomeres in the eight-cell stage.

2.2.5 Oocyte diameters

Following ovarian biopsies, ovarian tissue samples from each female were split into two sub-samples; one was immediately fixed for histological analysis, and the other used immediately to record fresh oocyte diameter and size frequency. Samples for fresh observation were dispersed in a petri dish using Serra's clearing solution. This was left to sit for a few minutes to allow for morphological classification by determining the position or presence of the germinal vesicle. Oocytes were inspected using an Olympus SZ61 stereo microscope and images of at least 50 oocytes from each sample taken with an Olympus EP50 microscope camera. Images of a 0.01 mm micrometer slide were taken at the same magnification as the oocyte images. Fresh oocyte diameters were measured using ImageJ software (bundled with Java 1.8.0_172). Micrometer slide photos were used to calibrate the measurements on ImageJ, and two diameter measurements were taken at perpendicular angles for oocytes of each fish on every sampling day. The average of the two measurements was taken and determined to be the diameter of each oocyte. Measurements of 50 oocytes from each fish were taken to ensure a representative sample size of oocytes for each fish was procured. The measurement of fresh oocyte samples prevents the underestimation of oocyte diameter resulting from post fixation shrinkage.

2.2.6 Histological analysis

Macroscopic staging of oocytes was verified through examination of ovarian tissue prepared for histology. Following ovarian biopsies, ovarian tissue was fixed in 10% buffered formalin for no longer than 24 hours. This was then replaced with 70% ethanol until being processed for light microscopy. Samples were progressively dehydrated with ethanol and xylene and then infiltrated and embedded in paraffin. They were sectioned at 3 µm and stained with haematoxylin and eosin. Examination and photography of histological slides was carried out using an Olympus CX23 binocular microscope with an Olympus EP50 microscope camera. Ovarian developmental stage was assessed according to the histological characteristics of the leading oocyte cohort and were classified into the following categories: 1) previtellogenic (PV), 2) cortical alveolar (CA), 3) early vitellogenic (EV), 4) late vitellogenic (LV), 5) germinal vesicle migration (GVM), 6) hydrated (HY) or 7) atretic (AT). Criteria for staging of oocytes into these classifications are given in Table 2.4**.** Leading cohort oocytes are defined as the most advanced stage of oocytes present within the ovaries.

2.2.7 Data analysis

All data analysis was done using R version 4.04. Graphs were created using the ggplot package. Single factor ANOVAs were used to detect differences in mortality, body weight and the occurrence of hydrated oocytes among the three treatment groups. A Tukey's HSD test was used for posthoc testing. Statistical tests were run with a confidence interval of <0.05

2.3 Results

2.3.1 Fish condition

Initial body weight of female yellowbelly flounder was not significantly different among treatments (ANOVA, p = 0.63). Individual body weight ranged between 826.94 grams and 254.2 grams. The highest mean weight was in the 100 µg/ kg^{-1} GnRHa treated group, whereas the sham treated group had the lowest mean body weight (Table 2.1.)

Table 2.1. Body weight of female yellowbelly flounder prior to injection with GnRHa. Mean ± SE.

Treatment	Number of females	Initial body weight (g)
Control	9	612.3 ± 157.1
GnRHa 50 µg/kg	9	665.6 ± 111.6
GnRHa 100 µg/kg	9	667.5 ± 109.9

2.3.2 Ovulation, fertilization, and egg viability

As evidenced by ovulation, hormonal induction of ovulation for yellowbelly flounder was successful with GnRHa at different doses of 50 or 100 µg/kg. During the study, a total of seven batches of eggs were collected, representing four fish which successfully responded to GnRHa treatment. During the study, three females in the 50 µg/kg treatment group successfully ovulated, and one fish from the 100 µg/kg group, while ovulation was absent in the control group. The average time to ovulation post-injection was considerably different between the 50 µg/kg and 100 µg/kg fish, being 4 ± 0.58 days and 28 days, respectively. All but one fish that ovulated during the experiment ovulated twice, with the period between first ovulations and all repeat ovulations being three days. Histological imaging of oocytes revealed 55% of the 50 µg/kg treated fish and 22% of 100 µg/kg treated fish had HY oocytes at some stage throughout

the experiment. The number of GnRHa treated fish that developed hydrated oocytes was significantly more than that reported in control fish (ANOVA p = 0.02), as there was no evidence of HY oocytes in control fish (Figure 2.6.). Hydrated oocyte diameter ranged in size from 545 to 805 µm (Table 2.4.). Throughout the 4-week experiment, 16 female mortalities were recorded, which were not significantly related to GnRHa treatment (ANOVA p = 0.335).

Fecundity (the number of eggs produced per ovulation), and fertilization rates were highly varied throughout this experiment. Fecundity ranged from 12-105ml in the 50 µg/kg treated group, with the volume of viable eggs ranging from 0-97ml (Table 2.2.). Within this group the maximum viable egg volume recorded was 97mL, correlating to approximately 339, 500 viable eggs (Table 2.2.). From both initial and repeat ovulations, mean total fecundity (100 g BW) was considerably higher for the 50 µg/kg treatment group (33,915 ± 7435 and 10854 ± 4047, respectively) than for the 100 µg/kg group (17,877 and 3465, respectively) (Table 2.3.). In both treatment groups, total fecundity in repeat ovulations was considerably less than for initial ovulations (Table 2.3.), and buoyancy decreased from the first to second ovulation (Table 2.3.). Fertilization rates for initial ovulations ranged from 0-84% in the 50 µg/kg treatment group (Table 2.2.), with a mean of 30 ± 27 (% ± SE)(Table 2.3.), and 80% in the 100 µg/kg treatment group (Table 2.2.), with a mean of 80% (Table 2.3.). In all instances, fertilization was absent in repeat ovulations (Table 2.2.).

Table 2.2. Fish that ovulated in each treatment group and the number of days after treatment that ovulation occurred. Data on egg volume, viable egg quantity, fertilization rate and time to hatch is included.

Fish #	Treatment µg/kg	Days after treatment	Fecundity (ml)	Viable egg volume (ml)	Est. number of viable eggs	Fertilization rate	Hatch time (Hours after fertilization)
18	50	4	50	33	115,500	6%	48-60
		7	25	1	3,500	0	N/A
47	50	5	105	97	339,500	84%	52
		8	12	0	0	0	N/A
5	50	3	50	42	147,000	0	N/A
44	100	28	45	N/A	N/A	80%	N/A
		31	8	N/A	N/A	N/A	N/A

Table 2.3. Effect of GnRHa treatment on total fecundity, egg buoyancy and fertilization rate from initial and repeat ovulations. Mean ± SE.

Treatment	Fecundity (100 g BW) (Initial ovulation)	Buoyancy (%)	Fertilization (%)	Fecundity (100 g BW) (Repeat ovulation)	Buoyancy (%)	Fertilization (%)
Control	-	-	-	-	-	-
50 µg/kg GnRHa	33,915 ± 7435	80.7 ± 7.7	30 ± 27	10854 ± 4047	2 ± 2	0
100 µg/kg GnRHa	17,877	-	80	3465	-	-

2.3.3 Fresh oocyte-size frequency

Throughout the 4-week experimental period, fresh oocyte size frequency distributions indicate distinct heterogeneous sized cohorts of oocytes developing simultaneously. Unfortunately, day 0 ovarian biopsies for fresh oocyte size-frequency analysis were not collected due to the available catheters proving unsuitable. During the study, oocyte diameters between 0-100 µm, corresponding to PV oocytes (Table 2.4.) were highly prevalent in both GnRHa treated and control groups (Figure 2.4.). In general, the majority of oocytes, particularly from control females, were below 600 µm in diameter (Figure 2.4.), corresponding to stages of PV, CA, EV, LV and GVM (Table 2.4.). On all days, the distribution for control females was highly positively skewed, peaking at 1-100 µm with a continuous distribution from the smallest to the largest oocytes (Figure 2.4a-e). The oocyte size distribution of the 50 µg/kg group on day 3 was bimodal, with peaks at both 0-100 µm and 400-500 µm (Figure 2.4f), corresponding to a high incidence of PV and GVM stage oocytes (Table 2.4.). On all following sampling days, the oocyte diameter distribution was positively skewed with the peak at 0-100 µm (Figure 2.4g-j). In the 100 µg/kg group, the size frequency distribution on all days was unimodal and positively skewed, with the peak at 0-100 µm and a continuous distribution from the smallest to largest oocytes (Figure 2.4k-o).

Maximum oocyte diameters between 800-900 µm were observed in the 50 µg/kg group on day 7 and day 28, which corresponded to HY oocytes (Table 2.4.), and oocytes larger than 800 µm were observed only in GnRHa treated females. From day 21 onwards, oocytes larger than 700µm, consisting of HY oocytes, were present only in the GnRHa treated groups (Figure 2.4.). On day 28, the oocyte size distribution of GnRHa treated groups is clearly different relative to controls.

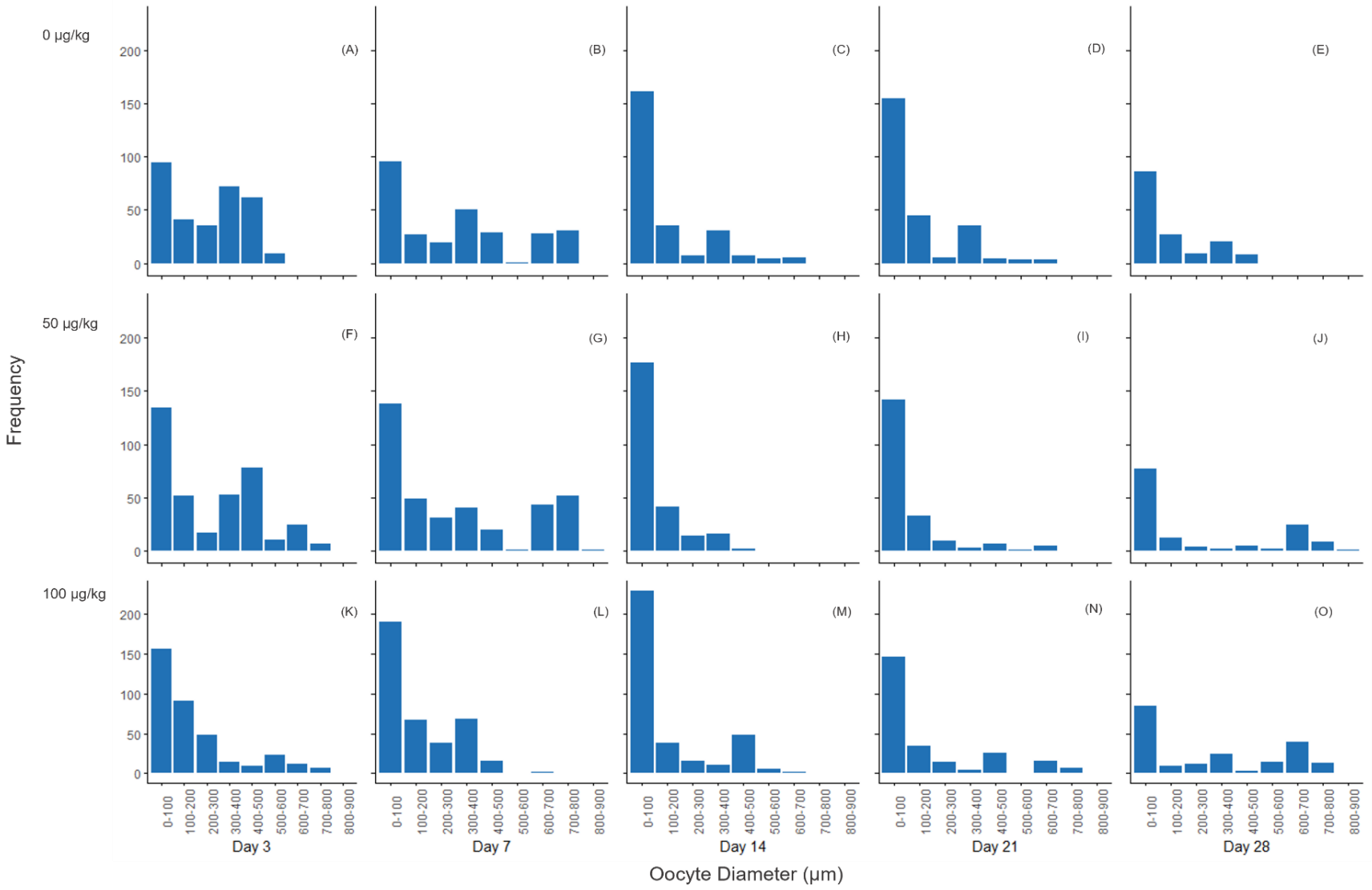

Figure 2.4. Fresh oocyte diameter size frequencies in female yellowbelly flounder treated with sham (0 µg/kg) or GnRHa injection at 50 or 100 µg/kg throughout the 28-day experimental period. Each histogram corresponds to the diameters of 50 oocytes from each fish on each sampling day within each treatment group.

Table 2.4. Stages of ovarian development and oocyte maturation in yellowbelly flounder (*Rhombosolea leporina)* based on the histological and fresh appearance of the leading cohort oocytes. Modified from Koverman (2018).

Ovarian development stage	Oocyte maturation classification	Description of histological appearance of oocyte	Oocyte diameter (µm)	Description of fresh oocyte appearance	Fresh Oocyte diameter (µm)
Previtellogenesis	1.Previtellogenic (PV)	Multiple nucleoli present at the periphery of centrally located germinal vesicle. Ooplasm is basophilic and zona radiata not yet visible in most oocytes, however, can be seen in the most mature.	15-117	Centrally located germinal vesicle takes up much of the oocyte, appearing dark. Ooplasm is translucent. No evidence of oil droplets.	16-152
Cortical alveolar phase	2.Cortical Alveolar (CA)	Oocyte increased significantly in size. Cortical alveoli granules now present surrounding ooplasm periphery and small oil droplets present perinuclearly in some cases. Nucleoli remain present at the periphery of centrally located germinal vesicle. Ooplasm remains basophilic and zona radiata now visible.	97-187	Oocyte increased significantly in size. In some cases, small oil droplets may be visible at germinal vesicle periphery.	155-264
Vitellogenesis	3.Early Vitellogenesis (EV)	Oocyte increased in size. Small vitellogenin granules are now present within the ooplasm, originating at the ooplasm periphery. Cortical alveoli granules and oil droplets may increase in number and size. Nucleoli may now be seen throughout germinal vesicle. Zona radiata continues to thicken and is clearly visible.	110-240	Oocyte increased significantly in size. Germinal vesicle centrally located. Multiple large oil droplets now visible surrounding centrally located germinal vesicle. Zona radiata can be seen. In later stage oocytes, vitellogenin granules may become visible.	191-401
	4.Late Vitellogenesis (LV)	Oocyte increased in size. Vitellogenin granules have increased in size and number, occupying most of the ooplasm. Remaining visible cortical alveoli granules remain located at the ooplasm periphery. Oil droplets remain perinuclear, but may have coalesced to form fewer, larger oil droplets. Germinal vesicle remains centrally located with nucleoli present throughout. Increase in thickness of zona radiata.	204-365		

Ovarian development stage	Oocyte maturation classification	Description of histological appearance of oocyte	Oocyte diameter (µm)	Description of fresh oocyte appearance	Fresh Oocyte diameter (µm)
Final oocyte maturation	5.Germinal Vesicle Migration (GVM)	Oocyte increased in size. Vitellogenin granules may begin coalescing into large masses. Few large oil droplets remain within the ooplasm. Germinal vesicle has begun migrating from its centrally located position to the periphery of the ooplasm.	209-396	Germinal vesicle located off centre, migrating towards oocyte periphery. Coalescence of vitellogenin granules may be visible in some cases. Large oil droplets may also be visible surrounding germinal vesicle.	311-539
	6.Hydrated (HY)	Oocytes increase in size further and are irregular in shape. Vitellogenin granules and oil droplets have all coalesced into one large mass taking up the entirety of the oocyte. Zona radiata has detached.	344-586	Oocyte increased significantly in size. Germinal vesicle no longer present. Oocyte appears smooth and translucent with visible oil droplets.	545-805
Atresia	7.Atretic (AT)	Oocytes appear misshapen and ooplasm and vitellogenin granules are degrading and breaking down. Breakdown and eventual disappearance of zona radiata visible.	69-337	Oocyte appears misshapen and deflated. May also appear cloudy. Germinal vesicle is no longer visible.	59-579

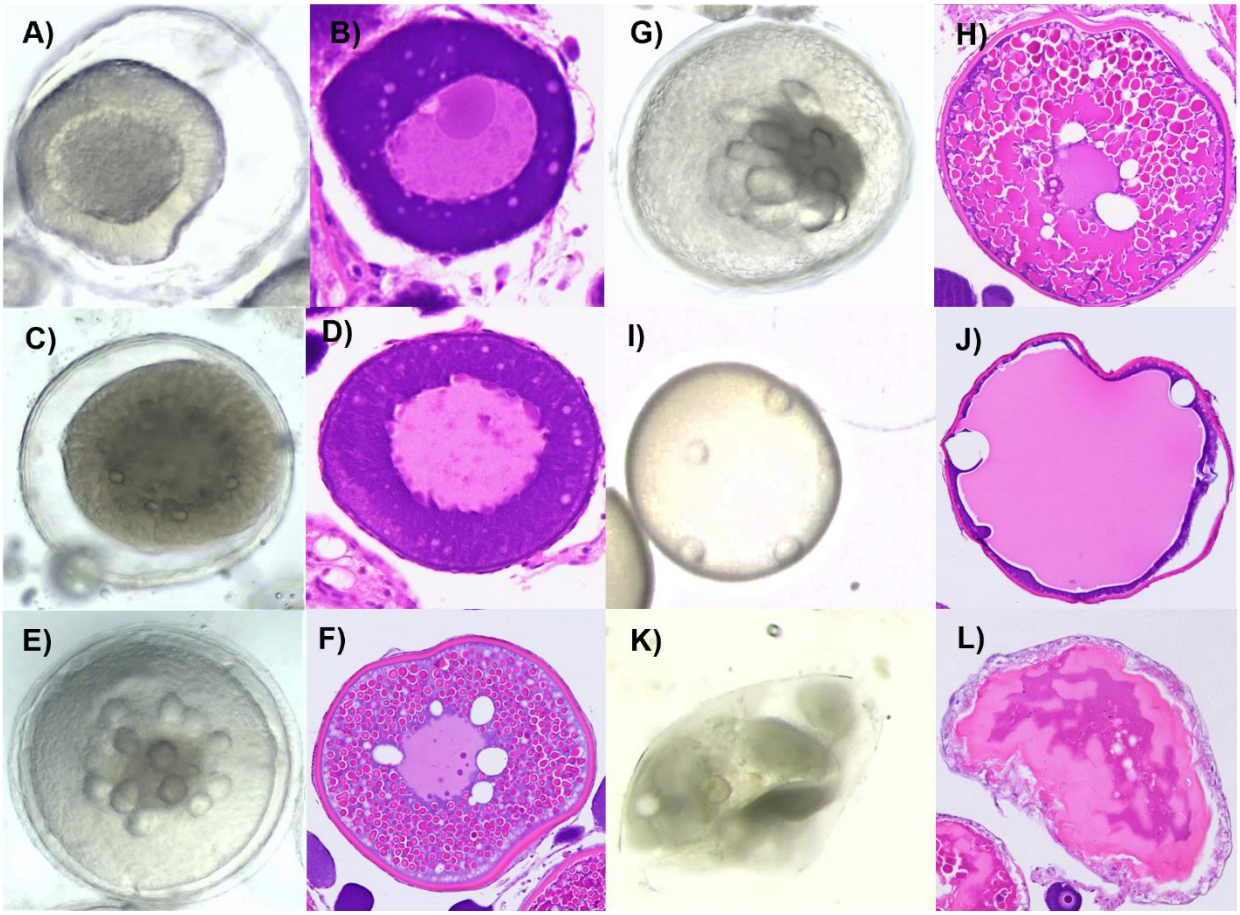

Figure 2.5. Oocyte development stages of adult yellowbelly flounder ovaries observed as fresh oocytes and in histological slides: (A) previtellogenic, (B) previtellogenic, (C) cortical alveoli, (D) cortical alveoli, (E) vitellogenic, (F) vitellogenic, (G) germinal vesicle migration, (H) germinal vesicle migration, (I) hydrated, (J) hydrated, (K) atretic and (L) atretic.

2.3.4 Histology

Based upon morphological characteristics from fresh oocyte appearance, histological analysis and the findings of Koverman (2018), a seven-stage oocyte development and maturation classification index was developed for yellowbelly flounder (Table 2.4.). All oocyte developmental stages could be easily distinguished and classified through histological analysis using light microscopy (Figure 2.5.).

On the day of hormone administration (Day 0), ovarian biopsies were successfully collected from only five fish. The leading cohort oocytes for all five fish were classified at GVM stage. On all subsequent sampling days, cohorts of different oocyte stages were obtained from every female regardless of treatment. The only exception to this was in a few individuals where only PV oocytes were present. While non-treated fish did mature to the GVM stage, seven GnRHa-

treated fish contained oocytes that had progressed to become fully hydrated (five and two fish each from the 50 µg/kg and 100 µg/kg groups, respectively) (Figure 2.6.). Therefore, oocytes from PV to GVM were present in non-GnRHa treated fish (including atretic oocytes), whereas oocytes at all stages from PV to HY were present in some GnRHa treated fish. GVM stage oocytes were present on all sampling days in the control group, although oocyte maturation past GVM did not occur (Figure 2.6.). HY oocytes were present in fish treated with 50 µg/kg on days 3, 7 and 28, compared to days 21 and 28 in 100 µg/kg treated fish (Figure 2.6.). Histological evidence of oocyte development beyond day 21 is lacking in the control group, whereas oocyte development was continued in both GnRHa treatment groups on day 28, confirmed by the presence of HY oocytes (Figure 2.6.).

2.3.5 **Follicular atresia**

AT oocytes were present in the ovaries of many fish, regardless of treatment (Figure 2.6.). Five control females had oocytes that progressed through to GVM (Figure 2.6.), however histological observations revealed that partway through GVM the oocytes became atretic and failed to mature further. This pattern of oocytes undergoing GVM and regressing before maturation was also observed in four of the GnRHa treated fish.

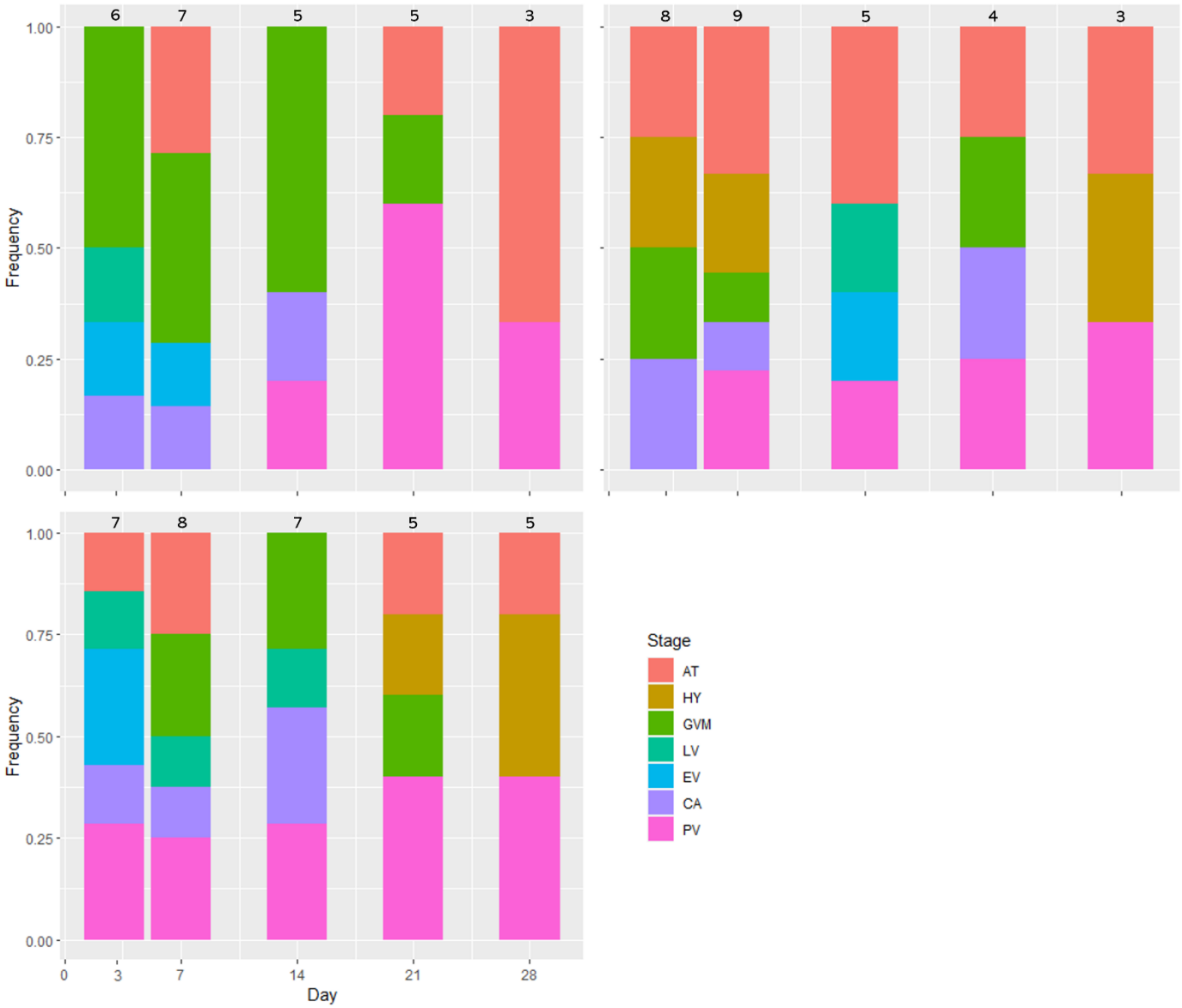

Figure 2.6. Proportion of yellowbelly flounder treated with a) 0 µg/kg, b) 50 µg/kg and c) 100 µg/kg at each leading cohort oocyte stage versus sampling day. Legend shows leading cohort oocyte stage. Numbers above bars represent the number of fish sampled.

2.4 Discussion

This study investigated the efficacy of induced ovulation in captive yellowbelly flounder using treatment with a gonadotropin releasing hormone analogue. Treatment with a single GnRHa injection successfully induced ovulation in adult female yellowbelly flounder.

2.4.1 Reproductive strategies

Throughout evolution, a variety of reproductive strategies have arisen in finfishes, including multiple spawning strategies and patterns of ovarian development (Lubzens et al., 2010;

Mañanós et al., 2009). The reproductive strategy that a species utilizes will have implications for its aquaculture. The frequency of ovulation of the fish in this study confirmed that yellowbelly flounder are indeed multiple batch spawners. At least three fish were observed to ovulate twice during the course of the experiment, confirming the suggestion by Koverman (2018) that multiple batch spawning is the likely spawning strategy in yellowbelly flounder based on histology. This also disproves past suggestions that they are capable of only a single spawning event each season (Colman, 1973; Mutoro, 2001). Multiple batch spawning is prevalent among a range of other flatfishes including greenback flounder (Earl, 2014), starry flounder (Lim, 2016), New Zealand turbot and brill (Poortenaar et al., 2001). The occurrence of multiple ovulations in response to GnRHa treatment is also similar to other cultured flatfishes (Lim, 2016). In this study, the second ovulation occurred almost exactly three days after the first, indicating that the ovulatory periodicity of yellowbelly flounder may be as short as 72 hours. This was comparable to the 72-80 hour ovulatory periodicity reported for Atlantic halibut (Norberg et al., 1991). However, it should be recognized that this ovulatory period may differ in unmanipulated wild fish. The fact that yellowbelly flounder are multiple batch spawners means that an appropriate protocol for the administration of GnRHa will need to be optimized if it is to be used as a reproductive therapeutic. It is commonly reported that certain sustained release hormones can be beneficial for optimizing the quality, quantity and occurrence of multiple ovulations in species that display multiple batch spawning (Guzmán et al., 2009; Zohar & Mylonas, 2001).

Past histological analysis of yellowbelly flounder ovaries has reported the existence of multiple group synchronous ovarian development (Koverman, 2018). This conclusion is further corroborated in the ovarian biopsies from the current study. Fresh oocyte frequency distributions showed the occurrence of distinct cohorts of heterogeneously sized oocytes. Histological analysis validated that these cohorts of oocytes were at different stages of development. In all instances, the greatest frequency of oocytes ranged between 0-100 µm, which corresponds to PV oocytes. This implies the existence of a larger reserve of PV oocytes from which cohorts of oocytes are recruited to undergo development and ultimately yield batches of ovulated eggs. Collectively, this evidence confirms that ovarian development in yellowbelly flounder is multiple group synchronous, as previously indicated by Koverman (2018).

2.4.2 Spawning in captivity

Despite the successful induction of ovulation in this study, there was a complete absence of spontaneous spawning. This indicates that similar to Atlantic Halibut (Skaalsvik et al., 2015), hand stripping is likely to be the dominant method of egg collection for the culture of this species. Although some species do undergo spontaneous spawning in captivity, this is often in F1 and later generations, and less frequently in wild caught broodstock (Martín et al., 2019; Pankhurst & Fitzgibbon, 2006). Further, spontaneous spawning in some flatfishes, such as greenback flounder (Pankhurst & Fitzgibbon, 2006), requires specific husbandry conditions that were not applied in the present study. Courtship and spawning behaviours have been reported in captive southern flounder (*Paralichthys lethostigma*) and greenback flounder. In both species, males swim alongside the female, with their genital pores aligned. The male directs the female to the water's surface, following which sperm is released and eggs spawned simultaneously at the surface (Pankhurst & Fitzgibbon, 2006; Smith et al., 1999). Thus, the lack of spontaneous spawning in yellowbelly flounder may relate to the absence of critical courtship due to unsuitable tank conditions. Further, spontaneous spawning occurred in southern flounder held in a water depth of 1.7m (Smith et al., 1999), compared to 0.6m in our study. Correspondingly, Colman (1973) suggested that wild yellowbelly flounder require a certain water depth before successful spawning can take place, with fish moving from shallow benthic flats to depths between 12-30m for spawning. In addition, for natural spawning in some captive finfishes, sex ratios greater than 1 male per female has been recommended (Emata, 2003; Morretti, 1999), compared to the 0.44:1 sex ratio in the current study. Collectively, these suggest that increased water depth and an increased ratio of males to females may be beneficial for captive yellowbelly flounder to complete courtship, although current data cannot assess whether this is the case. Therefore, this could form part of future investigations.

Spawning in numerous finfishes reportedly takes place during the scotophase, where daylight is absent, as the risk from visual predators to eggs is reduced. For example, spawning in captive greenback flounder occurs around 2 hours prior to dawn (Pankhurst & Fitzgibbon, 2006), and evening spawning has been observed in captive red spotted grouper (*Epinephelus akaara*) (Okumura et al., 2002) and wild New Zealand turbot (Poortenaar et al., 2001). Further, a nocturnal spawning rhythm has been reported for Senegalese sole (Oliveira et al., 2009).

Observations from the current study suggest that ovulation in yellowbelly flounder may also exhibit daily rhythms. Fish that appeared imminently ready for hand-stripping at midday failed to easily release eggs until the following morning, when eggs could be freely stripped with little effort. This has implications for aquaculture as reduced egg viability has been reported for many species when eggs remain in the oviduct for extended periods after ovulation. Reduced egg viability may occur within 3-6 hours post ovulation in some marine species (Hobby & Pankhurst, 1997; Rasines et al., 2012). Determining the daily timing of ovulation in yellowbelly flounder, as well as the time before reduced egg viability occurs will be critical for optimizing egg collection protocols.

2.4.3 Reproductive dysfunction in captivity

There were no ovulations recorded in the control group. This is not unusual as reproductive dysfunction is common in cultured finfishes (Mehdi & Ehsan, 2011). Despite this, considerable abdominal swelling was observed in a single control fish. Histological analysis proved that extensive atresia had occurred once the oocytes had progressed to GVM. Furthermore, histological staging of oocytes revealed that an additional four control females, and a few females in both GnRHa treatment groups, displayed extensive atresia of GVM oocytes. Follicular atresia in teleost fishes has been described as a degenerative process where granulosa cells begin to phagocytose the oocyte (Corriero et al., 2021). Atresia of GVM oocytes suggests that oocyte development in captive yellowbelly flounder proceeds normally until entering GVM. Presumably, stress inhibits the reproductive axis resulting in a failure of oocyte hydration. Although these results are only at a pilot scale, they imply that treatment with an exogenous GnRHa of at least 50 µg/kg enables GVM to be completed and oocyte hydration and ovulation to occur. Failure to complete final oocyte maturation is the most common form of reproductive dysfunction reported in finfish aquaculture (Zohar & Mylonas, 2001). Similar results have been reported in captive Senegalese sole, which was linked to lower circulating levels of E2 and T and/or inadequate environmental factors resulting in the pituitary failing to release LH, and subsequently the MIS (García-López et al., 2007). Furthermore, it is well documented that stress-induced secretion of cortisol can have deleterious effects on the reproductive axis, particularly through induced follicular atresia (Corriero et al., 2021). Previous studies on captive flatfishes have reported that high stocking density and netting through chasing, may induce a heightened stress response (Barnett & Pankhurst, 1998). It is

therefore likely that the stress of capture and captivity in addition to the repeated handing during the experiment has induced reproductive dysfunction in the yellowbelly flounder from this study. This is further supported by the prolonged time observed for many of the fish to start feeding in captivity. Reduced cortisol, and the associated stress response, is commonly reported for captive reared fish, such as F1 generation greater amberjack (Jerez et al., 2018). Consequently, the development of an F1 generation broodstock is an important objective in aquaculture. Cortisol levels and the stress response of captive yellowbelly flounder are yet to be characterised and this is therefore an avenue of future research interest.

Although successful ovulation occurred only in GnRHa treated fish, treatment did not always lead to successful ovulation. Ovulation occurred in 33% of fish treated with 50 µg/kg GnRHa and 11% of fish injected with 100 µg/kg GnRHa. Nonetheless, it is recognized that unobserved ovulation may have occurred in some of the fish as they were passively observed through the water surface rather than being handled between sampling days. Histological analysis from sampling days revealed HY oocytes in seven of the GnRHa treated females. Although only four were observed to have ovulated so that manual stripping was possible, oocytes of 55% and 22% of the 50 µg/kg and 100 µg/kg GnRHa treated fish, respectively, reached the HY stage. This was a significant increase compared to the lack of HY oocytes in control fish. This data suggests that GnRHa is effective at inducing yellowbelly flounder to complete final oocyte maturation and enter hydration. In addition, biopsies from some 50 µg/kg treated fish on day 14 and 100 µg/kg treated fish on day 28 were dominated by a mass of AT oocytes. The presence of large volumes of resorbing oocytes may indicate prior unspawned ovulations. Fish that require stripping in captivity may resorb oocytes through atresia (Mañanós et al., 2009). Collectively these findings may indicate a greater number of unobserved ovulations in both treatment groups due to sampling protocol. The ovulation rates in this study were greatly reduced compared to those of GnRHa treated southern flounder (Wright-Moore et al., 2019) and European turbot (Mugnier et al., 2000), where ovulation occurred in 100% of the fish. However, F1 generation broodstock were used in these studies, and GnRHa administered via sustained-release pellets. Contrastingly, this study used wild-caught broodstock and intra-peritoneal injections. This may indicate that F1 generation broodstock are desirable for high ovulation success and sustained-release pellets may be a more effective means of inducing ovulation than a single injection in flatfishes.

The efficacy of GnRHa treatment is related to both the hormone dosage and the stage of ovarian development. The optimal dosage for consistent ovulation varies greatly among species. Doses of GnRHa greater than 50 µg/kg have been reported to result in fewer ovulations in other captive flatfishes (Poortenaar & Pankhurst, 2000), although the ideal dosage is generally species-specific. For example, injection of GnRHa at 4 µg/kg has been reported as successful for inducing ovulation in African catfish *(Clarias gariepinus*) (Shokr, 2020), whereas repeated doses at 20 µg/kg has shown success in longfin yellowtail (*Seriola rivoliana*) (Fernández-Palacios et al., 2015). Dosage may also negatively impact egg quality (Avery et al., 2004; Duncan et al., 2012; Setiawan et al., 2016).

Reduced efficacy of GnRHa treatment has also been observed in fish where oocyte development is insufficient to respond to the subsequent LH signalling. In some species, GnRHa has been found to induce only LH production (Mateos et al., 2002; Nyuji et al., 2019). This may be contributing to the low rates of ovulation observed in the present study. In summer flounder (*Paralichthys denatus*), for instance, GnRHa was ineffective at inducing ovulation and growth of oocytes where the initial oocyte diameter was <500 µm (Berlinsky et al., 1997). Further, in studies with high rates of GnRHa induced ovulation success, fish are often treated when vitellogenic or post-vitellogenic (Mugnier et al., 2000; Wright-Moore et al., 2019). Thus, in yellowbelly flounder it would be beneficial to GnRHa treat fish with oocytes at late vitellogenic/germinal vesicle migration stage, at a minimum size of 311 µm, to ensure advanced ovarian maturity at the time of hormone administration. The present study was hindered by the lack of ovarian biopsies collected on the first sampling day, due to issues with the catheters. This made assessment of oocyte development and the corresponding suitability of individual fish to GnRHa treatment impossible. A few individuals had ovaries with only PV oocytes present throughout the entire sampling period, which indicates that these fish were not undergoing reproductive development at the time of the study. Further, anecdotal observations indicate the possibility of a bimodal spawning pattern in yellowbelly flounder populations. Thus, we suggest that multiple spawning groups may exist in wild populations across their reported 7-month spawning season (Koverman, 2018). This could contribute to the complete lack of reproductive development in some fish and failure to respond to GnRHa treatment. Colman (1973) reported early and late spawning groups for sand flounder (*Rhombosolea plebia*), where spawning coincides with falling and rising water temperatures, respectively. Thus, yellowbelly flounder may exhibit a similar spawning pattern, however as

this is anecdotal, confirmation requires further investigation. One of the goals of this study was to develop a classification index to determine ovarian developmental based on the macroscopic appearance of fresh oocytes. This will greatly assist similar studies in the future.

The delayed ovulation observed in a 100 µg/kg treatment fish raises the question of excessive GnRHa dosing. The fish ovulated 28 days after treatment, with histological analysis confirming hydrated oocytes did not develop in this fish until day 21. Although, similar response periods have been observed in GnRHa treated Senegalese sole, which first spawned 21 days post-injection, dose did not affect ovulation time (Guzmán et al., 2009). In contrast, yellowbelly flounder injected with 50 µg/kg of GnRHa ovulated in approximately 72 hours. An additional individual from the 100 µg/kg treatment also showed abdominal swelling shortly after the conclusion of the experiment (28 days). Consequently, these observations may be indicative of higher GnRHa dosages inhibiting the reproductive axis until becoming reduced to lower physiological levels. Perhaps the level of GnRHa overstimulated the receptor in the pituitary, resulting in this receptor becoming temporarily desensitized. Continuous GnRHa receptor stimulation and its subsequent desensitization from exogenous GnRH treatment has been reported previously (Neill, 2002). Furthermore, Habibi (1991) reported excessive GnRHa induced desensitization in goldfish pituitary. Whilst the delayed ovulation reported in the present study is anecdotal due to low numbers, and the mechanisms cannot be determined, doses of GnRHa below 100 µg/kg are advisable for yellowbelly flounder. Further research to characterize the circulating levels of plasma GnRHa following treatment would be of value.

2.4.4 Fecundity and fertilization

Fecundity reported in this study was considerably greater than that reported in the literature for other flatfishes. For example, fecundity (100 g BW) for 100 µg/kg GnRHa treated starry flounder was 3,907 ± 1302 eggs, compared to 17,877 eggs from yellowbelly flounder injected with the same volume and 33,915 ± 7435 eggs for 50 µg/kg GnRHa treated yellowbelly flounder in the present study. However, egg volume produced in the second ovulations of yellowbelly flounder were considerably less than in the initial ovulations. This may be due to the method of hormone administration. It is well recognized in the literature that both administration of GnRHa via sustained release pellets or multiple injections have been beneficial in increasing egg volumes in batch spawning flatfishes (Guzmán et al., 2009;

Mugnier et al., 2000). It remains to be determined whether alternate methods of hormone administration methods might increase fecundity in repeat ovulations in this species.

It was not possible to compare the effect of GnRHa treatments on fecundity and fertilization due to the limited ovulations that occurred during this experiment. Egg quality was, however, variable with fertilization rates ranging between 0-84%. Hormone induced spawning can reduce fertilization, such as in southern flounder where fertilization was reported in 40% of viable eggs (Watanabe et al., 2001), and 38.2% in 50 µg/kg GnRHa treated southern flounder (Wright-Moore et al., 2019). Contrastingly, the current study showed that high fertilization rates (>80%) are achievable in yellowbelly flounder using GnRHa treatment. Although this study recorded low fertilization rates in some instances, the exact timing of ovulation is not yet known for yellowbelly flounder, therefore post-ovulatory oocytes may have contributed to this.

It is important to note that the size of hydrated oocytes in yellowbelly flounder in this study were very small, with a maximum size of 805 µm. Therefore, these eggs are likely to yield very small larvae, which will have implications for larval production protocols. In particular this may pose a challenge for first feeding. Rotifers are a common live feed provided for first feeding in marine finfish larvae (Hamre, 2016), although the small mouth size of some species' larvae may require their prey at first feeding to be smaller than rotifers (Leu et al., 2010). In such cases, an alternate smaller live feed such as copepod nauplii is likely to be required (Gopakumar & Santhosh, 2009; Leu et al., 2010). This will be an important consideration for larval rearing.

2.4.5 Mortalities

Within the last two weeks of the experiment, an unidentified fungal infection resulted in the loss of a large proportion of the broodstock. This is not uncommon when working with recently wild-caught broodstock. Broodstock mortality due to infection and disease is a recurrent problem in many cultured finfishes (Sampaio et al., 2008). Stress experienced by fishes, such as that experienced by wild caught broodstock, increases vulnerability to infection and disease through immune suppression (Wendelaar Bonga, 1997). Further, due to the nature of aquaculture systems, wild-caught broodstock may be infected with parasites and diseases that result in high mortalities under culture conditions (Walker & Winton, 2010). To

overcome this limitation in future research, wild caught broodstock should be prophylactically treated for parasite removal and prevention of diseases which can result in high mortalities in culture systems.

2.4.6 Summary

This study provides the first attempt at induced reproduction using exogenous hormone treatment in a New Zealand flatfish. This research confirmed for the first time that yellowbelly flounder are multiple batch spawners. Yellowbelly flounder successfully ovulated in response to GnRHa and showed that both high fecundity and fertilization rates are achievable using this method. GnRHa treatment yielded a significant increase in fish completing final oocyte maturation. The frequency of ovulation, however, was inconsistent, and this was likely due to some fish having an inappropriate stage of ovarian development at the time of GnRHa treatment. Failure to ovulate in the higher 100 µg/kg GnRHa treatment may have been compounded by excessive doses. At least one individual in the 100 µg/kg group ovulated after 28 days. It is also possible that some ovulations in the GnRHa treatment groups were not observed and recorded during the course of the experiment. Further refinement is required to identify an optimal dose to maximize the number of ovulations as well as egg quality and fecundity for repeat ovulations. Overall, these results indicate that GnRHa administration should be a viable methodology to induce ovulation in captive yellowbelly flounder.

Chapter 3

Transcriptomic analysis and characterisation of key genes involved in the reproductive axis

3.1 Introduction

Within vertebrates, teleost fish have evolved considerable diversity in their reproductive strategies, having either external or internal fertilization and with certain lineages displaying hermaphroditism (Smith & Wootton, 2016). However, despite this exceptional diversity, the physiological and morphological changes that occur during oogenesis and spermatogenesis are largely consistent. Most studies to date regarding reproduction in teleosts have either focused on: 1) the levels of reproductive hormones present at different stages of reproductive maturity, or 2) reproductive success based on spawning performance, measured through egg and sperm quality and quantity (García-López et al., 2007; Hong et al., 2021; Lim, 2016; Setiawan et al., 2016). More recently, investigations have been carried out to understand the molecular and cellular mechanisms involved in reproductive processes. Some of this work has been particularly focused on the genes involved in teleost reproductive processes and their expression patterns at different stages of reproductive development (Hollander-Cohen et al., 2021; Jia et al., 2021; Wang et al., 2018). Determining the genes involved during reproduction within cultured finfish species and their expression patterns will provide important insights into the physiological events that occur throughout reproduction. This information will be invaluable for the successful development and optimization of existing and new fish species for aquaculture.

3.1.1 Omics in aquaculture

'Omic' technologies, such as genomics, transcriptomics, proteomics and metabolomics, have been utilized in recent years to better characterize aspects of reproduction, immunity, growth, nutrition and toxicology of many cultured finfishes (Jia et al., 2021; Natnan et al., 2021). The first marine fish species to undergo full genome sequencing, using whole-genome shotgun sequencing, was the pufferfish (*Fugu rubripes*). This species was originally chosen due to the

small size of its genome and for the purposes of comparison with the human genome sequence (Aparicio et al., 2002). Two decades later, the genomes of over 200 fish species have now been sequenced (Lu & Luo, 2020), and this is growing rapidly, with genomes of key cultured marine fish species having been fully sequenced in recent years, such as the olive flounder (*Paralichthys olivaceus*) (Shao et al., 2017). The fact that fish genomes are generally smaller than the human genome, ranging in size from 342 Mb (*Tetraodon nigroviridis*) to 2967 Mb (*Salmo salar*) helps in the sequencing of fish genomes (Yuan et al., 2018). However, it is next generation sequencing (NGS) technologies that have truly transformed genome sequencing and provided researchers with new ways to understand genes involved in physiological processes, through transcriptomics. This has allowed the identification of key genes whose functions contribute to the reproductive process in economically important fish species (Bar et al., 2016; Hu et al., 2016; Marín-Juez et al., 2011), and a better understanding of their regulation of gonadal development.

3.1.2 Transcriptomics

Transcriptomic approaches are being increasingly used within aquaculture research, due to their ease of use and the significant decrease in their cost over the last decade. The term 'transcriptomics' first started to be used in the 1980's, but only gained popularity within the 1990's due to the development of a number of new technologies. The first was expression microarrays (Wheelan et al., 2008), where a large number of gene probes were immobilized on a membrane or slide, and the sample to be queried was labelled and hybridized to the surface. The second was Expressed Sequence Tags (ESTs), which are fragments of mRNA sequences that were derived through a single sequencing reaction, performed on randomly selected clones from a cDNA library (Bouck & Vision, 2007). With the availability of these approaches, studies into the transcriptome of fish began in the late 1990's, and considerable effort was directed towards species such as olive flounder (Inoue et al., 1997), channel catfish (*Ictalurus punctatus*) (Ju et al., 2000; Kocabas et al., 2002) and zebrafish (*Danio rerio*) (Zeng & Gong, 2002). However, these early transcriptomic technologies have become replaced by modern next generation sequencing (NGS) approaches such as RNA-seq. This allows the direct sequencing of whole transcriptomes from any living organism and uses high-throughput sequencing technologies, such as Illumina IG, Roche 454 Life Science and Applied Biosystems SOLiD (Morozova et al., 2009; Wang et al., 2009). Resulting reads can be assembled *de novo*

where a genome has not yet been determined for a particular organism, or aligned to a reference genome or available gene transcripts (Wang et al., 2009). This more modern approach exhibits expansive benefits over its more basic and outdated counterparts, including significantly lower costs accompanied by higher throughputs (Qian et al., 2014; Wang et al., 2009). This has led to a rapid expansion of transcriptome data available for a wide range of organisms, including those used for aquaculture.

Transcriptomic technologies are primarily aimed at gene expression at the mRNA level and provide useful information regarding how individual genes are differentially expressed and their functional roles (Lowe et al., 2017). This makes it possible to identify and characterize critical genes involved in particular physiological processes or traits of commercial interest for cultured fish (Wang et al., 2009). Having these genes and pathways identified will allow for the improvement of current aquaculture techniques. A transcriptome can be defined as the complete set of transcripts in a specific cell, tissue or organism at any given time, which includes both the protein-coding messenger RNA (mRNA) and non-coding RNAs such as ribosomal RNA (rRNA) and transfer RNA (tRNA) (Qian et al., 2014). Unlike the genome, the transcriptome is not static and instead varies according to numerous factors, including environmental condition and developmental stage. The potential to utilize NGS technologies for analysis of the transcriptome and the differential regulation of critical genes under specified experimental or culture conditions, has been central to the identification of transcriptomics as a powerful tool for the future development of aquaculture (Cerda & Manchado, 2013).

Transcriptomic approaches and sequencing of the genes involved in reproduction have provided valuable information in studies of numerous cultured fish species. For example, transcriptome analysis in male European turbot (*Scophthalamus maximus)* has provided evidence for the differential expression of genes between developmental stages of spermatogenesis and allowed for key genes and pathways regulating spermatogenesis to be identified (Wang et al., 2018). Further, gene expression patterns are reported to correspond to specific stages of ovarian development and maturation in female teleosts (Martyniuk et al., 2013). Moreover, studies utilizing transcriptomic analysis of ovaries in teleosts have provided insight into how the transcriptional response can be correlated to the morphological and physiological changes taking place within the ovary. For instance, in Senegalese sole (*Solea*

senegalensis) oligonucleotide microarrays have been used to assess the transcriptome profile during ovarian development in adults and have helped identify a number of genes involved in ovarian development (Tingaud-Sequeira et al., 2009).

3.1.3 Vertebrate oogenesis

Among vertebrate groups, major differences in reproductive strategies are prevalent, as is evident between mammals and teleosts (Jalabert, 2005). However, across all groups, spermatogenesis and oogenesis are dynamic developmental processes which require complex regulation of gene expression. In all vertebrates, the brain-pituitary gonad (BPG) axis is well recognized for its pivotal role in the neuroendocrine regulation of reproduction (Dubois et al., 2002). Thus, gene expression across the BPG axis is central to reproduction. Some genes identified to have considerable importance and differential expression depending on reproductive status are conserved across species and taxonomic groups, such as the *VASA* gene, which is reported to play an essential role in germ cell development in mammals as well as fish (Castrillon et al., 2000; Qu et al., 2020).

Certain pathways within the reproductive axis are essential for oogenesis in vertebrates. One such pathway is the neuroendocrine pathway involved in pituitary gonadotropin release. Although the hypothalamic decapeptide, gonadotropin releasing hormone (GnRH), is generally considered the initial component of the BPG axis, evidence now supports the upstream regulation of GnRH via the kisspeptin system in mammals (Gahete et al., 2016). Once in circulation, GnRH stimulates the pituitary secretion of two essential gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in all vertebrate groups to induce sex steroid biosynthesis (Dubois et al., 2002). In teleosts however, the kisspeptin system is known to play a role in regulating reproduction, but the specific actions of kisspeptins are currently unclear (Somoza et al., 2020). Further, *Kiss* genes have reportedly been lost through evolution in birds (Pasquier et al., 2014). Following pituitary gonadotropin release, the sex steroid biosynthesis pathway is an essential component of the reproductive axis. Estradiol is the key estrogen involved in oocyte development in female mammalian and non-mammalian vertebrates, and the reactions involved in the estradiol biosynthesis pathway are catalyzed by various enzymes (Magoffin, 2005; Tenugu et al., 2021). Thus, differential expression of a vast array of genes is pivotal for the functioning of the reproductive axis in all vertebrates.

3.1.4 Genes involved in teleost reproduction

During teleost reproduction, different genes are involved with different processes. Many of these genes are reported to be highly conserved across fish species, and thus play a consistent and predictable role throughout the reproductive process. With the development of transcriptomic technologies, patterns of gene expression throughout reproduction and their specific functions through development have been explored in some finfish species (Jia et al., 2021; Martyniuk et al., 2013; Tingaud-Sequeira et al., 2009). Studies utilizing transcriptomic analysis of ovaries in teleosts have provided insight into gene expression patterns that correspond to specific stages of ovarian development and maturation in female teleosts. In largemouth bass, analysing ovaries at eight morphologically diverse stages of development identified the differential expression of 552 and 2070 genes during ovulation and oocyte atresia, respectively (Martyniuk et al., 2013). In female topmouth (*Culter alburnus*), an important aquaculture species in China (Jia et al., 2021), RNA-seq technology was recently applied to identify genes and pathways related to reproduction. This investigation gave important insights into the differential expression of candidate genes seen in fish with malformed gynogenic ovaries versus healthy ovaries.

The characterization and expression of genes present across two key pathways involved in teleost oogenesis, the neuroendocrine pathway for pituitary gonadotropin synthesis (Figure 3.1.) and the estradiol biosynthesis pathway (Figure 3.2.), have been extensively studied to provide an understanding of the genes controlling oogenesis. These pathways are essential for regulating oogenesis in all vertebrates, and among teleosts the genes involved are highly conserved. The pathway responsible for pituitary gonadotropin production occurs across the entire reproductive axis (Figure 3.1.). In teleosts, the kisspeptin system is suggested to be a key regulator of this pathway. These kisspeptins, encoded by KISS genes, act via their receptor *GPR54*. The exact mechanisms through which kisspeptins act is currently unclear in fish, and in some teleosts, such as striped bass, *Gpr54* is expressed on the GnRH neuron located within the hypothalamus (Zmora et al., 2012). In other species, co-expression of *Gpr54* on GnRH neurons is absent, and instead kisspeptins may act directly on the pituitary to stimulate gonadotropin release (Espigares et al., 2015). In contrast to the single *Kiss1* gene found in mammalian vertebrates, two kisspeptins have been identified in teleosts, encoded by *Kiss1* and *Kiss2* (Felip et al., 2009; Selvaraj et al., 2010), which arose from a whole genome

duplication event (Steinke et al., 2006). The pituitary gonadotropins, FSH and LH, synthesized within this pathway, are heterodimers each composed of a common glycoprotein α subunit (*GTHα*) and an individual gonadotropin hormone β subunit, *Fshβ* and *Lhβ*, encoded by separate genes (Levavi-Sivan et al., 2010). The downstream targets of these hormones are their respective receptors, *Fshr* and *Lhr*, expressed on the gonads, where they mediate steroidogenesis through regulating sex steroid production (Jia & Lei, 2019).

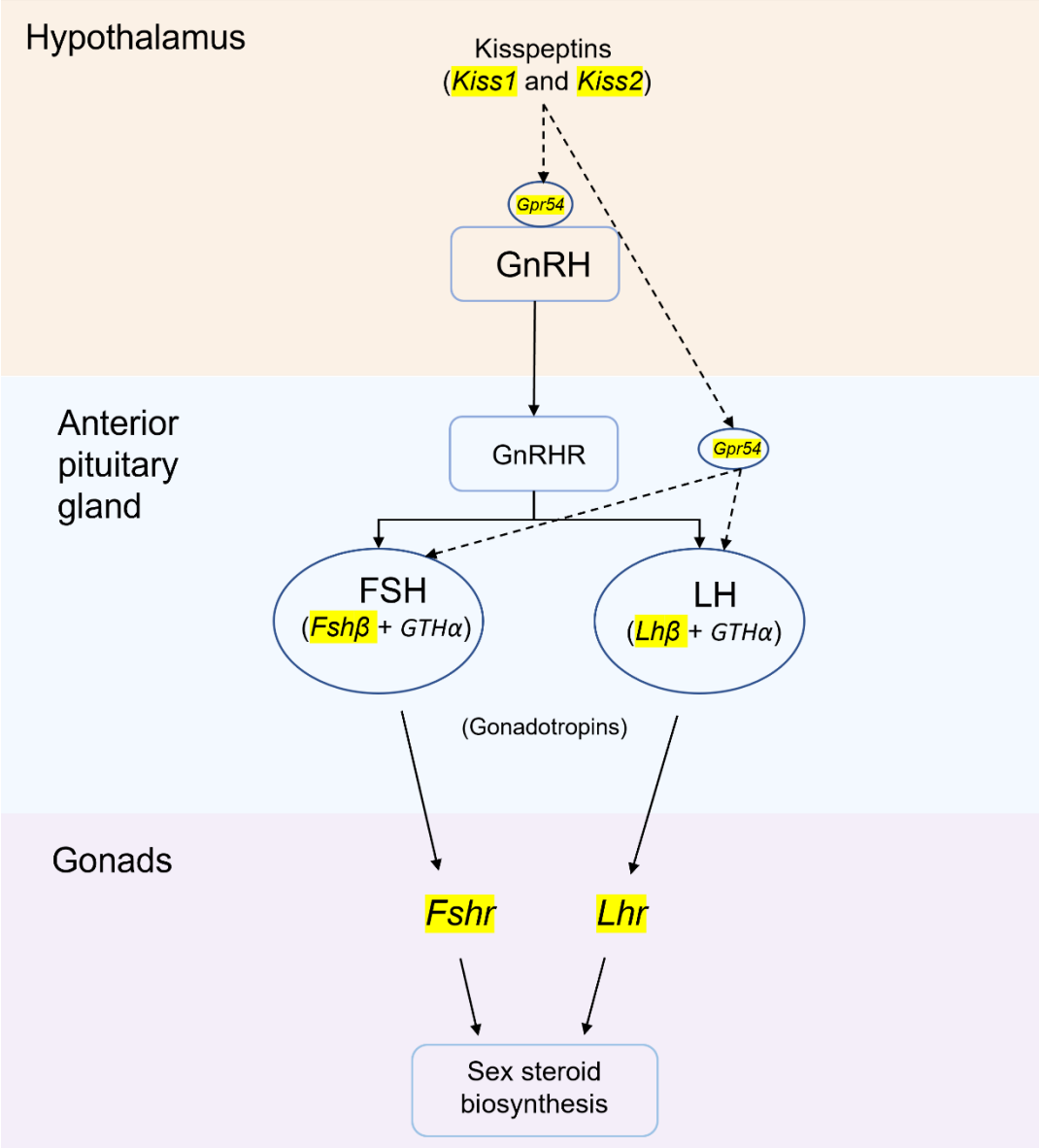

Figure 3.1. Simplified hypothetical pathway for pituitary gonadotropin synthesis. Solid lines represent confirmed pathways that remain consistent among species. Dotted lines represent two potential kisspeptin pathways which differ among species. Target genes for the present study are highlighted in yellow.

Estradiol is one of the key sex steroids in female teleosts (Yaron & Levavi-Sivan, 2011). The steps of the estradiol biosynthesis pathway in the ovaries occur within the follicular cell layers. Initiation of the pathway occurs via the steroidogenic acute regulatory protein (*StAR*) initiated mobilization of cholesterol within the thecal cells, from the outer mitochondrial membrane to the inner mitochondrial membrane (Tenugu et al., 2021). Subsequently, each step in the estradiol biosynthesis pathway is catalysed by steroidogenic enzymes, including cytochrome p450 side-chain cleavage (*cyp11a1*), steroidogenic cytochrome P450 17-hydroxylase/lyase (*cyp17*), hsd3b1, hydroxysteroid 17β-dehydrogenase 1, (*hsd17b1*) and cytochrome P450, family 19, subfamily a, polypeptide 1 (*cyp19a1a/b*) (Tenugu et al., 2021)**.** Thus, this pathway involves the complex expression of various genes encoding these enzymes (Figure 3.2.).

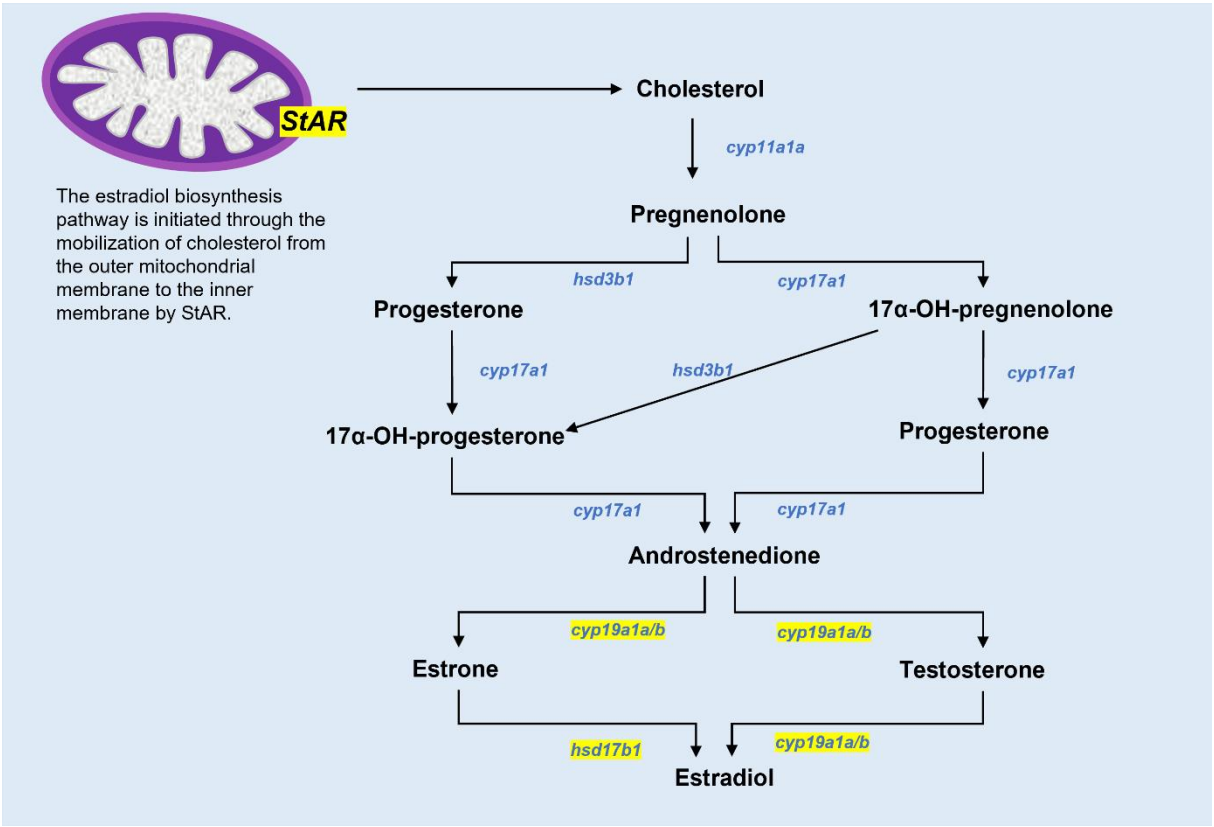

Figure 3.2. Schematic representation of the steroidogenic pathway for estradiol (E2) biosynthesis in teleosts. The initiation of the pathway by the *StAR* mediated mobilisation of cholesterol to the inner mitochondrial membrane in the thecal cells and the major enzymes that catalyze the reactions for steroid synthesis. Target genes for the present study are highlighted in yellow. Abbreviations are: cyp, cytochrome P450; OH-, hydoxy-; hsd, hydroxysteroid dehydrogenase; StAR, steroidogenic acute regulatory protein. Modified from Tenugu et al. (2021).

3.1.5 Genes involved in flatfish reproduction

Flatfishes are a diverse family of fishes, and several studies sequencing the genome and transcriptome of commercially important cultured flatfishes have already been undertaken. In some flatfish, such as European turbot (Figueras et al., 2016) and tongue sole (*Cynoglossus semilaevis)* (Chen et al., 2014), whole genomes are now available. This will help improve the quality of research possible for these species, while at the same time provide reference genomes to assist with the identification of genes in closely related commercially valuable flatfish, until their genomes are sequenced. Similarly, transcriptomic data is not available regarding sexual reproduction in many flatfishes, with the majority of transcriptomic studies having largely focused on sex differentiation, metamorphosis, disease and immune-related genes (Labella et al., 2018; Louro et al., 2020; Ribas et al., 2016; Ronza et al., 2021; Shao et al., 2017). A considerable amount of transcriptomic analyses of reproduction has been completed for Senegalese sole, where the differential expression and characterization of genes involved in both spermatogenesis (Forne et al., 2011) and oogenesis (Tingaud-Sequeira et al., 2009) have been identified, including the differential expression of 118 genes during ovarian development, maturation and atresia. In male European turbot, transcriptome analysis has provided evidence for the differential expression of genes between developmental stages of spermatogenesis and allowed for key genes and pathways regulating spermatogenesis to be identified (Wang et al., 2018).

3.1.6 Oogenesis in yellowbelly flounder

Despite significant advances in transcriptomic technologies, limited knowledge exists regarding the important genes that control the processes involved in the reproduction of many cultured marine fish. This presents an obstacle to the optimization of breeding programs in cultured fishes and therefore the identification and characterization of these genes is often acknowledged as an important area of focus for aquaculture research. However, with the increased use of transcriptomic approaches, it is now possible to start investigations into the reproduction of any cultured fish species, where previously no gene information had existed. Despite the mass of transcriptomic studies that have been conducted on many flatfishes, there is currently no transcriptomic information available for any species of New Zealand flatfish. This is due to the lack of commercial production or development of any flatfish species for culture in New Zealand. Yellowbelly flounder (*Rhombosolea leporina*) is a flatfish that has been

targeted as a candidate species for aquaculture. To date, no studies have been carried out in in this species using NGS technologies, and consequently there have not been any genes characterized involved in the reproduction of this species. To establish this species within the New Zealand aquaculture industry, such information is essential for advancing the understanding of their reproduction. This investigation will use RNA-seq within selected tissues of reproductively mature female yellowbelly flounder, to help identify important genes involved in the oogenesis. The availability of these genes within this species will allow for the development of critical tools for the advancement of this species for aquaculture and inform the further optimization of reproduction under culture conditions.

3.2 Aims and objectives

This chapter presents the discovery of genes involved in reproduction using the first available transcriptome data obtained from female yellowbelly flounder (*Rhombosolea leporina*). Here we characterize and identify important genes involved in the reproductive process which will be invaluable in developing a greater understanding of the reproductive physiology of yellowbelly flounder. This study aims to use known protein sequences from other flatfish species, to mine the transcriptome from yellowbelly flounder to identify functional genes that are present and expressed within the gonad, brain, and pituitary. Genes we attempted to characterize in this study are previously highlighted in Figure 3.1. and Figure 3.2.

3.3 Methods

3.3.1 Capture

Three mature yellowbelly flounder (>30 cm TL) were caught by a commercial fisherman in May 2022, within the Manukau Harbour, Auckland, via set netting. Following capture, fish were held in an outdoor tank until relocation to the Toi Ohomai Institute of Technologies' indoor aquaculture facility one week later. Once transported to Toi Ohomai, fish were housed in 1600L tanks on a recirculating aquaculture system. Sex of each fish was determined to be female through backlighting the gonad (see Chapter 2, Section 2.2.2.). Fish were dissected 2 days following their arrival at Toi Ohomai Institute of Technology.

3.3.2 Dissection

Following anaesthetization with 1 ml/L 2-phenoxyethanol, fish were weighed to the nearest gram, measured from the tip of the snout to the end of the caudal fin, and then quickly dispatched through decapitation. The brain, pituitary, ovary, spleen and head kidney tissues were dissected out of each fish under sterile conditions. Sections of each tissue were immediately fixed in RNA*later®* (Thermo Fisher, USA) in RNase-free 1.5ml centrifuge tubes. Tissues larger than 5mm were cut into smaller pieces <5mm to ensure sufficient penetration of the solute. Samples were refrigerated at 4°C for 24 hours before storage at -80°C until transportation for transcriptomic analysis. Tissues were transported on dry ice (-80°C) one week following sample collection for transcriptome sequencing by BGI Genomics, China.

At the time of dissection, small samples of ovarian tissue from each fish were also fixed in 1ml buffered formalin for no longer than 24 hours and stored in 70% ethanol for histological analysis. Samples were stored at room temperature until processing for light microscopy (Otago). Samples were progressively dehydrated with ethanol and xylene and then infiltrated and embedded in paraffin. They were sectioned at 3 µm and stained with haematoxylin and eosin.

3.3.3 Histology

Oocyte development stage for each fish was classified according to the stage of the leading cohort. Criteria for oocyte developmental stages are outlined in Chapter 2, Table 2.4. This was carried out using an Olympus CX23 binocular microscope with an Olympus EP50 microscope camera.

3.3.4 Transcriptome sequencing, *de novo* assembly and bioinformatics

Transcriptomic sequencing was carried out by BGI, China. Total RNA was extracted from the brain, pituitary and gonad tissues from the three different female flounder using the RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. The extracted RNA was then quantified and assessed for integrity using the NanoDrop (Thermo, USA) and 2100 Agilent Bioanalyzer (Agilent, USA) prior to library preparation. A PE150 strand-specific library was prepared using an MGIEasy RNA Library Prep Set (MGI, China). Briefly, poly-A containing mRNA molecules were purified from 10 ng -1 µg of total RNA using poly-T oligo-attached magnetic beads. Next, using divalent cations under elevated temperature, the mRNA was

fragmented into small pieces and the fragments transcribed into cDNA using reverse transcriptase and random primers, followed by a second strand synthesis. A single 'A' base was added to each cDNA fragment and an adaptor subsequently ligated to it. These ligated fragments were purified and enriched using PCR amplification to produce the cDNA library. After quantification, each enriched library was subjected to single strand circularized DNA molecule (ssDNA circle) preparation to produce the final transcriptome library. The generation of DNA nanoballs (DNBs), to intensify the fluorescent signals during the sequencing process was achieved using the ssDNA circle and rolling circle replication (RCR). These DNBs were loaded onto a patterned nanoarray and >20M clean pair-end reads of 150 bp were read through on the BGISEQ-500 platform for each tissue. Raw sequencing reads were subsequently filtered to obtain clean reads and the raw FASTQ file used for subsequent analysis.

3.3.5 Database searching and sequence analysis

A range of key reproductive genes involved in the estradiol biosynthesis pathway and pituitary gonadotropin synthesis pathway were identified in the published literature and selected as genes to target within the yellowbelly flounder brain, pituitary and gonad tissues. These genes were *StAR, Hsd17b1, Cyp19a1a, Cyp19a1b, Gpr54, Kiss1, Kiss2, Fshβ, Lhβ, Fshr* and *Lhr.* Sequences for these genes were identified within a closely related species (order Pleuronectiformes) from the Universal Protein Resource (UniProt) database to act as a reference protein sequence for searching the yellowbelly flounder brain, pituitary and gonad transcriptomes.

Geneious Prime 2022.2.2 (https://www.geneious.com) software was used to identify genes involved in the estradiol biosynthesis pathway and pituitary gonadotropin synthesis pathway from transcriptomes, constructed from the brain, pituitary and gonad of female yellowbelly flounder. Each transcriptome sequence database was uploaded into Geneious prime and set up so that each database could be searched using the BLAST (Altschul et al., 1990) program tBLASTn. Known protein sequences of *StAR, Hsd17b1, Cyp19a1a, Cyp19a1b, Gpr54, Kiss1, Kiss2, Fshβ, Lhβ, Fshr* and *Lhr* from closely related species (order Pleuronectiforms) retrieved from the UniProt database were used to search each RNA-seq library. The resulting hits, which covered as much of the gene of interest as possible, were downloaded and used to construct a consensus sequence. Consensus sequences were translated into amino acid sequences using

the ExPASy translate tool (https://web.expasy.org/translate/) and put through a BLAST search to confirm the proteins' identity.

Once protein sequences were obtained for genes of interest, they were compared with homologous sequences from a range of vertebrate species obtained by searching their non-redundant protein sequence database, using the tBLASTn program at National Center for Biotechnology Information (NCBI) (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Protein sequences for genes of interest included one representative from Mammalia, Reptilia, Aves and Chondrichthyes and four different teleosts, including a Perciform, Pleuronectiform, Salmoniform and Cypriniform. Resulting sequences from this search, selected to contain as much of the gene of interest as possible, were retrieved and aligned to the selected reference protein sequence. To construct each multiple alignment, Clustal Omega 1.2.4 (https://avermitilis.ls.kitasato-u.ac.jp/clustalo/) (Sievers et al., 2011) was used. Locations of protein signatures and important functional sites were identified using what has been published and predicted using the ScanProsite tool (https://prosite.expasy.org/scanprosite/). Phylogenetic trees were also constructed by first aligning selected vertebrate sequences using Clustal Omega 1.2.4 using a pairwise distance matrix for all the sequences and a guide tree was created using the neighbour joining algorithm, which was bootstrapped 1000 times. The Interactive Tree Of Life (iTOL) v6 (https://itol.embl.de/) was used for the actual construction and annotation of the phylogenetic trees.

3.4 Results

3.4.1 Histology

Stages of oocyte development of each wild caught flounder were confirmed based on oocyte diameter and morphology from ovarian histology (Table 3.1.). Histological classification of yellowbelly flounder ovaries is outlined in Chapter 2.

Table 3.1. Reproductive status (stage of ovarian development) of three wild-caught female yellowbelly flounder sampled in May 2022 for transcriptomic analysis.

Fish #	Length (cm)	Stage of ovarian development
1	34.5	Late vitellogenic
2	33	Atretic
3	35.5	Atretic

3.4.2 Sequences identified

Using tBLASTn, the raw FASTQ file for each yellowbelly flounder gonad, pituitary and brain transcriptomic library, was BLAST-searched using amino acid sequences obtained from the non-redundant database from other Pleuronectiforms for the reproductive genes *StAR, Lhβ, Fshβ, Lhr, Fshr, Cyp19a1a, Cyp19a1b, Kiss1, Kiss2, Gpr54 and Hsd17b1*. Using this approach, sufficient nucleotide sequences were able to be assembled into the complete or partial CoDing Sequence (CDS) for the yellowbelly flounder *StAR, Lhβ, Fshβ, Lhr, Fshr, Cyp19a1a, Cyp19a1b, Gpr54 and Hsd17b1*. The identified nucleotide consensus sequences, alignments and translated CDS sequence for each gene is included in detail in the Appendices.

3.4.3 *StAR*

The Senegalese sole (*Solea senegalensis)* amino acid sequence for *StAR* was used to BLAST search the yellowbelly flounder brain, pituitary and ovary transcriptome libraries. Expression of *StAR* transcripts was detected in the ovarian and brain tissues of yellowbelly flounder The resulting nucleotide sequences from each tissue were aligned and a consensus nucleotide sequence determined, which was 861 bp in length (Appendix 1). The CDS encoded a predicted protein sequence of 286 amino acids which was aligned to selected vertebrate *StAR* sequences (Figure 3.3.). Conserved functional regions were highlighted based on sites identified in earlier studies which revealed two potential sites for protein kinase A-mediated phosphorylation (Figure 3.3.). Overall, homology was high between the yellowbelly flounder StAR protein sequence and the selected vertebrate sequences, especially within the two potential sites for protein kinase A-mediated phosphorylation. Only a single amino acid substitution is present in the yellowbelly flounder amino acid sequence for the first phosphorylation site. Phylogenetic analysis also confirms that this sequence is the yellowbelly flounder *StAR* homologue, due to it branching closely with other known flatfish *StAR* sequences (Figure 3.4.).

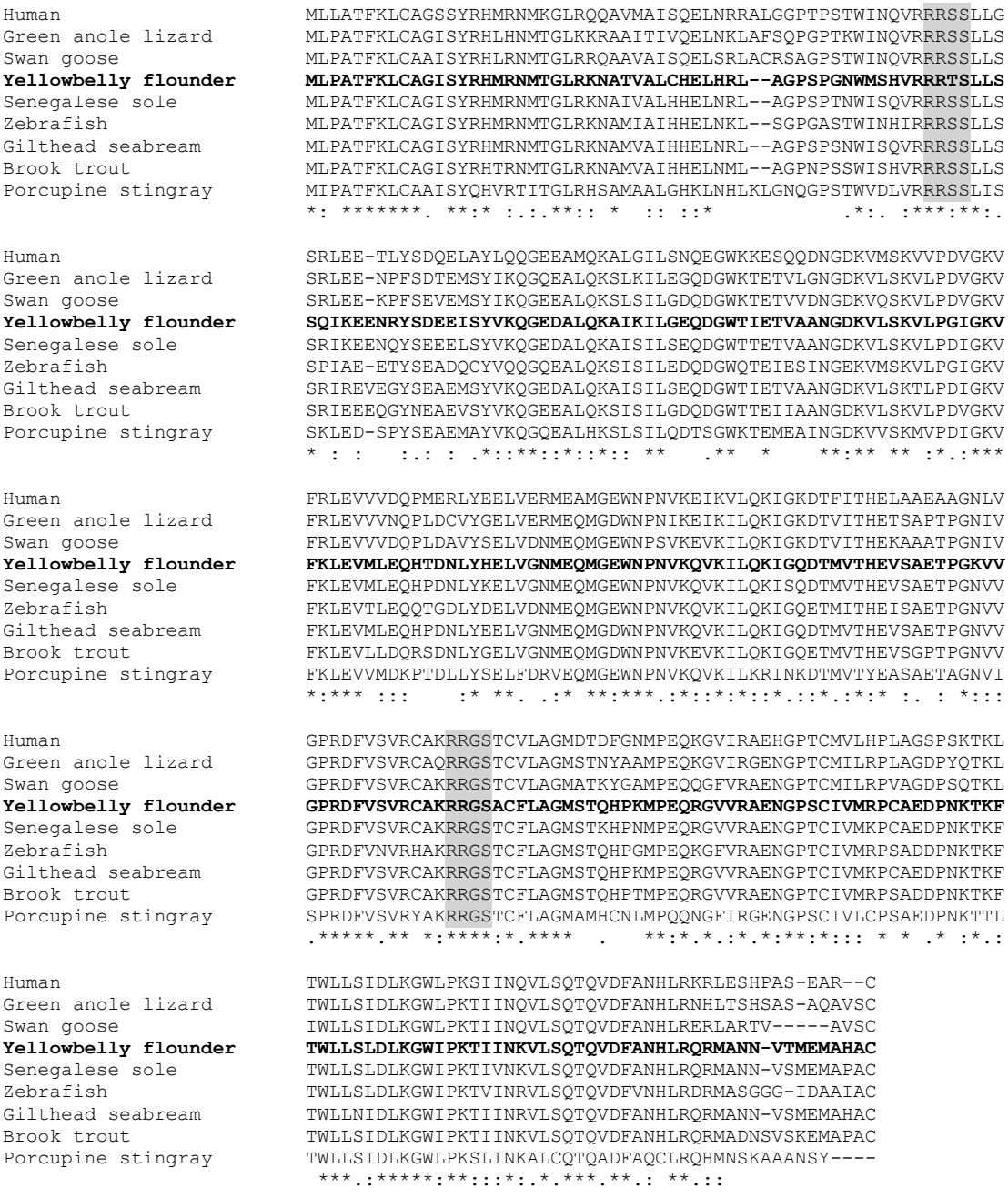

Figure 3.3. Multiple alignment of amino acid sequence of yellowbelly flounder *StAR* with selected vertebrate sequences. Two potential phosphorylation sites are highlighted in grey (Arakane et al., 1997). The *StAR* sequence accession numbers are: Human (AAC50141.1), green anole lizard (GAFD01005116.1), swan goose (QXO36676.1), Senegalese sole (ADW08748.1), zebrafish (AAG28593.1), gilthead seabream (ABV25959.1), brook trout (AAG39689.1) and porcupine stingray (AAS64370.1).

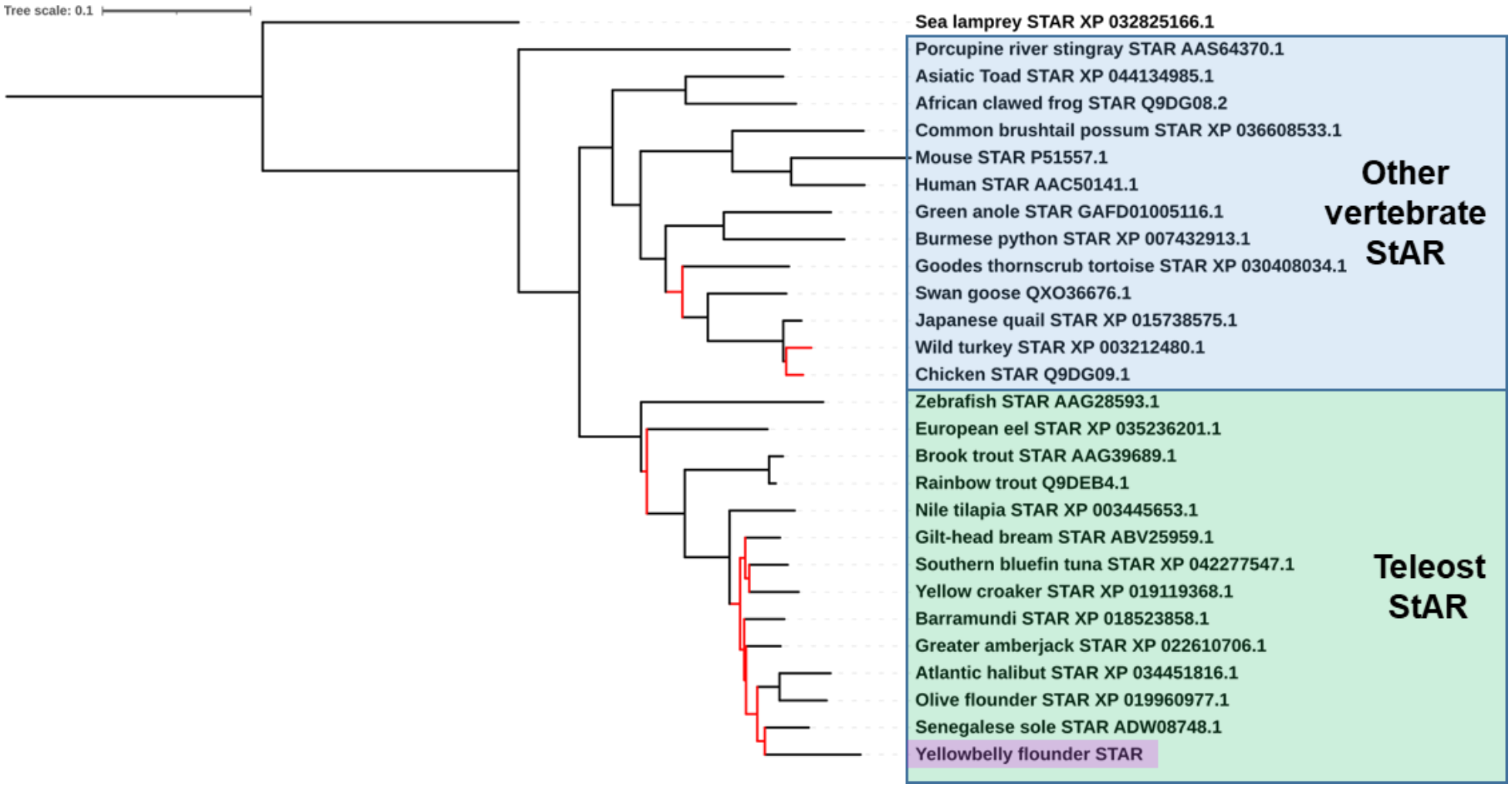

Figure 3.4. Phylogenetic tree constructed in iTOL v6 using the neighbour joining method from an alignment in Clustal Omega 1.2.4, showing the relationship between the yellowbelly flounder predicted *StAR* protein with selected vertebrate *StAR* sequences. Black lines represent genetic distance, with those in red indicating bootstrap values less than 75%. Accession numbers for each sequence used are included in the tree.

3.4.4 *Hsd17b1*

The Senegalese sole (*Solea senegalensis*) amino acid sequence for *Hsd17b1* was used to BLAST search the yellowbelly flounder brain, pituitary and ovary transcriptome libraries. Expression of *Hsd17b1* transcripts were detected in the brain and ovarian tissues of yellowbelly flounder. The resulting nucleotide sequence from each tissue were aligned and a consensus nucleotide sequence determined, which was 876 bp in length (Appendix 2). The CDS encoded a predicted protein sequence of 291 amino acids, which was aligned to selected vertebrate *Hsd17b1* sequences (Figure 3.5.). Conserved functional regions were highlighted based on those identified in earlier studies, revealing a co-factor binding site motif, catalytic site and an NAG structural motif (Figure 3.5.). Overall, homology was high between the yellowbelly flounder *Hsd17b1* protein sequence and the selected vertebrate sequences, and the co-factor binding site motif, catalytic site and NAG structural motif were all conserved. Phylogenetic analysis also confirms that this sequence is the yellowbelly flounder *Hsd17b1* homologue, due to it branching closely with other known flatfish *Hsd17b1* sequences (Figure 3.6.).

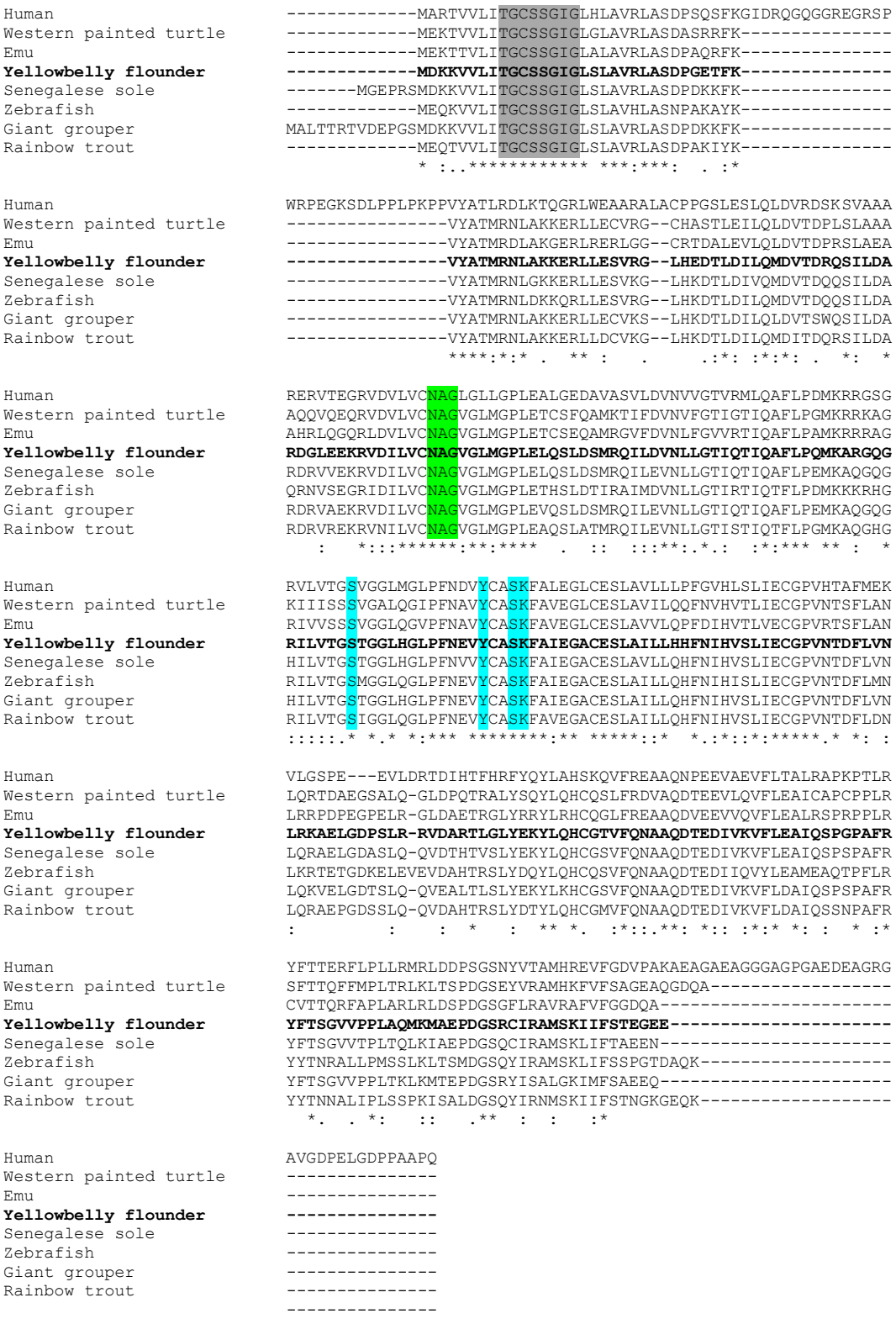

Figure 3.5. Multiple alignment of amino acid sequence of yellowbelly flounder *Hsd17b1* with selected vertebrate sequences. The NAG-structural motif is highlighted green. The co-factor binding site motif is highlighted grey and the catalytic site is highlighted blue (Koyama et al., 2019; Mindnich et al., 2004). The *Hsd17b1* sequence accession numbers are: Human (AAB19917.2), western painted turtle (XP_005313978.1), emu (XP_025973604.1), Senegalese sole (XP_042907423.1), zebrafish (AAP74564.1), giant grouper (XP_033501630.1) and rainbow trout (XP_021480528.1).

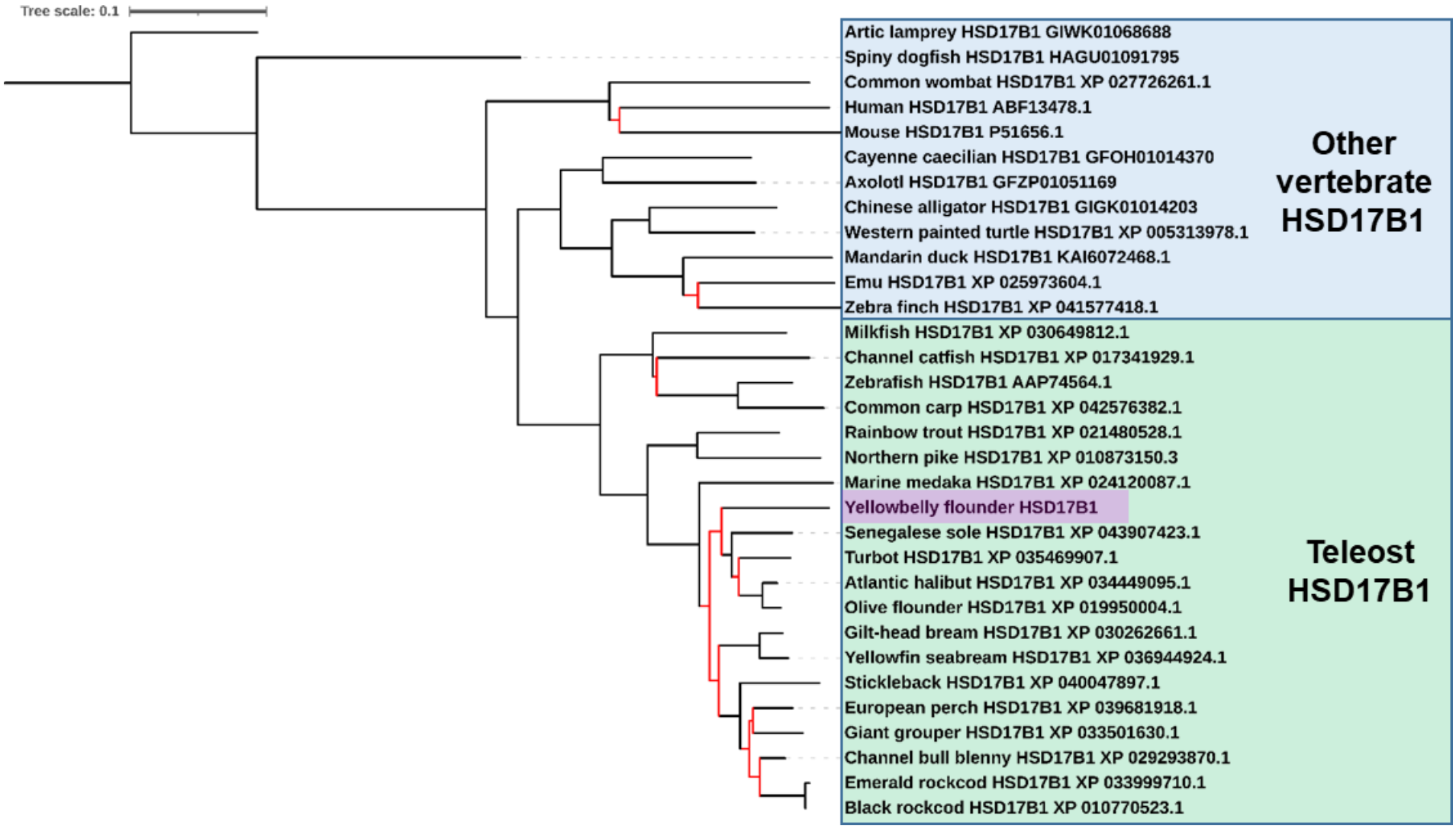

Figure 3.6. Phylogenetic tree constructed in iTOL v6 using the neighbour joining method from an alignment in Clustal Omega 1.2.4, showing the relationship between the yellowbelly flounder predicted *Hsd17b1* protein with selected vertebrate *Hsd17b1* sequences. Black lines represent genetic distance, with those in red indicating bootstrap values less than 75%. Accession numbers for each sequence used are included in the tree.

3.4.5 *Cyp19a1a*

The olive flounder (*Paralichthys olivaceus*) amino acid sequence for *Cyp19a1a* was used to BLAST search the yellowbelly flounder brain, pituitary and ovary transcriptome libraries. Expression of *Cyp19a1a* transcripts in yellowbelly flounder were detected only in the ovarian tissues. The resulting nucleotide sequence from each tissue were aligned and a consensus nucleotide sequence determined, which was 1581 bp in length (Appendix 3). The CDS encoded a predicted protein sequence of 528 amino acids, which was aligned to the selected vertebrate *Cyp19a1a* sequences (Figure 3.7.). Conserved regions were highlighted based on sites identified in earlier studies, which revealed a membrane region, substrate-binding region, steroid-binding region, aromatic region and heme-binding region (Figure 3.7.). Overall, homology was high between the yellowbelly flounder *Cyp19a1a* protein sequence and the selected vertebrate sequences, especially within the membrane region, substrate-binding region, steroid-binding region, aromatic region and heme-binding region. Phylogenetic analysis also confirms that this sequence is the yellowbelly flounder *Cyp19a1a* homologue, due to it branching closely with other known flatfish *Cyp19a1a* sequences (Figure 3.9.).

```
Human                   --------------------MVLEMLNP-IHYNITSIVPEAMPAATMPVLLLTGLFLLVW
Leopard gecko           --------------------MIEESLNP-GHY-ISKVVSETMPAATLPLLLLMGFLFLMW
Chicken                 --------------------MIPETLNP-LNY-FTSLVPDLMPVATVPIIILICFLFLIW
Yellowbelly flounder    --MDLMPARDLPMTPMRFDAIAADL-VAMSPNATTVGSRGIS-VASRTLLLLICLLLVVW
Olive flounder          --MDRIPACDLAMTPVGLGAALGDL-VSTSPNATAVRTPGIS-VASRTLILLVCVLLVAW
Zebrafish               MAGDLLQPC--GMKPVRLGEAVVDLLIQRAHNGTERAQDNACGATATILLLLLCLLLAIR
Hong Kong grouper       --MDLISACERAMTPVGLDDMVADL-ATMSPNATAVGSPGIS-IATRLLILLICVLLAAW
Brown trout             -------VCGRVMAAVCLDTVIADLLVSESRNATATRSEGVS-LATGSLLLLLCLVVATW
Atlantic stingray       ----------MEMKEFAPDKNLLQMSQPAFQNATAEVVPRMASRATVPLLLLLGLLLIFL
                                                :                     ::  :::*  ...

Human                   NY-EGTSSIPGPGYCMGIGPLISHGRFLWMGIGSACNYYNRVYGEFMRVWISGEETLIIS
Leopard gecko           NF-EETSSIPGPGYCMGIGPLISHWRFLWMGVGSACNYYNETYGEFVRVWINGEETLVTS
Chicken                 NH-EETSSIPGPGYCMGIGPLISHGRFLWMGVGNACNYYNKTYGEFVRVWISGEETFIIS
Yellowbelly flounder    SHT-DKRPVPGPSFCLGLGPLLSYLRFIWTGIGTASNYYNNKYGDIVRVWINGEETLVIS
Olive flounder          SHT-DRRTVPGPPFCLGLGPLLSYVRFIWTGIGTACNYYNKRYGDIVRVWIDGEETLILS
Zebrafish               HHRPHKSHIPGPSFFFGLGPIVSYCRFIWSGIGTASNYYNSKYGDIVRVWINGEETLILN
Hong Kong grouper       SHT-DKKTVPGPSFCLGLGPLLSYVRFIWTGIGTASNYYNNKYGDIVRVWINGEETLILS
Brown trout             RHT-DNNSVPGPFFCLGVGPLLSYLRFIWTGIGTASNYYNSKYGDIVRVWINGEETLILS
Atlantic stingray       KLSQKKATLPGPSFCLGIGPLISYGRFLWMGIGSASNYYNEKYGGIVRVWIHGEETLIIS
                                :*** : :*:**::*: **:* *:*.*.****  ** ::**** ****:: .

Human                   KSSSMFHIMKHNHYSSRFGSKLGLQCIGMHEKGIIFNNNPELWKTTRPFFMKALSGPGLV
Leopard gecko           KSPSMFHVMKHGHYICRFGSKLGLKCIGMHENGIIFNKNPALWKEIRPFFTKALSGPGLV
Chicken                 KSSSVFHVMKHWNYVSRFGSKLGLQCIGMYENGIIFNNNPAHWKEIRPFFTKALSGPGLV
Yellowbelly flounder    RPSAVQHVLKNGHYTSRFGSKQGLSCIGMHERGIIFNNNVTLWKKIRAYFAKALTGPGLQ
Olive flounder          RASAIYHVLKNGHYTSRFGSKQGLSCIGMYERGIIFNNNVSLWKKIRTHFTRALTGPGLQ
Zebrafish               RSSAVYHVLRKSLYTSRFGSKLGLQCIGMHEQGIIFNSNVALWKKVRAFYAKALTGPGLQ
Hong Kong grouper       RASAVHHVLKNGNYTSRFGSKQGLSCIGMNERGIIFNNNVELWKKIRTYFSKALTGPGLQ
Brown trout             SSSAVHHVLRQGRYTSRFGSKQGLSCIGMDERGIIFNSNMALWKKTRTYFAKALTGPGLQ
Atlantic stingray       RSSAVNHVMKKGHYISRFGSKHALQCIGMNENGIIFNNNPSIWKQTRSYFAKALTGPILQ
                           :: *::::  * .***** .*.**** *.*****.*   **  * .: :**:** *

Human                   RMVTVCAESLKTHLDRLEEVTNE-SGYVDVLTLLRRVMLDTSNTLFLRIPLDESAIVVKI
Leopard gecko           KMIAICVDSTREHLDHLQEVTTE-LGHINALNLMRCIMLDTSNRLFLGIPLDENAIVLKI
Chicken                 RMIAICVESTIVHLDKLEEVTTE-VGNVNVLNLMRRIMLDTSNKLFLGVPLDESAIVLKI
Yellowbelly flounder    KTVEVCVTSTQSHLDDLDTL-----GYVDFLILLRCTVVDISNRLFLDVPINEKELMVKI
Olive flounder          KTVEVCVSSTQTHLDDLDGL-----GHVDVLSLLRCTVVDISNRLFLDVPINEKELLVKI
Zebrafish               RTMEICTTSTNSHLDDLSQLTDA-QGQLDILNLLRCIVVDVSNRLFLGVPLNEHDLLQKI
Hong Kong grouper       QTVEVSVSATQTHLDDLDGL-----GHVDVLSLLRCTVVNISNRLFLDVPVNEKELLLKI
Brown trout             RTVDVCVSSTQTHLDALQGLDGLMGGQVDVLSLLRCTVVDISNRLFLGVPLNEKELLQKI
Atlantic stingray       RTLAMTVESTRDHLEKLLGGNNN-TTKVDVLLFLRAITLDIANRLFLRVPLHEGEIVAKV
                        : : : . :   **: *          :: * ::*   :: :* *** :*:.*  :: *:

Human                   QGYFDAWQALLIKPDIFFKISWLYKKYEKSVKDLKDAIEVLIAEKRRRISTEEKLEECMD
Leopard gecko           QNYFDAWQALLLKPDIFFKISWLYKKYEKSAKDLKEAIEILIEQKRQKLSTAEKLEEHMD
Chicken                 QNYFDAWQALLLKPDIFFKISWLCKKYEEAAKDLKGAMEILIEQKRQKLSTVEKLDEHMD
Yellowbelly flounder    EKYFDTWQSVLIKPDIYFKFDRIHQRHKTAFQELHDAIGDLVEQKRKEVEQAEKLD-DIN
Olive flounder          LKYFDTWQTVLIKPDIYFKFDWIHQRHKAAVQELHDAIGDLVEQKRRDVEQADKLD-NIN
Zebrafish               HKYFDTWQTVLIKPDVYFRLDWLHKKHKRDAQELQDAITALIEQKKVQLAHAEKLD-HLD
Hong Kong grouper       FKYFDTWQTVLIKPDVYFKFDWIHQRHKTAAQELQDAIESLVEQKRRDMEQADKLD-NIN
Brown trout             QKYFDTWQTVLIKPDIYFKLDWIQQKHRRAAQELQDAIESLVDQKRRGLQEADKLD-HIN
Atlantic stingray       QKYFDTWQSLLLKPDIFFKFKCMYKKYEKAAQDLQDAVEELLLKKQQELRDTEKLHDITD
                          ***:**::*:***::*::. : :::.   ::*: *:  *: :*:  :   :**.   :

Human                   FATELILAEKRGDLTRENVNQCILEMLIAAPDTMSVSLFFMLFLIAKHPNVE-EAIIKEI
Leopard gecko           FASQLIFAQCRGELTGENVNQCVLEMLIAAPDTLSVTLFFMLALIAEHPQCGRGTVMKEI
Chicken                 FASQLIFAQNRGDLTAENVNQCVLEMMIAAPDTLSVTLFIMLILIADDPTVE-EKMMREI
Yellowbelly flounder    FTTELIFAQGHGELSAENVIQCVLEMVIAAPDTLSVSLFFMLLLLKQNPNVE-LQLLQEI
Olive flounder          FTTGLIFAQNHGELSAENVVQCVLEMVIAAPDTLSVSLFFMLLLLKQNPDVE-LQLLREI
Zebrafish               FTAELIFAQSHGELSAENVRQCVLEMVIAAPDTLSISLFFMLLLLKQNPDVE-LKILQEM
Hong Kong grouper       FTAELIFAQNHGELSAENVRQCVLEMVIAAPDTLSISLFFMLLLLKQNPDVE-LQLLQEI
Brown trout             FTADLIFAQSHGELSAENVRQCVLEMVIAAPDTLSISLFFMLLLLKQNPDVE-LQLLEEI
Atlantic stingray       FATDLIFAQTHGELTPDNVRQSLLEILIAGPDTMSVSIYFMLLLIAQHPEVE-KKILEEI
                        *:: **:*: :*:*: :** *.:**::**.***:*::::::** *: ..*      ::.*:

Human                   QTVIGERDIKIDDIQKLKVMENFIYESMRYQPVVDLVMRKALEDDVIDGYPVKKGTNIIL
Leopard gecko           QAVMGDRDIESEDMPKLKVVESFIYESMRYQPVVDLVMRKALQDDVIDGYAVKKGTNIIL
Chicken                 ETVMGDREVQSDDMPNLKIVENFIYESMRYQPVVDLIMRKALQDDVIDGYPVKKGTNIIL
Yellowbelly flounder    DTVLGDRRLQRGDLPKLHVLENFINESLRFHPVVDFTMRRALSDDIIEGYSVPKGTNIIL
Olive flounder          DTVVGERQLQNGDLQKLQVLESFINECLRFHPVVDFSMRRALSDDIIDGYRVPKGTNIIL
Zebrafish               DSVLAGQSLQHSHLSKLQILESFINESLRFHPVVDFTMRRALDDDVIEGYNVKKGTNIIL
Hong Kong grouper       DTVVGERQLQNADLQKLQVLESFINECLRFHPVVDFTMRRALSDDIIDGYRVSKGTNIIL
Brown trout             DTAIGERELHNSDLQNLRVLESFINESLRFHPVVDFTMRRALSDDVISGYRVPKGTNIIL
Atlantic stingray       QTVTGKREVQNNDLQQLKILENFINESMRYQPVVDITMRKALKDDMIDGFLVKKGTNIIL
                        ::. . : :.  .: :*:::*.** *.:*::****: **:**.**:*.*: * *******
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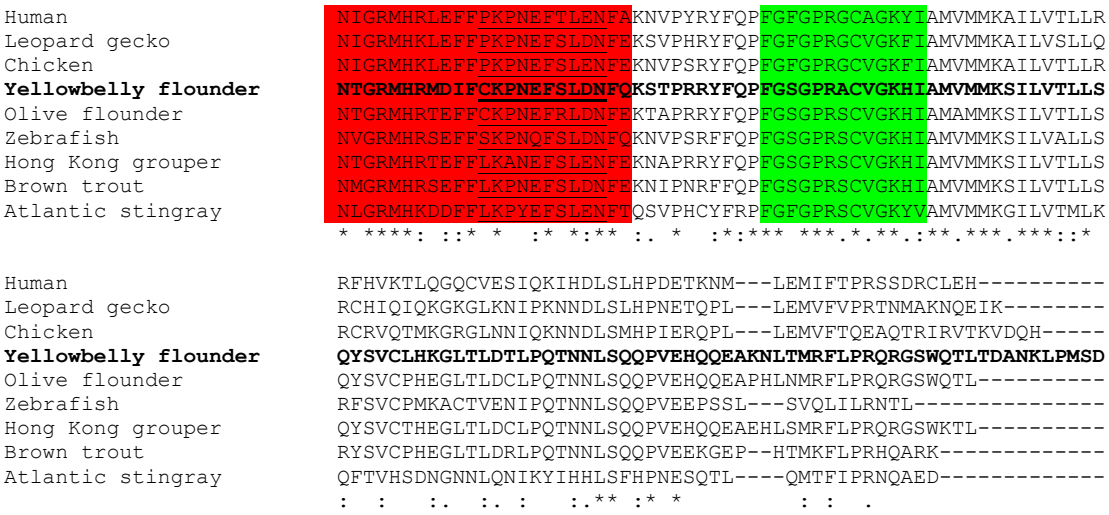


Figure 3.7. Multiple alignment of amino acid sequence of yellowbelly flounder *Cyp19a1a* with selected vertebrate species. The membrane region is highlighted in grey. The substrate-binding region is highlighted yellow, steroid-binding region highlighted red and heme-binding region highlighted green. The aromatic region is underlined (Böhne et al., 2013). The *Cyp19a1a* sequence accession numbers are: Human (CAA31929.1), leopard gecko (BAE20061.1), chicken (AAA48738.1), olive flounder (BAA74777.1), zebrafish (AAG12243.1), Hong Kong grouper (AAS58448.1), brown trout (AAR04775.1) and Atlantic stingray (AAF04617.1).

3.4.6 *Cyp19a1b*

The amino acid sequence from Atlantic halibut (*Hippoglossus hippoglossus*) *Cyp19a1b* was used to BLAST search the yellowbelly flounder brain, pituitary and ovary transcriptome libraries. Expression of *Cyp19a1b* transcripts were detected in both the brain and pituitary tissues of yellowbelly flounder. The resulting nucleotide sequence from each tissue were aligned and a consensus nucleotide sequence determined, which was 1473 bp in length (Appendix 4). The CDS encoded a predicted protein sequence of 490 amino acids which was aligned to selected fish *Cyp19a1b* sequences (Figure 3.8.). Conserved functional regions were highlighted based on sites identified from earlier studies, which revealed four conserved regions, the membrane-spanning region, Ozol's peptide region, aromatase-specific region and heme-binding region (Figure 3.8.). Overall, homology was high between the yellowbelly flounder *Cyp19a1b* sequence and the selected vertebrate sequences, especially within the membrane-spanning region, Ozol's peptide region and aromatase-specific region (Figure 3.8.). Phylogenetic analysis also confirms that this sequence is the yellowbelly flounder *Cyp19a1b* homologue, due to it branching closely with other known flatfish *Cyp19a1b* sequences (Figure 3.9.).

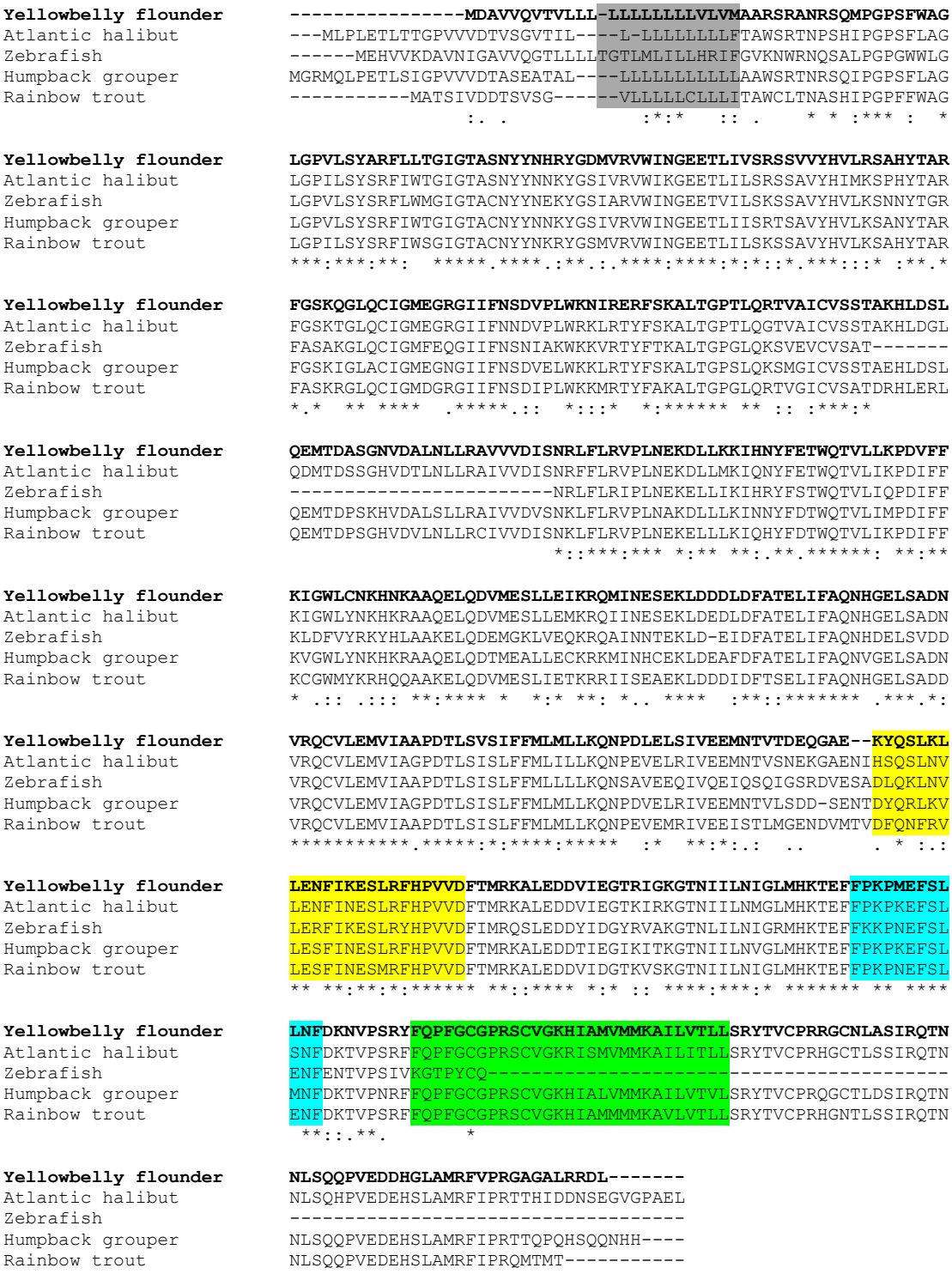


Figure 3.8. Multiple alignment of amino acid sequence of yellowbelly flounder *Cyp19a1b* with selected fish sequences. The membrane-spanning region is highlighted in grey. Ozol's peptide region is highlighted yellow, and the heme-binding region is highlighted green. The aromatase-specific region is highlighted blue (Zhang et al., 2008). The *Cyp19a1b* sequence accession numbers are: Atlantic halibut (AAY6901.1), zebrafish (AAH76104.1), humpback grouper (AAV91181.1) and rainbow trout (CAC84574.1).

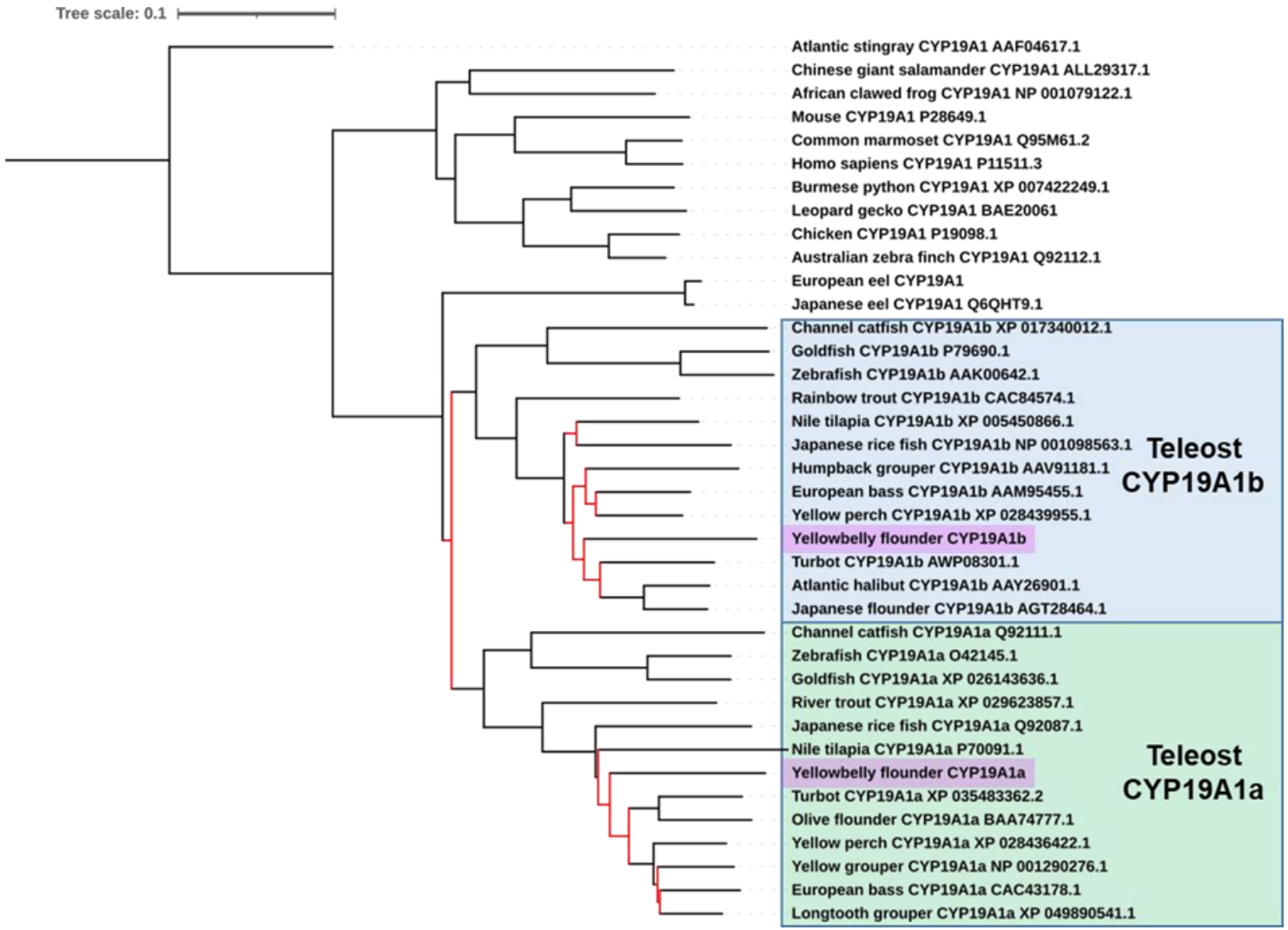


Figure 3.9. Phylogenetic tree constructed in iTOL v6 using the neighbour joining method from an alignment in Clustal Omega 1.2.4, showing the relationship between the yellowbelly flounder predicted *Cyp19a1a* and *Cyp19a1b* proteins with selected vertebrate *Cyp19a1a and Cyp19a1b* sequences. Black lines represent genetic distance, with those in red indicating bootstrap values less than 75%. Accession numbers for each sequence used are included in the tree.

3.4.7 *Gpr54*

The Senegalese sole (*Solea* senegalensis) amino acid sequence for *Gpr54* was used to BLAST search the yellowbelly flounder brain, pituitary and ovary transcriptome libraries. Expression of *Gpr54* transcripts were detected in the brain tissues of yellowbelly flounder. The resulting nucleotide sequence from each tissue were aligned, and a consensus nucleotide sequence determined, which was 986 bp in length (Appendix 5). The CDS encoded a predicted protein sequence of 329 amino acids, which was aligned to selected vertebrate *Gpr54* sequences (Figure 3.10.). Conserved functional regions were highlighted based on sites identified from earlier studies, which revealed seven conserved transmembrane domain regions (Figure 3.10.). Overall, homology was high between the yellowbelly flounder *Gpr54* sequence and the selected vertebrate sequences, especially within the seven transmembrane domain regions. Phylogenetic analysis also confirms this sequence is the yellowbelly flounder *Gpr54* sequence, due to it branching closely with other known flatfish *Gpr54* sequences (Figure 3.11.).

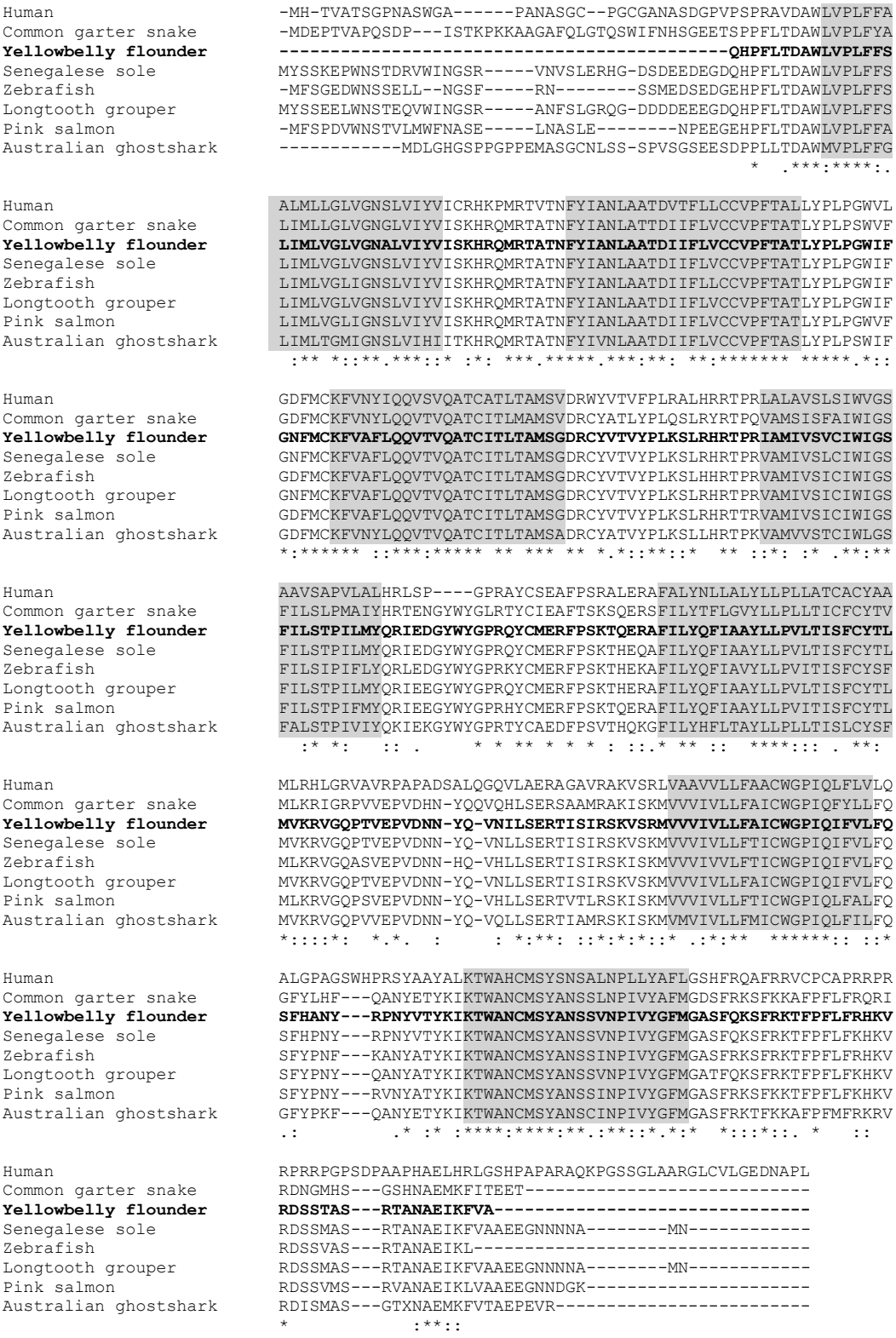


Figure 3.10. Multiple alignment of amino acid sequence of yellowbelly flounder *Gpr54* with selected vertebrate sequences. Conserved transmembrane regions are highlighted in grey (Zhang et al., 2018). The *Gpr54* sequence accession numbers are: Human (AAB19917.2), common garter snake (XP_013930854.1), Senegalese sole (ABW96362.1), zebrafish (ABV44612.1), longtooth grouper (AEN14559.1), pink salmon (XP_046178411.1) and Australian ghostshark (XP_007898035.2). Note: currently no homologue of *Gpr54* has been found in any bird species (Sivalingam & Parhar, 2022).

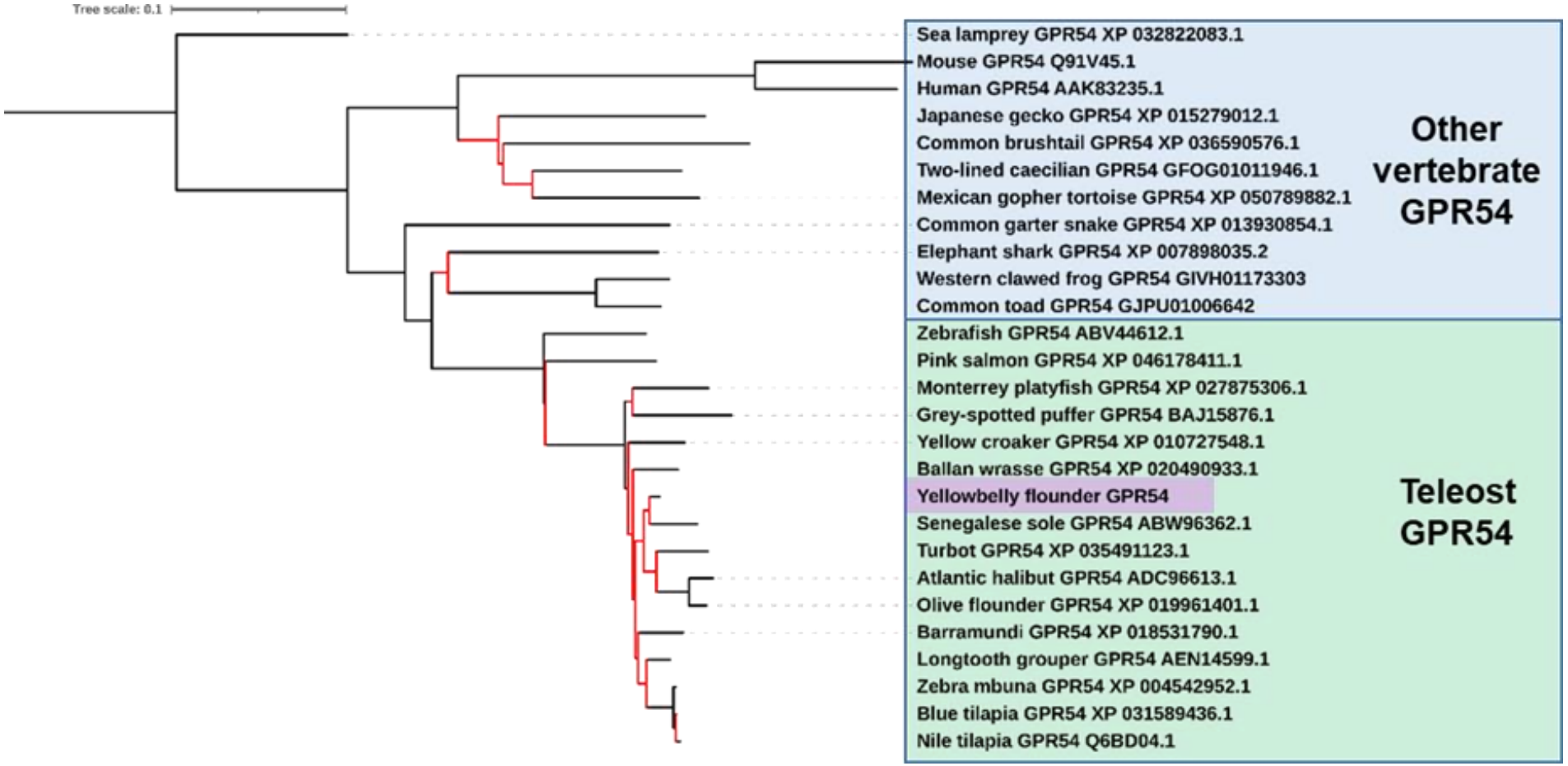


Figure 3.11. Phylogenetic tree constructed in iTOL v6 using the neighbour joining method from an alignment in Clustal Omega 1.2.4, showing the relationship between the yellowbelly flounder predicted *Gpr54* protein with selected vertebrate *Gpr54* sequences. Black lines represent genetic distance, with those in red indicating bootstrap values less than 75%. Accession numbers for each sequence used are included in the tree.

3.4.8 *Fshβ*

The European turbot (*Scophthalamus maximus*) amino acid *sequence for Fshβ* was used to BLAST search the yellowbelly flounder brain, pituitary and ovary transcriptome libraries. Expression of *Fshβ* transcripts were detected in the brain, pituitary and ovarian tissues of yellowbelly flounder. The resulting nucleotide sequence from each tissue were aligned and a consensus nucleotide sequence determined, which was 363 bp in length (Appendix 6). The CDS encoded a predicted protein sequence of 120 amino acids, which was aligned to selected vertebrate *Fshβ* sequences (Figure 3.12.). Conserved functional regions were highlighted based on sites identified in earlier studies, which revealed two putative N-glycosylation sites and 12 conserved cystine residues (Figure 3.12.). Overall, homology was high between the yellowbelly flounder *Fshβ* protein sequence and the selected vertebrate sequences, especially within one of the putative N-glycosylation sites, and all 12 cysteine regions were conserved. Phylogenetic analysis also confirms that this sequence is the yellowbelly flounder *Fshβ* sequence, due to it branching closely with other known flatfish *Fshβ* sequences (Figure 3.14.).

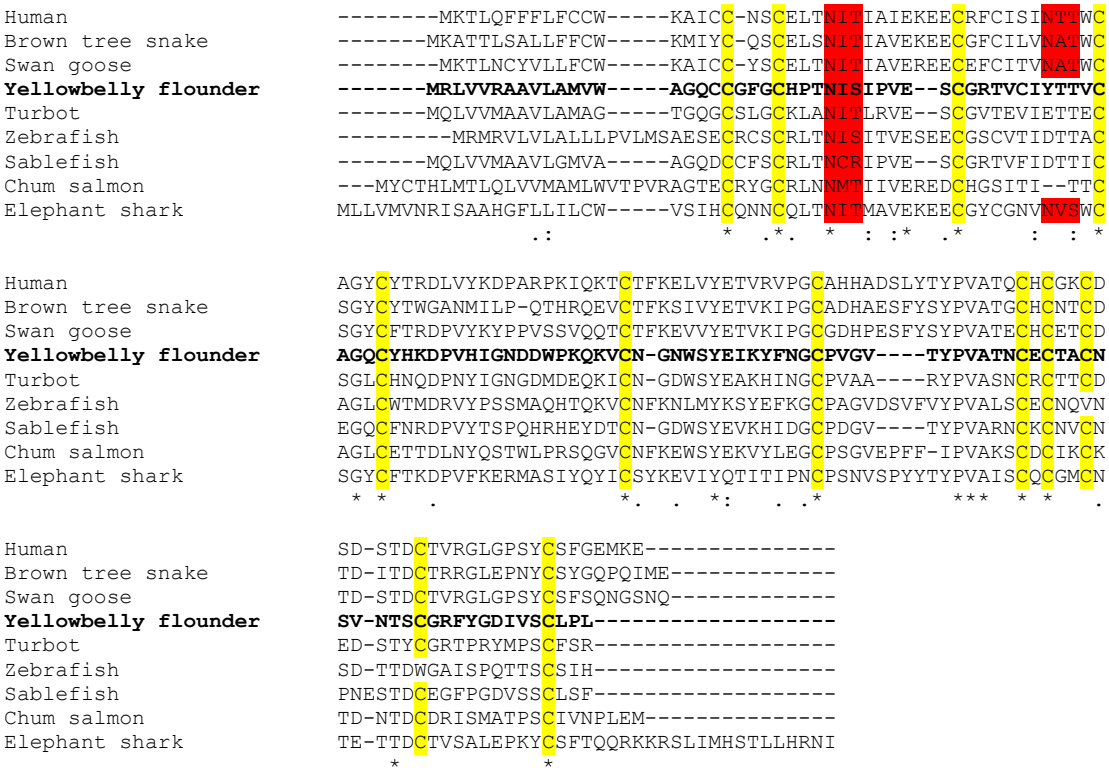


Figure 3.12. Multiple alignment of amino acid sequence of yellowbelly flounder *Fshβ* with selected vertebrate sequences. The putative N-glycosylation sites are highlighted in red and conserved cystine residues are highlighted yellow (Li et al., 2021). The *Fshβ* sequence accession numbers are: Zebrafish (AAV31152.1), European turbot (QNU12868.10), sablefish (AGS55583.1), chum salmon (AAA49408.1), elephant shark (NP_001279311.1), human (CP37869.1), swan goose (ACC97141.1) and brown tree snake (BAJ14510.1).

3.4.9 *Lhβ*

The European turbot (*Scophthalamus maximus*) amino acid sequence for *Lhβ* was used to BLAST search the yellowbelly flounder brain, pituitary and ovary transcriptome libraries. Expression of *Lhβ* transcripts were detected in the ovarian and brain tissues of yellowbelly flounder. The resulting nucleotide sequences from each tissue were aligned and a consensus nucleotide sequence determined, which was 417 bp in length (Appendix 7). The CDS encoded a predicted protein sequence of 138 amino acids, which was aligned to selected vertebrate *Lhβ* sequences (Figure 3.13.). Conserved functional regions were highlighted based on sites identified in earlier studies, which revealed one putative N-glycosylation site and 12 conserved cystine residues (Figure 3.13.). Overall, homology was high between the yellowbelly flounder *Lhβ* protein sequence and the selected vertebrate *Lhβ* sequences, especially within the putative N-glycosylation site, and all 12 cysteine regions were conserved. Phylogenetic analysis also confirms that this sequence is the yellowbelly flounder *Lhβ* homologue, due to it branching closely with other known flatfish *Lhβ* sequences (Figure 3.14.).

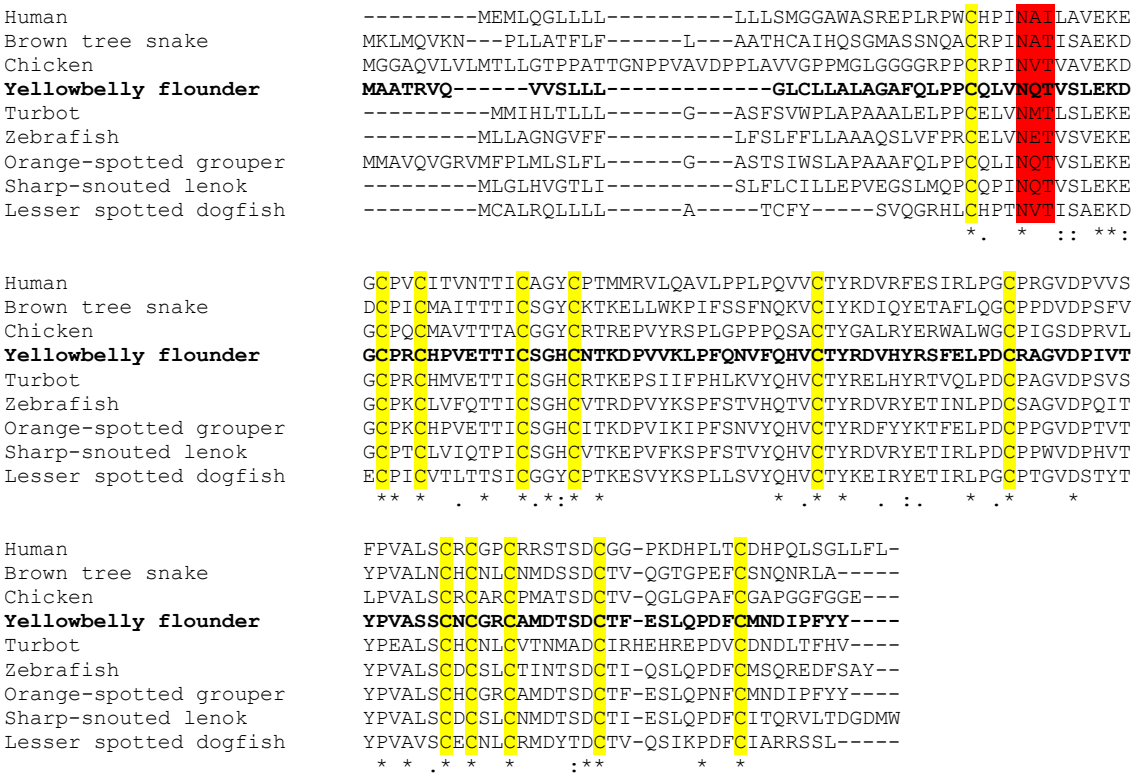


Figure 3.13. Multiple alignment of amino acid sequence of yellowbelly flounder *Lhβ* with selected vertebrate sequences. The putative N-glycosylation site is highlighted in red and conserved cystine residues are highlighted yellow (Rather et al., 2016). The *Lhβ* sequence accession numbers are: Zebrafish (AAV31153.1), European turbot (QAU21463.1), orange-spotted grouper (AAM28896.1), sharp snouted lenok (AAR99811.1), lesser spotted dogfish (CAC43236.1), human (NP_000885.1), chicken (ADY03193.1) and brown tree snake (BAJ14511.1).

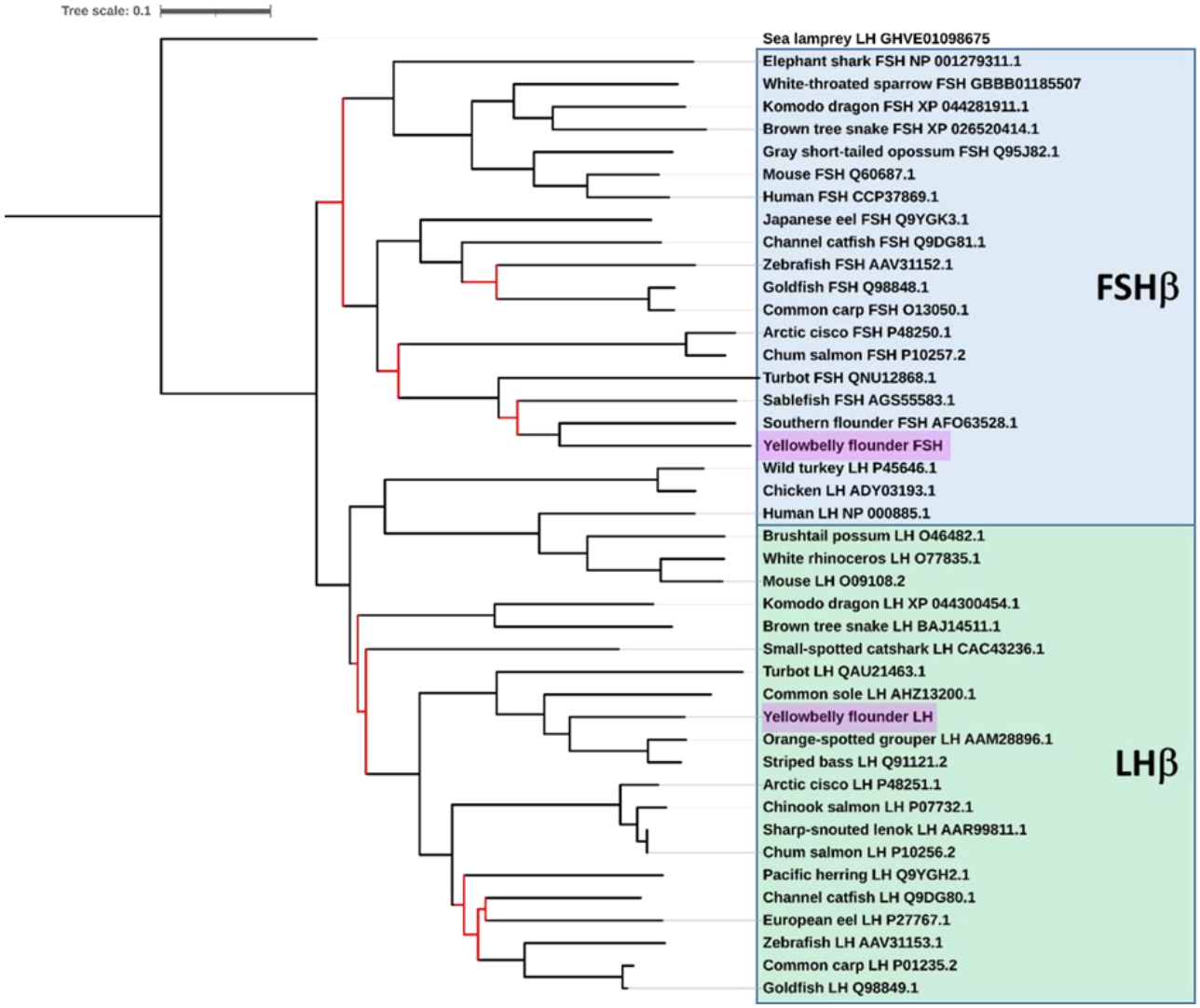
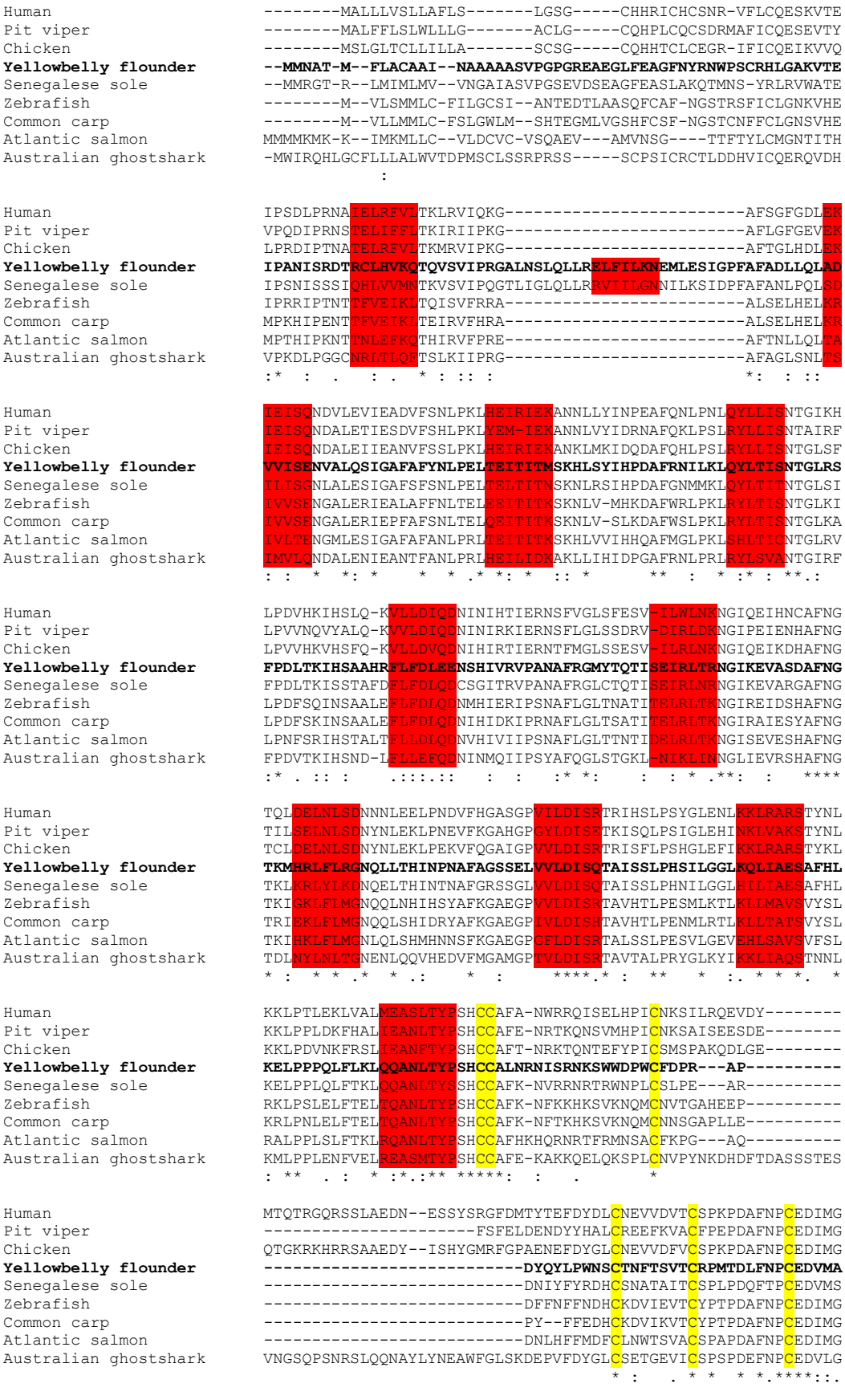


Figure 3.14. Phylogenetic tree constructed in iTOL v6 using the neighbour joining method from an alignment in Clustal Omega 1.2.4, showing the relationship between the yellowbelly flounder predicted *Fshβ* and *Lhβ* proteins with selected vertebrate *Fshβ and Lhβ* sequences. Black lines represent genetic distance, with those in red indicating bootstrap values less than 75%. Accession numbers for each sequence used are included in the tree.

3.4.10 *Fshr*

The Senegalese sole (*Solea senegalensis*) amino acid sequence for *Fshr* was used to BLAST search the yellowbelly flounder brain, pituitary and ovary transcriptome libraries. Expression of *Fshr* was detected in the brain, pituitary and ovary tissues of yellowbelly flounder. The resulting nucleotide sequence from each tissue were aligned and a consensus nucleotide sequence determined, which was 2112 bp in length (Appendix 8). The CDS encoded a predicted protein sequence of 703 amino acids, which was aligned to selected vertebrate *Fshr* sequences (Figure 3.15.). Conserved functional regions were highlighted based on sites identified in earlier studies, which revealed seven conserved transmembrane domain regions, 13 conserved cystine residues and 11 leucine rich motifs (Figure 3.15.). Overall, homology was high between the yellowbelly flounder *Fshr* sequence and selected vertebrate sequences, especially within the transmembrane domain regions, and the 13 cysteine residues were fully conserved. Ten of the leucine rich motifs were conserved across all selected vertebrates, and an additional motif conserved in both flatfish sequences (Figure 3.15.). Phylogenetic analysis also confirms that the sequence is the yellowbelly flounder *Fshr* homologue, due to it branching closely with other known flatfish *Fshr* sequences (Figure 3.16.).


Human
Pit viper
Chicken
Yellowbelly flounder
Senegalese sole
Zebrafish
Common carp
Atlantic salmon
Australian ghostshark

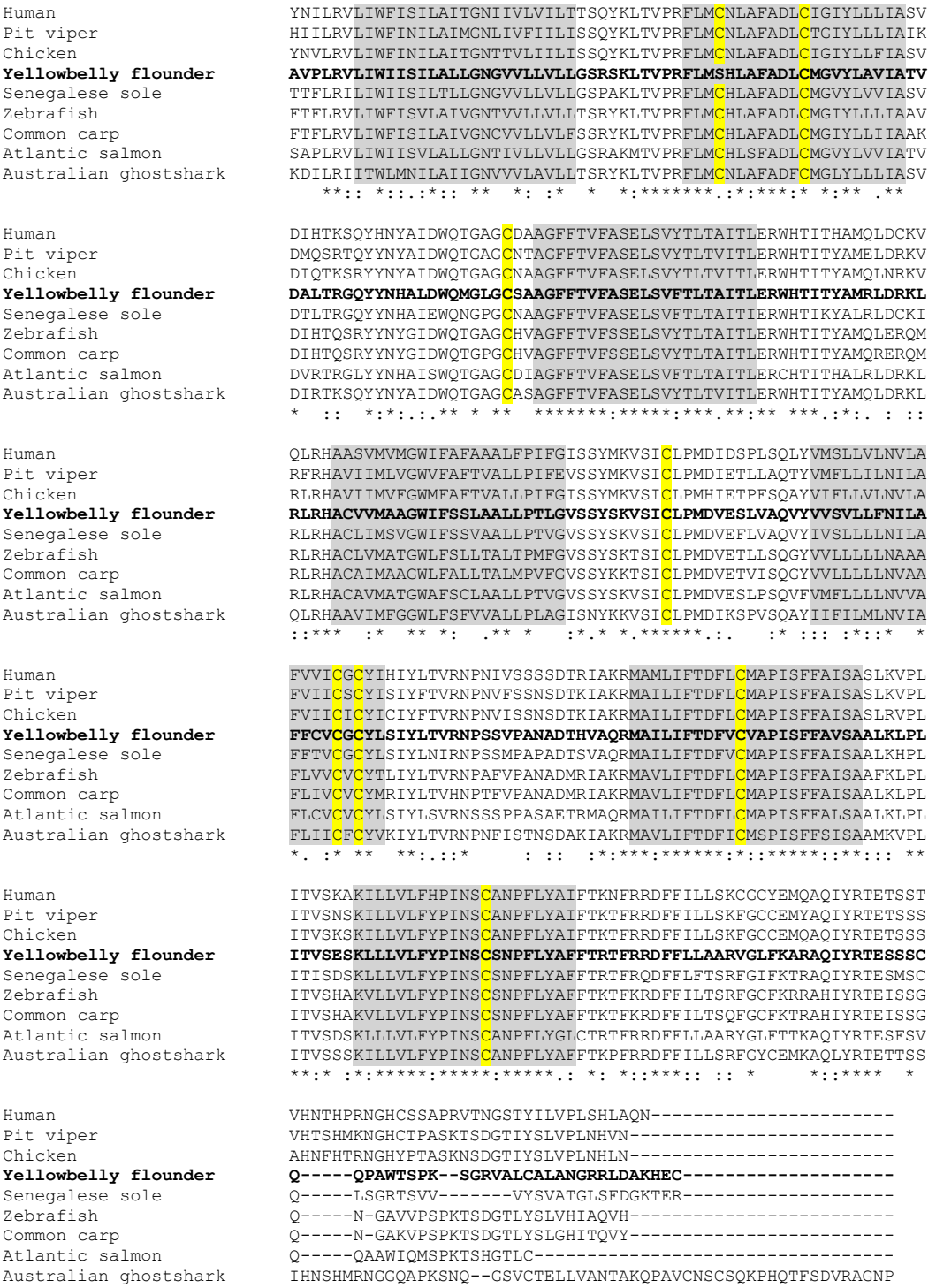


Figure 3.15. Multiple alignment of amino acid sequence of yellowbelly flounder *Fshr* with selected vertebrate sequences. Conserved transmembrane regions are highlighted in grey, conserved cystine residues highlighted yellow and leucine rich motifs highlighted red (Burow et al., 2020). The *Fshr* sequence accession numbers are: Human (AAA52478.1), pit viper (AAO72730.1), chicken (NP_990410.2), Senegalese sole (ADH51678.1), zebrafish (AAP33512.1), common carp (QDZ71264.1), Atlantic salmon (NP_001117082.1) and Australian ghostshark (XP_007903189.1*).*

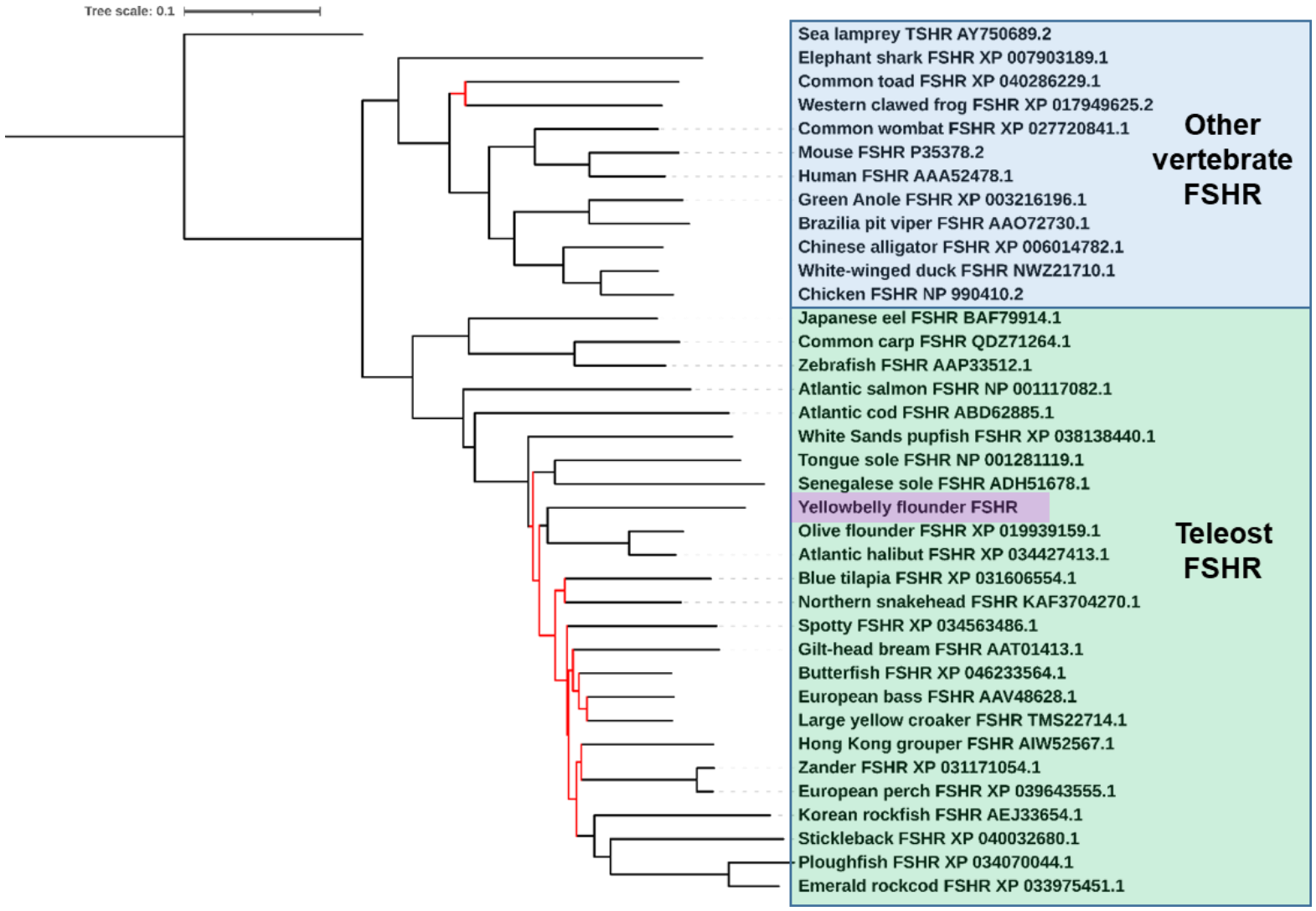
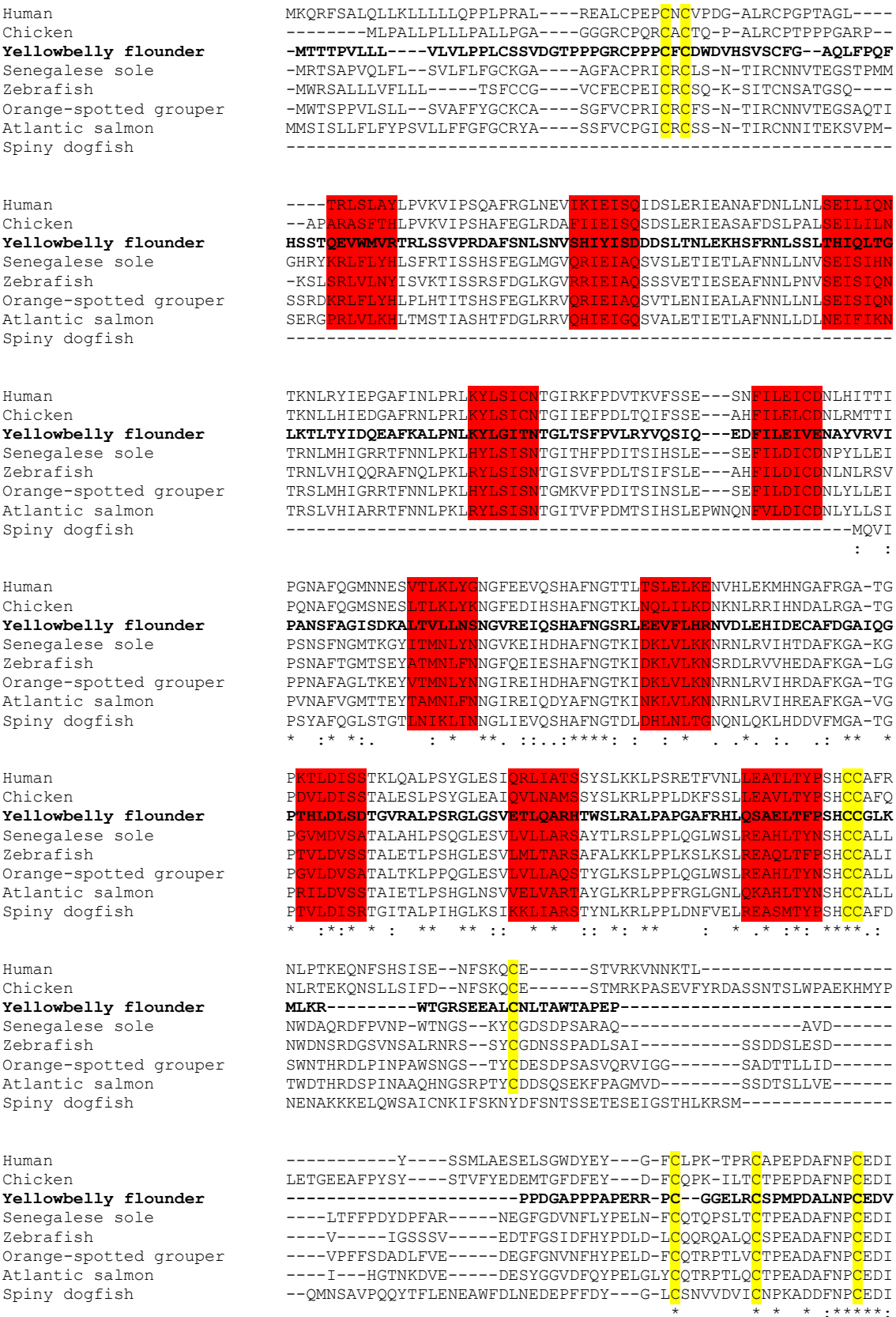


Figure 3.16. Phylogenetic tree constructed in iTOL v6 using the neighbour joining method from an alignment in Clustal Omega 1.2.4, showing the relationship between the yellowbelly flounder predicted *Fshr* proteins with selected vertebrate *Fshr* sequences. Black lines represent genetic distance, with those in red indicating bootstrap values less than 75%. Accession numbers for each sequence used are included in the tree.

3.4.11 *Lhr*

The Senegalese sole (*Solea senegalensis*) *Lhr* amino acid sequence was used to BLAST search the yellowbelly flounder brain, pituitary and ovary transcriptome libraries. Expression of *Lhr* transcripts were detected in the pituitary tissues of yellowbelly flounder. The resulting nucleotide sequence from each tissue was aligned, and a consensus nucleotide sequence determined, which was 1932 bp long (Appendix 9). The CDS encoded a predicted protein sequence of 653 amino acids, which was aligned to selected vertebrate *Lhr* sequences (Figure 3.17). Conserved functional regions were highlighted based on sites identified from earlier studies, which revealed seven transmembrane domain regions, 10 leucine rich motifs and 16 cysteine residues (Figure 3.17.). Overall, homology was good between the yellowbelly flounder *Lhr* protein sequence and the selected vertebrate sequences, especially within the transmembrane domain regions, leucine rich motifs, and 15 of the cysteine residues were conserved. Phylogenetic analysis clearly groups this sequence with the fish *Lhr1* sequences, but it does not group closely with the other flatfish *Lhr1* sequences (Figure 3.18.).


Human
Chicken
Yellowbelly flounder
Senegalese sole
Zebrafish
Orange-spotted grouper
Atlantic salmon
Spiny dogfish

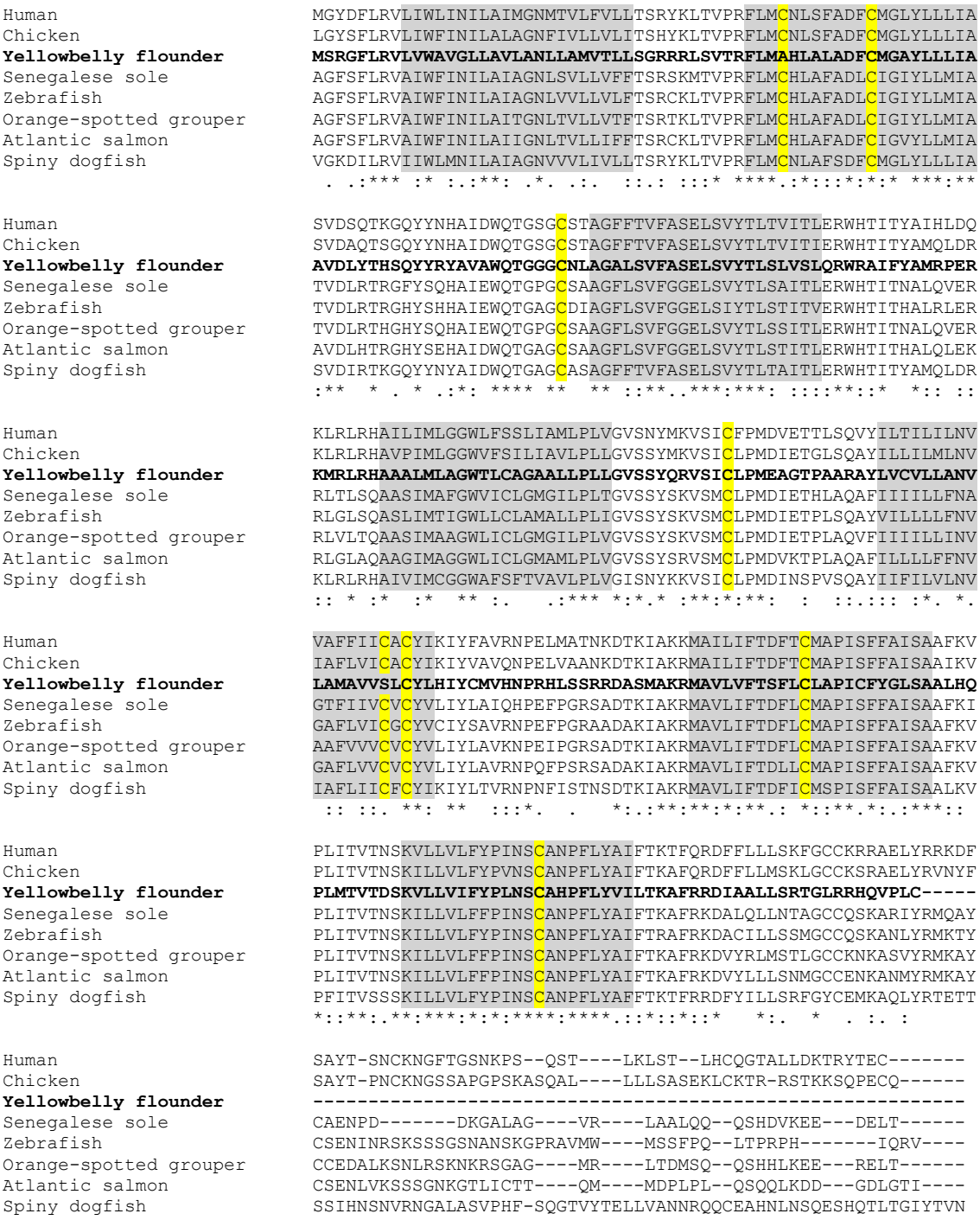


Figure 3.17. Multiple alignment of amino acid sequence of yellowbelly flounder *Lhr* with selected vertebrate sequences Conserved transmembrane regions are highlighted in grey, conserved cysteine residues highlighted yellow and leucine rich motifs highlighted red (Burow et al., 2020). The *Lhr* sequence accession numbers are: Human (AAB19917.2), chicken (NP_990267.1), Senegalese sole (ADH51679.1), zebrafish (AAR84281.1), orange-spotted grouper (AEG65827.1), Atlantic salmon (ABH10577.1) and spiny dogfish (HAGT01084345.1).

Figure 3.18. Phylogenetic tree constructed in iTOL v6 using the neighbour joining method from an alignment in Clustal Omega 1.2.4, showing the relationship between the yellowbelly flounder predicted *Lhr* proteins with selected vertebrate *Lhr* sequences, including fish *Lhr1* and *Lhr2* sequences. Black lines represent genetic distance, with those in red indicating bootstrap values less than 75%. Accession numbers for each sequence used are included in the tree.

3.5 Discussion

The main aim of this study was to identify yellowbelly flounder genes important in two key pathways involved in teleost oogenesis, which are the neuroendocrine production of pituitary gonadotropins pathway and the estradiol biosynthesis pathway. Currently, a lot has been done to characterise genes within a number of flatfish species globally, however very little genetic data exists for the yellowbelly flounder within the available databases. To enable the fast discovery of a number of important genes involved in reproduction, transcriptome libraries were generated from the brain, pituitary, and ovarian tissues of wild-caught New Zealand adult yellowbelly flounder. As these are the key tissues involved in the brain-pituitary-gonad (BPG) axis, it was expected that the targeted genes that are involved in oogenesis should be transcribed within these tissues and therefore nucleotide sequences should be able to be determined. Each transcriptome library for yellowbelly flounder was searched by BLAST, using protein sequences previously characterised from other Pleuronectiformes. This enabled the identification and characterization of transcripts for various genes of interest in the yellowbelly flounder, that are known to be important within reproduction.

The regulation of reproduction in teleosts is dependent on the neuroendocrine control of the brain-pituitary-gonad axis (Weltzien et al., 2004). The key pathway in this axis involves the hypothalamic production of a gonadotropin-releasing hormone which stimulates the secretion of two pituitary gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Zohar et al., 2010). These gonadotropins work via their receptors on the gonads which regulate gametogenesis and steroidogenesis through the secretion of gonadal sex steroids (Yaron & Levavi-Sivan, 2011). In this study, a number of genes were identified and assigned to the yellowbelly flounder reproductive axis. These genes were *StAR, Hsd17b1, Cyp19a1a, Cyp19a1b, Gpr54, Fshr, Lhr, Fshβ* and *Lhβ*. The transcriptome libraries were searched for two additional reproductive genes, *Kiss1* and *Kiss2*, however these genes were surprisingly not detected. This may be a result of low expression in the samples (García-Ortega & Martínez, 2015), which could be a factor of when the fish were actually sampled and where they were within the reproductive cycle, or sequencing errors, and does not imply that these genes are not present in the yellowbelly flounder transcriptome. Furthermore, the actual identification of *Gpr54* does indicate at least one of these genes should be present in this species, due to its function as a *Kiss* receptor (Elizur, 2009).

Some reproductive genes with notable importance were omitted due to the scope of the present study. One such example identified in the gonadotropin pathway is the GnRH receptor. However, there are multiple variants of the GnRH receptor genes found in teleosts (Luckenbach et al., 2010; Zohar et al., 2022), making it very difficult to clearly characterise each homologue using a transcriptome library that has been constructed with short reads (i.e. 150 bp). Also, characterisation of this group of genes would be more useful when the functions of each receptor has been determined and when the receptor, or receptors, specific to reproduction in teleosts has been determined. Below are the genes that were successfully found within the yellowbelly flounder brain, pituitary and gonad tissue and some analysis of the predicted protein sequences, to help in the characterisation of each gene:

3.5.1 Characterisation of *StAR*

Steroidogenic acute regulatory protein (*StAR*) is known for its critical role in steroidogenesis through facilitating the transportation of cholesterol within steroidogenic cells (Tenugu et al., 2021). Cholesterol is the precursor to all sex steroids, thus the first step in steroidogenesis involves the *StAR* mediated mobilization of cholesterol. In the yellowbelly flounder transcriptome, *StAR* transcripts were found expressed within both the brain and gonad. Previous work confirms the expression of *StAR* can occur in steroidogenic tissues, including the gonads (Ings & Van Der Kraak, 2006) and brain (Nagarajan et al., 2011). The consensus nucleotide sequence constructed for the yellowbelly flounder *StAR* homologue was 861 base pairs long and coded for 286 amino acids. This is similar to that reported for the homologous sequences in Atlantic cod (*Gadus morhua*) (Goetz et al., 2004) and Senegalese sole (Marín-Juez et al., 2011).

Multiple alignments of the yellowbelly flounder *StAR* predicted protein sequence with other selected vertebrate sequences, highlighted the conservation of two regions containing potential for protein kinase A-mediated phosphorylation sites (Arakane et al., 1997). Phosphorylation has been identified to have a significant role in regulating the biological activity of the *StAR* protein, leading to the regulation of steroid synthesis (Arakane et al., 1997; Castillo et al., 2015). The presence of these conserved regions provides evidence that this gene is the yellowbelly flounder *StAR* sequence and indicates the functional role of *StAR* in cholesterol mobilisation is conserved in this species. It should be noted, that contrary to previous reports where these regions have been conserved in other finfishes, a single amino

acid substitution is present at the first phosphorylation site in the yellowbelly flounder *StAR* sequence. However, single amino acid substitutions are highly common, and although they can adversely affect protein function, a substitution with similar amino acids can be functionally neutral (Ng & Henikoff, 2006). The site of the amino acid substitution was highly conserved here, changing from an 'S' to a 'T' indicating this phosphorylation site should remain functionally conserved in yellowbelly flounder. In addition to the alignment, phylogenetic analysis revealed the yellowbelly flounder *StAR* amino acid sequence was closely related to known flatfish *StAR* sequences, confirming its relatedness and the identify of this sequence as the yellowbelly flounder *StAR* homologue.

3.5.2 Characterisation of *Hsd17b1*

In all vertebrates, steroidogenesis is regulated by a series of reactions, each of which is catalysed by a specific enzyme. Two pathways exist for the synthesis of estradiol, through the reduction of estrone, or the aromatization of testosterone to estradiol (Piferrer & Blázquez, 2005; Steckelbroeck et al., 2003). One group of enzymes that has importance in steroidogenesis is the 17β-hydroxysteroid dehydrogenases (hsd17b), which catalyse the conversion of estrone to estradiol and reduction of androstenedione to testosterone (Koyama et al., 2019; Steckelbroeck et al., 2003). Among the genes in the hsd17b family, the hsd17b1 enzyme has been identified to play a role in the synthesis of estradiol (Hakkarainen et al., 2015; Zou et al., 2020). Research in olive flounder (*Paralichthys olivaceous*) suggested the role of the enzyme hsd17b1 was involved in both androgen and estrogen synthesis within the flounder (Zou et al., 2020). In the yellowbelly flounder transcriptome, the *Hsd17b1* gene was found expressed within both the brain and gonad. Previous investigations have shown *Hsd17b1* is strongly associated with steroidogenic tissues, due to its critical role in steroidogenesis (Filby et al., 2010; Xiao et al., 2020; Zou et al., 2020), so its expression within the yellowbelly flounder was expected due to the steroidogenic activity of both tissues (Piferrer & Blázquez, 2005).

The consensus nucleotide sequence constructed for the *Hsd17b1* homologue in yellowbelly flounder was 876 base pairs long, which encodes a 291 amino acid peptide. A similar peptide length for *Hsd17b1* has been reported for another Pleuronectiform, olive flounder (Zou et al., 2020). The *Hsd17b1* gene is a member of the short-chain dehydrogenase/reductase family, the protein sequences of which have typical conserved residues which have been elucidated in previous studies (Mindnich et al., 2004). The yellowbelly flounder *Hsd17b1* predicted

protein sequence was shown through multiple alignment with other selected vertebrate sequences to have three conserved residues with structural and functional importance. These are the NAG-structural motif, co-factor binding site motif and a catalytic site, and the essential amino acids in all three regions were fully conserved (Mindnich et al., 2004; Oppermann et al., 2003). These sites are closely linked to short chain dehydrogenases/reductases and the NAG-structural motif and co-factor binding site play a role in maintaining the central β-sheet, and therefore coenzyme binding (Oppermann et al., 2003), and the catalytic site is responsible for catalysis. The presence of these conserved regions provide evidence that this gene is the yellowbelly flounder *Hsd17b1* homologue. Furthermore, phylogenetic analysis showed that the predicted protein sequence for the yellowbelly flounder *Hsd17b1* was closely related to known flatfish *Hsd17b1* sequences, providing more confirmation of its relatedness.

3.5.3 Characterisation of *Cyp19a1a* and *Cyp19a1b*

Each step during steroidogenesis following the mobilization of cholesterol by *StAR* is catalyzed by specific steroidogenic enzymes. Thus, steroidogenesis is controlled through the expression of genes that code for steroidogenic enzymes (Sampath Kumar et al., 2000). One such enzyme is cytochrome P450 aromatase (cyp19a1), the enzyme responsible for catalysing the reaction that aromatizes androgens to form estrogens (Piferrer & Blázquez, 2005). Unlike most vertebrates, two genes, encoding two *Cyp19a1* isoforms, have been identified in teleosts, which arose from a whole genome duplication event that occurred when teleosts emerged (Steinke et al., 2006). Expression of the *Cyp19a1* genes in finfishes has been reported in both the ovaries and the brain (González & Piferrer, 2002; Sampath Kumar et al., 2000), with the two isoforms respectively specific to the ovaries (*Cyp19a1a*) and the brain (*Cyp19a1b*) (Doering et al., 2021). Both isoforms have been acknowledged to have distinct physiological relevance (Diotel et al., 2010), with *Cyp19a1b* contributing largely to the increased estrogen biosynthesis observed in the brains of teleosts, compared to other vertebrates (Callard et al., 2001). *Cyp19a1a* and *Cyp19a1b* are members of the cytochrome P450 family from the larger hemoprotein superfamily involved in catalysing mono-oxygenase reactions (Uno et al., 2012).

In the yellowbelly flounder transcriptome, the two isoforms have very distinct patterns of expression, with the *Cyp19a1a* gene found expressed within the ovaries, whereas Cyp19a1b was expressed within the brain and pituitary. This clearly indicates the respective roles of these isoforms in the brain and ovaries (Callard et al., 2001; Doering et al., 2021) are conserved

in this species. The consensus nucleotide sequence constructed for the yellowbelly flounder *Cyp19a1a* homologue was 1581 base pairs long and coded for 528 amino acids, and the *Cyp19a1b* homologue was 1473 base pairs long and coded for 490 amino acids. Multiple alignments of the yellowbelly flounder *Cyp19a1a* predicted protein sequence with other selected vertebrate sequences highlighted the conservation of five conserved functional regions, based on the structural information of the *Cyp19a1a* gene as reported by Böhne et al. (2013). These regions include the heme-binding region, membrane region, substrate-binding region, steroid-binding region and aromatic region. Similarly, multiple alignments of the yellowbelly flounder *Cyp19a1b* predicted protein sequence with other selected vertebrate sequences, highlighted the conservation of four conserved functional regions, including the membrane-spanning region, Ozol's peptide region, the aromatic region and the heme-binding region, as reported by Zhang et al. (2008). These regions are well conserved within the yellowbelly flounder amino acid sequences and are characteristic of all vertebrate aromatase genes (Böhne et al., 2013; Castro et al., 2005). Their identification provides evidence these genes are the yellowbelly flounder *Cyp19a1a* and *Cyp19a1b* homologues. Phylogenetic analysis also confirmed the identifies of the yellowbelly flounder *Cyp19a1a* and *Cyp19a1b* amino acid sequences, where they grouped closely to known flatfish *Cyp19a1a* and *Cyp19a1b*, providing more confirmation of their relatedness.

3.5.4 Characterisation of *Gpr54*

The G protein-coupled receptor 54, encoded by *Gpr54*, is an integral part of the KISS/GPR54 system in teleosts (Elizur, 2009). The system is known to play an important role in reproductive processes, however, the exact mechanisms in teleosts remain unclear. Currently it is known that the *Kiss* genes act to regulate pituitary gonadotropin synthesis via GPR54, through two potential pathways (Espigares et al., 2015; Ma et al., 2019). The consensus nucleotide sequence constructed for the yellowbelly flounder *Gpr54* homologue was 986 base pairs long and encoded 329 amino acids, which is notably shorter than the 390 amino acid protein of the lined seahorse (*Hippocampus erectus*) (Zhang et al., 2018) and 378 amino acid protein predicted for Senegalese sole (Mechaly et al., 2009). This indicates that only a partial sequence for *Gpr54* was obtained from the yellowbelly flounder transcriptome libraries.

In the yellowbelly flounder transcriptome, the *Gpr54* gene was found expressed only within the brain. Previous work confirms that the expression of *Gpr54* in other teleosts can be high

within multiple tissues, including the brain, pituitary and gonad, throughout different stages of development (Martinez-Chavez et al., 2008; Mechaly et al., 2009; Nocillado et al., 2007), and weakly expressed in somatic tissues (Martinez-Chavez et al., 2008). The finding that transcripts were only detected in the brain of the yellowbelly flounder is consistent with previous studies where expression of *Gpr54* in teleosts has been highly expressed in different regions of the brain (Li et al., 2009; Ma et al., 2019; Mechaly et al., 2009). However, the fact that *Gpr54* was undetected in the pituitary and ovary may be due to low expression levels corresponding to the stages of development the fish were sampled at. However, this result suggests that one of the functional regions of *Gpr54* in the BPG axis of adult yellowbelly flounder is in the brain. This indicates the potential for the KISS/GPR54 regulation of GnRH neuron activity, as has been suggested in blackhead seabream (*Acanthopagrus schlegeli)* (Ma et al., 2019), grass puffer (*Takifugu niphobles*) (Shahjahan et al., 2010) and zebrafish (*Danio rerio*) (Zhao et al., 2014). Nonetheless, as the exact functioning of the KISS/GPR54 system in teleosts remains undetermined, investigation into the exact locations of *Gpr54* expression in the yellowbelly flounder brain and its expression within other tissues could form part of future investigations.

Multiple alignments using the yellowbelly flounder *Gpr54* predicted protein sequence with other selected vertebrate sequences, highlighted seven highly conserved putative transmembrane regions, as reported in the lined seahorse (Zhang et al., 2018). These regions were almost fully conserved among the aligned sequences from teleosts. Transmembrane domains are characteristic of all membrane-bound G protein-coupled receptors (Breton et al., 2021; Muir et al., 2001), and the existence of seven putative transmembrane domains is highly reported for teleost *Gpr54* (Nocillado et al., 2007; Parhar et al., 2004; Zhang et al., 2018). The identification of these regions confirms the identity of this gene as *Gpr54* and indicates the functional role of this receptor is likely also conserved in this species. In addition to the alignment, phylogenetic analysis also confirmed that the predicted protein sequence for *Gpr54* in yellowbelly flounder was most similar to the known *Gpr54* sequences in other flatfishes, confirming its relatedness and the identity of this gene as the yellowbelly flounder *Gpr54* homologue.

3.5.5 Characterisation of *FSHβ* and *LHβ*

The two pituitary gonadotropins, FSH and LH, are key components in steroidogenesis and gametogenesis, through their primary role in the production of sex steroids. FSH is highlighted for its function in stimulating the production of androgens and estrogens in both females and males, where LH has importance in final maturation through the synthesis of the maturation inducing steroid (Arcand-Hoy & Benson, 1998; Mañanós et al., 2002; Yaron & Levavi-Sivan, 2011). Both gonadotropins, FSH and LH are glycoprotein hormones and as such are heterodimers, composed of two noncovalently bonded alpha and beta subunits, a common glycoprotein α subunit (GTHα) and their respective *Fshβ* and *Lhβ* subunits (Chi et al., 2015; Ulloa-Aguirre et al., 1999). Each beta-subunit is encoded by a specific gene, thus FSH and LH are identified through the characterisation of their unique beta subunits. It is well documented that both *Fshβ* and *Lhβ* are expressed within the brain, pituitary and gonads at different stages throughout reproduction in various teleosts (Chi et al., 2015; Kajimura et al., 2001; Li et al., 2005; Shi et al., 2015). Expression of the yellowbelly flounder *Fshβ* transcripts were detected in all three tissues and expression of the yellowbelly flounder *Lhβ* transcripts were detected in the brain and ovary, highlighting the importance of these genes throughout the reproductive axis in yellowbelly flounder. The coding region for the yellowbelly flounder *Lhβ* subunit gene was 417 base pairs long, encoding a 138 amino acid peptide. This is almost identical in length to the 139 amino acid peptide reported for European turbot (Gao et al., 2019). In addition, the nucleotide sequence for the *Fshβ* gene was determined to be composed of 363 base pairs, which coded for a peptide of 120 amino acids. This is identical in length to the *Fshβ* protein sequenced for olive flounder (Kajimura et al., 2001) and similar to that of half-smooth tongue sole (Cynoglossus semilaevis) (Shi et al., 2015). These similarities already provide evidence of relatedness of these yellowbelly flounder genes to known teleost *Fshβ* and *Lhβ* sequences.

Multiple alignments of the yellowbelly flounder *Lhβ* predicted protein sequence with other selected vertebrate sequences, highlighted the conservation of an N-linked glycosylation site and 12 cysteine residues. A single-N-linked glycosylation site in the amino acid sequence for the *Lhβ* subunit gene has been identified in previous studies on Indian grass carp (*Catla catla*) (Rather et al., 2016) and Japanese sea bass (*Lateolabrax japonicas*) (Chi et al., 2015). In addition, all 12 cysteine residues were fully conserved among all orders represented in the

multiple alignments and have been found to be conserved among vertebrates (Swanson et al., 2003). Multiple alignments of the yellowbelly flounder *Fshβ* predicted protein sequence with other selected vertebrate sequences, also highlighted the conservation of an N-linked glycosylation site and 12 cysteine residues. N-linked glycosylation sites located on the *Fshβ* gene are known to have notable importance for the biological functioning of follicle stimulating hormone (Ulloa-Aguirre et al., 1999). The conserved putative N-glycosylation site was identified in the amino acid sequence for the yellowbelly flounder *Fshβ* gene, based on the identification of this site in largemouth bass (*Micropterus salmoides*) (Li et al., 2021). However, similar to various other teleosts, yellowbelly flounder *Fshβ* lacks the second N-linked glycosylation site present in other vertebrate orders (Chi et al., 2015; Shen et al., 2006; Swanson et al., 2003). Additionally, the presence of 12 highly conserved cysteine residues for the *Fshβ* subunit, could be seen, which is also common with other teleost sequences (Li et al., 2021). Cysteine residues are structurally and functionally essential in proteins (Morand et al., 2004), and these conserved features of the yellowbelly flounder *Fshβ* and *Lhβ* protein sequences indicates that the structures and functions of these genes are well conserved and helps to confirm their identity as the *Fshβ* and *Lhβ* homologues. In addition to alignments, phylogenetic analysis with other known vertebrate *Fshβ* and *Lhβ* sequences revealed that both sequences in yellowbelly flounder were most closely related to known *Fshβ* and *Lhβ* sequences of other flatfishes, providing more confirmation of their relatedness.

3.5.6 Characterisation of *Fshr* and *Lhr*

The pituitary gonadotropin receptors, encoded by *Fshr* and *Lhr,* are membrane-bound G protein-coupled receptors from a rhodopsin-like family (Schulze et al., 2020). These receptors are central components of the reproductive axis, through their key role in receiving and transmitting signals from FSH and LH (Yaron & Levavi-Sivan, 2011). In the yellowbelly flounder transcriptome, the *Fshr* gene was found expressed within the brain, pituitary and ovary, whereas the *Lhr* gene was only expressed in the pituitary. Previous studies show *Fshr* and *Lhr* are primarily associated with the gonads in female and male teleosts (Andersson et al., 2009; Burow et al., 2020; Kobayashi et al., 2008; Yamaguchi et al., 2007). Expression of these receptors extra-gonadally has been reported in some species, including in the brain of Japanese medaka (Burow et al., 2020) and both brain and pituitary of Atlantic halibut (Kobayashi et al., 2008) and Atlantic salmon (Andersson et al., 2009). So, the observation that

Fshr and *Lhr* were seen within the pituitary is consistent with previous work, however the fact that yellowbelly flounder *Lhr* was only found expressed within the pituitary is different. The expression of *Fshr* in all three tissues is the same as observed in other teleosts (Andersson et al., 2009; Kobayashi et al., 2008). The pituitary expression of *Lhr* in some teleosts has been attributed to a paracrine role of LH on the expression of growth hormones, through Lhr mediation (Kobayashi et al., 2008; Zhou et al., 2005). However, it is still expected that *Lhr* would be expressed in the gonads due to the key role both *Fshr* and *Lhr* play in regulating gonadal development. The reason for this is unclear, although it could have something to do with the fact that there are two copies of *Lhr* (*Lhr1* and *Lhr2*) in some teleosts (Maugars & Dufour, 2015), making it difficult to obtain a reliable consensus sequence. The consensus nucleotide sequence constructed for the yellowbelly flounder *Fshr* homologue was 2112 base pairs long and coded for 703 amino acids and the *Lhr* homologue was 1932 base pairs long and coded for 653 amino acids.

Multiple alignments for the yellowbelly flounder *Fshr* and *Lhr* predicted protein sequences with other selected vertebrate sequences, highlighted the conservation of seven transmembrane domain regions that are characteristic of G protein-coupled receptors. These seven transmembrane regions have also been reported for the Japanese medaka (*Oryzias latipes)* (Burow et al., 2020). These transmembrane regions had high similarity in amino acid composition to those of orthologous sequences, providing good evidence of their identity. These are important in receptor function through facilitating signal transduction and activation of the receptor (Ulloa-Aguirre et al., 2007). Further, the G protein-coupled receptor superfamily can be characterised by multiple leucine rich motifs and cysteine residues (Hsu et al., 2000). The *Fshr* sequence was shown to contain 13 conserved cysteine residues, consistent with channel catfish (*Ictalurus* punctatus) (Kumar et al., 2001). Throughout the protein there are 11 leucine rich motifs, including an extra motif which is specific to the *Fshr* gene in Ancanthomorpha (spiny-ray-finned fishes) (Burow et al., 2020; Chauvigné et al., 2010). Leucine rich motifs in G protein-coupled receptors are critical for the functioning of the receptor, through hormone binding (Chauvigné et al., 2010). Similarly, the *Lhr* sequence contained ten leucine rich motifs and 15 conserved cysteine residues, lacking a 16^{th} cysteine which was conserved in all other selected vertebrate sequences. It is unclear why yellowbelly flounder *Lhr* lacks a conserved cysteine, although this may be due to the difficulty obtaining a reliable consensus sequence resulting from the potential presence of two gene copies. The

presence of these characteristic regions provides further evidence of the identity of these sequences from yellowbelly flounder. In addition to the alignment, phylogenetic analysis also confirmed that the yellowbelly flounder *Fshr* gene was closely related to known flatfish *Fshr* sequences, providing more confirmation of its relatedness. However, phylogenetic analysis did show something strange with the yellowbelly flounder *Lhr* sequence, where it did not group with either of the flatfish *Lhr1* or *Lhr2* sequences. The issue here could be with using the transcriptome to put together a consensus sequence, when two copies that are quite similar in sequence exist, meaning you obtain a hybrid of the two sequences rather than one of the copies. This is especially a risk as the size of the reads that were sequenced were only 150 bp in length, making it possible for sequences to be aligned incorrectly. One way around this in the future, would be to use a transcriptomic approach that sequences longer reads, such as PacBio or Oxford Nanopore Technologies (Weirather et al., 2017).

3.6 Summary

Using the transcriptomes obtained from the brain, pituitary and ovary of yellowbelly flounder, this study was able to identify the nucleotide sequences for a number of important genes involved in the reproductive axis. This approach was successful in characterising eight key reproductive genes in yellowbelly flounder and included: *StAR, Hsd17b1, Cyp19a1a, Cyp19a1b, Gpr54, Fshβ, Lhβ* and *Fshr*. Candidates for *Kiss1* and *Kiss2* were unable to be identified using this approach and the sequence for Lhr requires further investigation due to the fact that two Lhr genes potentially exist in this species. For those genes we could obtain and characterise, conservation of functional sites, clear homology with existing sequences from other flatfish species and phylogenetic analysis confirm these are clearly homologues and are likely to have similar functional roles within this species. The genes identified using this transcriptomic approach have generated information that will be highly valuable for the further development of this species for commercial aquaculture. Straight away, primers will be able to be designed to each of these genes, allowing an in-depth analysis of gene expression, using quantitative PCR (qPCR) during the reproductive development of yellowbelly flounder. Understanding the role these genes play during reproductive maturation and development will enable the optimization of reproductive success within this species, which will be a key advancement in making this species a successful aquaculture species within New Zealand.

Chapter 4

General Discussion

This thesis investigates reproduction in yellowbelly flounder, for the purposes of advancing the commercial culture of the species. Specific goals were to initiate the development of a protocol for induced reproduction and to generate a platform of information to support the further development of species-specific genetic tools. While the general function of the reproductive axis is relatively well conserved across finfishes, the individual reproductive strategy and reproductive success in captivity can differ greatly. Reproduction in New Zealand flounders, particularly for aquaculture, is largely unexplored. As the yellowbelly flounder, *Rhombosolea leporina*, has been identified as a potential candidate for the New Zealand aquaculture industry, furthering the current understanding and control of their reproduction is key to their development for aquaculture. Furthermore, diversification of farmed species is critical to ensure that the New Zealand aquaculture industry remains sustainable, resilient and economically viable toward the government's $3 billion goal.

4.1 Reproductive strategies

Despite the prevalence of external fertilization in finfishes (Benun Sutton & Wilson, 2019), their reproductive strategies, including spawning methods and gonadal development, are highly complex and variable (Ciannelli et al., 2015; Mañanós et al., 2009). Consequently, determination of the reproductive strategies of aquaculture target species is needed if an optimal breeding program is to be developed (Mañanós et al., 2009). It is documented for many flatfishes that oocyte development is multiple group synchronous (Agulleiro et al., 2006; Barnett & Pankhurst, 1999; Finn et al., 2002; Poortenaar et al., 2001). The current study has confirmed previous observations (Koverman, 2018) that yellowbelly flounder have multiple group synchronous ovarian development. Cohorts of oocytes at distinct stages of oocyte development were simultaneously present in the ovaries of every fish (Chapter 2). At least three of these fish ovulated twice during the study to provide the first definitive evidence that yellowbelly flounder are multiple batch spawners with an ovulatory periodicity of approximately 72 hours. This general reproductive strategy is consistent with that of other

flatfishes. The use of sustained-release methods or repeat doses of GnRHa therapies are typically better suited to group synchronous batch spawning fish (Guzmán et al., 2009). Further, it is important to note that this study found the size of hydrated oocytes for this species to be very small, which will likely yield small larvae. Therefore, it is possible that larval rearing protocols will need to utilize prey items such as small copepod nauplii for first feeding to ensure successful larval feeding. The current improved understanding of reproduction in female yellowbelly flounder will benefit further optimization of induced reproduction in this species.

4.2 Hormonal induction of reproduction

Yellowbelly flounder were successfully induced to ovulate following treatment with at least 50 µg/kg GnRHa (Chapter 2). Furthermore, fertilization rates of >80% were achieved following treatment. The frequency of spawning within the population was low however, with 33% of the 50 µg/kg GnRHa treated fish and 11% of the 100 µg/kg GnRHa treated fish ovulating. None of the control fish had hydrated or ovulated oocytes during the study. While, GnRHa treatment successfully induced ovulation, spontaneous spawning did not occur. Instead, eggs were manually stripped using gentle pressure on the gonad. Similar outcomes are common in hormonally treated fish (Skaalsvik et al., 2015). The lack of spontaneous spawning will have implications for the optimization of captive reproduction in this species. Spawning has been achieved in some cultured finfishes through manipulating male/female ratios and altering tank conditions (Emata, 2003; Morretti, 1999; Smith et al., 1999). Such factors should be considered for future development of this species. In the absence of spontaneous spawning, hand stripping and artificial fertilization will need to be implemented. In this eventuality, the use of GnRHa to synchronise timing of ovulation among broodstock fish will be valuable as egg quality is oftentimes degraded if ovulated oocytes remain in the oviduct for extended periods (Hobby & Pankhurst, 1997; Rasines et al., 2012). Beyond providing a consistent supply of offspring, the development of an artificial fertilization protocol would also present the opportunity for selectively bred trait enhancement.

Reproductive dysfunction appeared to be prevalent in control fish. Hydrated oocytes were absent in these fish and follicular atresia was observed in both control and some GnRHa treated fish following entry into germinal vesicle migration. This indicates that like many fish, captive yellowbelly flounder experience reproductive dysfunction where oocyte development

arrests after entering into GVM and oocytes fail to enter hydration (Zohar & Mylonas, 2001). Despite reproductive dysfunction in all treatments, hydrated oocytes were only observed in GnRHa treated individuals. Histology revealed a number of GnRHa treated fish completed final oocyte migration and developed hydrated eggs, which was a significant increase compared to the control group. Thus, the current study confirmed that exogenous hormones are a viable method for overcoming captivity-associated reproductive dysfunction in this species. There is a strong likelihood that the reduced number of fish responding to GnRHa treatment was related to inappropriate stage of development at the time of treatment (Berlinsky et al., 1997; Mugnier et al., 2000; Wright-Moore et al., 2019). This was unable to be screened properly due to an issue with the available biopsy catheters at the start of the experiment. Subsequent anecdotal observations have also indicated that yellowbelly flounder populations may have a bimodal spawning patten, with peaks in early winter and spring. Ovulated wild caught fish have been recorded from June through to December (Koverman, 2018). This increases the likelihood of a captured population having many individuals at different stages of development. As a result, this study has developed a classification table based on fresh oocyte samples to ensure that only late vitellogenic/GVM stage fish are GnRHa treated in future.

4.3 Characterization of reproductive genes

Transcriptomic libraries from the three principal tissues involved in the reproductive axis, the brain, pituitary and gonad, were constructed for this species, to enable the fast discovery of key reproductive genes. Characterisation of eight key reproductive genes was achieved in the present study, providing the first analysis of the yellowbelly flounder transcriptome (Chapter 3). A notable observation was the lack of sequences found relating to the kisspeptin system. *Kiss* peptides have been highlighted in a wide range of finfishes for their regulatory role in the reproductive axis, although the exact functioning is unclear, and the functional pathways differ among species (Espigares et al., 2015; Zmora et al., 2012). The two *Kiss* genes reported in teleosts, *Kiss1* and *Kiss2*, were undetected in the yellowbelly flounder transcriptome. Despite this, their receptor *Gpr54* was identified, indicating that at least one *Kiss* gene is present in this species. Thus, the fact that *Kiss* genes were not detected is likely a factor of low expression levels at the time fish were sampled. Further, the location of *Gpr54* in the brain also indicates that the kisspeptin system in this species may exert its influence on the reproductive axis through mediating GnRHa neuron activity (Ma et al.,

2019; Zhao et al., 2014). *In situ* hybridisation would reveal the locality of *Gpr54* expression within the brain to further elucidate this (Kanda et al., 2013). Further investigation into the kisspeptin system in this species will allow for a more comprehensive overview of the functioning of the reproductive axis to assist with protocol development for aquaculture. Furthermore, a reliable sequence was also unable to be obtained for the *Lhr* gene in yellowbelly flounder, and as such requires further investigation. This is likely due to the potential for two *Lhr* genes to exist in this species and therefore utilizing sequencing technologies that generate long reads, such as PacBio and Oxford Nanopore Technologies, may be of use.

The genes encoding the two unique beta-subunits that make up follicle-stimulating hormone (FSH) and luteinizing hormone (LH), and the FSH receptor, *Fshr* were shown to be expressed in this species. Conserved functional regions of *Fshβ* and *Lhβ* and phylogenetic analysis confirm the presence of these genes in yellowbelly flounder. LH is known to regulate the synthesis of the maturation inducing hormone (Arcand-Hoy & Benson, 1998; Yaron & Levavi-Sivan, 2011) and the confirmed presence of this gene indicates that it has the same role in this species. It is likely that the failure of control fish to complete GVM and enter hydration in this study is related to LH signalling and reduced maturation inducing hormone production. Although speculative, it seems reasonable to conclude that the GnRHa treatment upregulated the LH expression in fish that successfully ovulated (Chapter 2), as reported for other species (Mateos et al., 2002; Nyuji et al., 2019). Further investigation into the expression of *Fshβ* and *Lhβ* transcript levels in response to GnRHa treatment would help clarify the function of the reproductive axis in yellowbelly flounder.

The elucidation of these gene sequences enables the development of sensitive research tools. Not only can qPCR primers be designed for gene expression, but these sequences can also be used to develop recombinant gonadotropins. For example, enzyme-linked immunosorbent assays (ELISA) are useful to characterise plasma concentrations of FSH and LH during finfish reproduction (Burow et al., 2019; Molés et al., 2012; Nyuji et al., 2019). Gonadotropins are species specific and therefore require bespoke antibodies (Chauvigné et al., 2015; Molés et al., 2020). The availability of the *Fshβ* and *Lhβ* protein sequences for this species opens up the opportunity for recombinant gonadotropins to be generated and antibodies produced to develop specific ELISAs (Molés et al., 2020). In combination with GnRHa treatment,

development of an ELISA for FSH and LH quantification would enable assessment of gonadotropin signalling for determining the effects of GnRHa treatment, as well as characterising reproduction in this species.

Gonadal steroids in teleosts drive gametogenesis and are themselves produced by steroidogenic enzymes (Tenugu et al., 2021). Four genes encoding enzymes involved in the estradiol biosynthesis pathway were identified in yellowbelly flounder. These include *StAR*, encoding the steroidogenic acute regulatory protein, noted for its importance in initiating steroidogenesis (Tenugu et al., 2021), as well as *Hsd17b1* and *Cyp19a1a*. These were functionally conserved in their role in gonadal steroidogenesis as evidenced by important functional regions. In addition, the brain isoform of the aromatase gene, *Cyp19a1b* was also characterised.

Future work on yellowbelly flounder reproduction may now include qPCR assays for key genes based on these sequences. qPCR is widely used to assess GnRHa regulated expression of reproductive genes (An et al., 2008; Anderson et al., 2017; Chi et al., 2015). Thus, one of the primary benefits for optimization of GnRHa administration will be through assessing the effects of different GnRHa doses on the expression of key genes to contribute to the fine tuning of the GnRHa protocol.

4.4 Recommendations for future research

While this study indicates reproductive dysfunction occurs in captive yellowbelly flounder, the stress response of yellowbelly flounder to captive conditions is yet to be characterised. Measurement of plasma corticosteroid levels in captive fish under differing conditions would be useful to assess the role of stress in the observed reproductive dysfunction. The fact that fish generally failed to complete GVM suggests low maturation inducing hormone concentrations, possibly linked to reduced LH production and secretion. To enable quantification of gonadotropins in response to GnRHa treatment, development of ELISAs specific to the gonadotropins, using recombinant gonadotropins from the generated FSH and LH protein sequences would be of value. Further, to avoid broodstock mortalities, it is recommended to prophylactically treat the wild-caught fish against pathogens and disease immediately post capture.

Successful induction of ovulation in finfishes is dependent in part on exogenous hormone dosage. While GnRHa successfully induced ovulation in some fish, the data indicated dosages under 100 µg/kg are advisable. Therefore, further investigation into the optimal GnRHa dose to administer in cultured yellowbelly flounder would greatly benefit the development of this species for aquaculture. Future studies would also benefit from trialling doses of lower concentrations than those used in the current study, as lower doses may result in a greater incidence of ovulations as well as potentially improve fertilization rates. The use of lower doses in conjunction with appropriate verification of oocyte maturity prior to GnRHa treatment would help to determine the most effective dose. It would also be interesting to further investigate the mechanism of GnRHa overdose and the possible temporary desensitization of the GnRH receptor in the pituitary. This could be done in part using qPCR to assess the expression of the genes involved in the GnRH pathway that were characterised in this study.

Sequencing the transcriptome of yellowbelly flounder and characterising key genes involved in reproductive pathways presents a valuable opportunity for the future development of tools to advance the aquaculture of this species. The sequences generated in this study can be used to design primers and probes specific to yellowbelly flounder. This would allow the quantitative assessment of gene expression using qPCR across the reproductive axis as well as the precise localisation of gene expression using *in situ* hybridisation. Depending on budget constraints, further transcriptomic analysis could be used to effectively assess the effects of different culture conditions on gene expression. Only select reproductive genes were characterised in this study, although thousands of additional genes play important roles in reproduction. From the transcriptomic libraries compiled here, there are a significant number of genes that could be characterised. It is worth noting that from a quantitative perspective, such transcriptomic analysis would still need to be supported with qPCR assays of key reproductive targets. This study provides the first data from which these tools can be developed.

4.5 Conclusion

Globally, aquaculture is becoming an increasingly important food production industry, and the diversification of the New Zealand aquaculture industry is acknowledged as a critical component for its further development. Yellowbelly flounder present themselves as a

promising novel candidate species for the New Zealand aquaculture industry. In order to effectively culture a new species, a comprehensive understanding of their reproduction is required, alongside establishing successful broodstock management protocols. This study has paired physiology and genetics to advance the current understanding of reproduction in this species. In particular, it demonstrates that treatment with an exogenous GnRHa may induce ovulation in female yellowbelly flounder and doses of at least 50 µg/kg GnRHa can significantly increase the number of fish completing final oocyte maturation and entering hydration. In this instance the number of fish responding to the treatment was lower than expected, but this was likely a function of inappropriate ovarian maturity at the time of treatment in many of the fish. Evidence from gonadal histology, fresh oocyte morphology and incidence of ovulation confirmed that yellowbelly flounder are multiple batch spawners. In future studies, it is recommended to use fish with oocytes of a proposed minimum size of 311 µm, corresponding to late vitellogenic and germinal vesicle migration stage oocytes. We can conclude good fecundity, fertilization and egg viability rates can be obtained using at least 50 µg/kg GnRHa. Transcriptomic analysis allowed for the characterisation of eight key genes involved in the reproductive axis of female yellowbelly flounder. These genes were highly conserved, therefore building upon the current knowledge of reproduction in this species. This research provides a stepping-stone in terms of the current knowledge base and genetic tools to further optimize a broodstock management protocol based on GnRHa induced reproduction in yellowbelly flounder.

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Appendices

Appendix 1: *StAR*

A) Nucleotide sequences for yellowbelly flounder *StAR* from each tissue where it was expressed

>GND2A_TR3571
Gttcacaaattaaagaggagaatcgatacagcgacgaagagatctcctacgtgaagcaaggtgaggatgcactgcagaaggccatcaaaatcctcggcgagcaggat
ggctggaccattgaaactgtagctgccaatggagacaaagtcctaagtaaggtgctgcctggcatcgggaaggtgttcaagctggaagtgatgcttgagcaacatac
ggacaatctttatcatgagctggtggggaacatggagcaaatg

>GND2A_TR35090
Tagatttaagaaaagcttgtttgcaaaagccctttgttattatacaaatcggggagttaggggagtaatgaggccctgtgctgaagacccaaataagaccaagttca
cctggttactgagtctcgatctaaagggttggatcccgaagacaatcataaacaaagtgctctcccagacacaggtggactttgccaaccacctcaggcaaaggatg
gctaataacgttactatgga

>GND2A_TR23868
Gaatcttctcagttctcttggagttgttgtaaagtcctgctcaaggaaatgctgcctgcaacttttaaactgtgcgctgggatctcctaccggcatatgagaaacat
gacaggtttaaggaaaaacgccacggtggccctttgccatgagctgcacagactggcaggtccaagccccggaaactggatgag

>PIT2A_TR17642
Ccctaatgtcaaacaggtcaagatcttgcaaaagattggccaggatacaatggtcacccacgaggtgtctgcggagacacctggcaaagtggtgggaccaagggact
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>PIT2A_TR9047
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>PIT3A_TR38994
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>PIT4A_TR66088
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tcagttcacaaattaaagaggagaatcgatacagcgacgaagagatctcctacgtgaagcaaggtgaggatgcactgcagaaggccatcaaaatcctcggcgagcag
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ctgacaccgatctttcacaacgtgcgacccattcatccacaccactccaaggctaagccagtgtaaacagtcaaacatgtaacaaaaaacttcacttttttttttta
ccgataacaccccgagttattgttcttgcgtgacttttatttt

B) Alignment including all deduced nucleotide sequences for yellowbelly flounder *StAR*

```
GND2A_TR23868      cttggagttgttgtaaagtcctgctcaaggaaatgctgcctgcaacttttaaactgtgcg      76
PIT3A_TR38994      cttggagttgttgtaaagtcctgctcaaggaaatgctgcctgcaacttttaaactgtgcg      110
GND2A_TR35090      ------------------------------------------------------------      0
PIT2A_TR17642      ------------------------------------------------------------      0
PIT4A_TR66088      cttggagttgttgtaaagtcctgctcaaggaaatgctgcctgcaacttttaaactgtgcg      180
GND2A_TR3571       ------------------------------------------------------------      0
PIT2A_TR9047       ------------------------------------------------------------      0

GND2A_TR23868      ctgggatctcctaccggcatatgagaaacatgacaggtttaaggaaaaacgccacggtgg      136
PIT3A_TR38994      ctgggatctcctaccggcatatgagaaacatgacaggtttaaggaaaaacgccacggtgg      170
GND2A_TR35090      ------------------------------------------------------------      0
PIT2A_TR17642      ------------------------------------------------------------      0
PIT4A_TR66088      ctgggatctcctaccggcatatgagaaacatgacaggtttaaggaaaaatgccacggtgg      240
GND2A_TR3571       ------------------------------------------------------------      0
PIT2A_TR9047       ------------------------------------------------------------      0

GND2A_TR23868      ccctttgccatgagctgcacagactggcaggtccaagccccggaaactggatgag-----      191
PIT3A_TR38994      ccctttgccatgagctgcacagactggcaggtccaagccccggaaactggatgagccatg      230
GND2A_TR35090      ------------------------------------------------------------      0
PIT2A_TR17642      ------------------------------------------------------------      0
PIT4A_TR66088      ccctttgccatgagctgcacagactggcaggtccaagccccggaaactggatgagccatg      300
GND2A_TR3571       ------------------------------------------------------------      0
PIT2A_TR9047       -Cctttgccatgagctgcacagactggcaggtccaagccccggaaactggatgagccatg      59

GND2A_TR23868      ------------------------------------------------------------      191
PIT3A_TR38994      tgcgcagacggacctcccttctcagttcacaaattaaagaggagaatcgatacagcgacg      290
GND2A_TR35090      ------------------------------------------------------------      0
PIT2A_TR17642      ------------------------------------------------------------      0
PIT4A_TR66088      tgcgcagacggacctcccttctcagttcacaaattaaagaggagaatcgatacagcgacg      360
GND2A_TR3571       ------------------------Gttcacaaattaaagaggagaatcgatacagcgacg      36
PIT2A_TR9047       tacgcagacggacctcccttctcagttcacaaattaaagaggagaatcgatacagcgacg      119

GND2A_TR23868      ------------------------------------------------------------      191
PIT3A_TR38994      aagagatctcctacgtgaagcaaggtgaggatgcactgcagaaggccatcaaaatcctcg      350
GND2A_TR35090      ------------------------------------------------------------      0
PIT2A_TR17642      ------------------------------------------------------------      0
PIT4A_TR66088      aagagatctcctacgtgaagcaaggtgaggatgcactgcagaaggccatcaaaatcctcg      420
GND2A_TR3571       aagagatctcctacgtgaagcaaggtgaggatgcactgcagaaggccatcaaaatcctcg      96
PIT2A_TR9047       aagagatctcctacgtgaagcaaggtgaggatgcactgcagaaggccatcaaaatcctcg      179

GND2A_TR23868      ------------------------------------------------------------      191
PIT3A_TR38994      gcgagcaggatggctggaccattgaaactgtagctgccaatggagacaaagtcctaagta      410
GND2A_TR35090      ------------------------------------------------------------      0
PIT2A_TR17642      ------------------------------------------------------------      0
PIT4A_TR66088      gcgagcaggatggctggaccattgaaactgtagctgccaatggagacaaagtcctaagta      480
GND2A_TR3571       gcgagcaggatggctggaccattgaaactgtagctgccaatggagacaaagtcctaagta      156
PIT2A_TR9047       gcgagcaggatggctggaccattgaaactgtagctgccaatggagacaaagtcctaagta      239
```

```
GND2A_TR23868      ------------------------------------------------------------      191
PIT3A_TR38994      aggtgctgcctggcatcgggaaggtgttcaagctggaagtgatgcttgagcaacatacgg      470
GND2A_TR35090      ------------------------------------------------------------      0
PIT2A_TR17642      ------------------------------------------------------------      0
PIT4A_TR66088      aggtgctgcctggcatcgggaaggtgttcaagctggaagtgatgcttgagcaacatacgg      540
GND2A_TR3571       aggtgctgcctggcatcgggaaggtgttcaagctggaagtgatgcttgagcaacatacgg      216
PIT2A_TR9047       aggtgctgcctggcatcgggaaggtgttcaagctggaagtgatgcttgagcaacatacgg      299

GND2A_TR23868      ------------------------------------------------------------      191
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GND2A_TR35090      ------------------------------------------------------------      0
PIT2A_TR17642      ----------------------------------------------------Ccctaatg      8
PIT4A_TR66088      acaatctttatcatgagctggtggggaacatggagcaaatgggggagtggaaccctaatg      600
GND2A_TR3571       acaatctttatcatgagctggtggggaacatggagcaaatg--------------------      257
PIT2A_TR9047       acaatctttatcatgagctggtggggaacatggagcaaatggg------------------      342


GND2A_TR23868      ------------------------------------------------------------      191
PIT3A_TR38994      tcaaacaggtcaagatcttgcaaaagattggccaggacacaatggtcacccacgaggtgt      590
GND2A_TR35090      ------------------------------------------------------------      0
PIT2A_TR17642      tcaaacaggtcaagatcttgcaaaagattggccaggatacaatggtcacccacgaggtgt      68
PIT4A_TR66088      tcaaacaggtcaagatcttgcaaaagattggccaggatacaatggtcacccacgaggtgt      660
GND2A_TR3571       ------------------------------------------------------------      257
PIT2A_TR9047       ------------------------------------------------------------      342


GND2A_TR23868      ------------------------------------------------------------      191
PIT3A_TR38994      ctgcggagacacctggcaaagtggtgggaccaagggactttgtcagcgtccgctgtgcca      650
GND2A_TR35090      ------------------------------------------------------------      0
PIT2A_TR17642      ctgcggagacacctggcaaagtggtgggaccaagggactttgtcagcgtccgctgtgcca      128
PIT4A_TR66088      ctgcggagacacctggcaaagtggtgggaccaagggactttgtcagcgtccgctgtgcca      720
GND2A_TR3571       ------------------------------------------------------------      257
PIT2A_TR9047       ------------------------------------------------------------      342


GND2A_TR23868      ------------------------------------------------------------      191
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GND2A_TR35090      Tagatttaagaaaagcttgtt----tgc---aaaag-ccctttgttat-----tatacaa      47
PIT2A_TR17642      aacgtagaggctccgcctgcttcctggctggaatgtccactcagcatccgaagatgccgg      188
PIT4A_TR66088      aacgtagaggctccgcctgcttcctggctggaatgtccactcagcatccgaagatgccgg      780
GND2A_TR3571       ------------------------------------------------------------      257
PIT2A_TR9047       ------------------------------------------------------------      342


GND2A_TR23868      ------------------------------------------------------------      191
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GND2A_TR35090      ---atcggggag------------------------ttaggggagtaatgaggccctgtg      80
PIT2A_TR17642      agcagcggggtgtggtcagggccgagaatgggccttcctgtatcgtaatgaggccctgtg      248
PIT4A_TR66088      agcagaggggtgtggtcagggccgagaatgggccttcctgtatcgtaatgaggccctgtg      840
GND2A_TR3571       ------------------------------------------------------------      257
PIT2A_TR9047       ------------------------------------------------------------      342


GND2A_TR23868      ------------------------------------------------------------      191
PIT3A_TR38994      ctgaagacccaaataagaccaagttcacctggttactgagtctcgatctaaagggttgga      830
GND2A_TR35090      ctgaagacccaaataagaccaagttcacctggttactgagtctcgatctaaagggttgga      140
PIT2A_TR17642      ctgaagacccaaataagaccaagttcacctggttactgagtctcgatctaaagggttgga      308
PIT4A_TR66088      ctgaagacccaaataagaccaagttcacctggttactgagtctcgatctaaagggttgga      900
GND2A_TR3571       ------------------------------------------------------------      257
PIT2A_TR9047       ------------------------------------------------------------      342


GND2A_TR23868      ------------------------------------------------------------      191
PIT3A_TR38994      tcccgaagacaatcataaacaaagtgctctcccagacacaggtggactttgccaaccacc      890
GND2A_TR35090      tcccgaagacaatcataaacaaagtgctctcccagacacaggtggactttgccaaccacc      200
PIT2A_TR17642      tcccgaagacaatcataaacaaagtgctctcccagacacaggtggactttgccaaccacc      368
PIT4A_TR66088      tcccgaagacaatcataaacaaagtgctctcccagacacaggtggactttgccaaccacc      960
GND2A_TR3571       ------------------------------------------------------------      257
PIT2A_TR9047       ------------------------------------------------------------      342

GND2A_TR23868      ------------------------------------------------------------      191
PIT3A_TR38994      tcaggcaaaggatggctaataacgttactatggagatggctcatgcctgctgacaccagc      950
GND2A_TR35090      tcaggcaaaggatggctaataacgttactatgga----------------------------      234
PIT2A_TR17642      tcaggcaaaggatggctaataacgttactatggagatggctcatgcctgctgacaccagc      428
PIT4A_TR66088      tcaggcaaaggatggctaataacgttactatggagatggctcatgcctgctgacaccagc      1020
GND2A_TR3571       ------------------------------------------------------------      257
PIT2A_TR9047       ------------------------------------------------------------      342
```

C) Yellowbelly flounder *StAR* consensus nucleotide sequence and translation

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acaggtttaaggaaaaacgccacggtggccctttgccatgagctgcacagactggcaggt
 T  G  L  R  K  N  A  T  V  A  L  C  H  E  L  H  R  L  A  G
ccaagccccggaaactggatgagccatgtgcgcagacggacctcccttctcagttcacaa
 P  S  P  G  N  W  M  S  H  V  R  R  R  T  S  L  L  S  S  Q
attaaagaggagaatcgatacagcgacgaagagatctcctacgtgaagcaaggtgaggat
 I  K  E  E  N  R  Y  S  D  E  E  I  S  Y  V  K  Q  G  E  D
gcactgcagaaggccatcaaaatcctcggcgagcaggatggctggaccattgaaactgta
 A  L  Q  K  A  I  K  I  L  G  E  Q  D  G  W  T  I  E  T  V
gctgccaatggagacaaagtcctaagtaaggtgctgcctggcatcgggaaggtgttcaag
 A  A  N  G  D  K  V  L  S  K  V  L  P  G  I  G  K  V  F  K
ctggaagtgatgcttgagcaacatacggacaatctttatcatgagctggtggggaacatg
 L  E  V  M  L  E  Q  H  T  D  N  L  Y  H  E  L  V  G  N  M
gagcaaatgggggagtggaaccctaatgtcaaacaggtcaagatcttgcaaaagattggc
 E  Q  M  G  E  W  N  P  N  V  K  Q  V  K  I  L  Q  K  I  G
caggatacaatggtcacccacgaggtgtctgcggagacacctggcaaagtggtgggacca
 Q  D  T  M  V  T  H  E  V  S  A  E  T  P  G  K  V  V  G  P
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ttactgagtctcgatctaaagggttggatcccgaagacaatcataaacaaagtgctctcc
 L  L  S  L  D  L  K  G  W  I  P  K  T  I  I  N  K  V  L  S
cagacacaggtggactttgccaaccacctcaggcaaaggatggctaataacgttactatg
 Q  T  Q  V  D  F  A  N  H  L  R  Q  R  M  A  N  N  V  T  M
gagatggctcatgcctgctga
 E  M  A  H  A  C  -
```

Appendix 2: *Hsd17b1*

A) Nucleotide sequences for yellowbelly flounder *Hsd17b1* from each tissue where it was expressed

```
>TR27673_BR2A
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>TR26936_GND2A
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gatgaggaacctggccaagaaggagcgcctcctggagagcgtcaggggactgcacgaggacaccttggacatcctgcaaatggacgtgacggaccgacagtccattc
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>TR36520_GND3A
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gatgaggaacctggccaagaaggagcgcctcctggagagcgtcaggggactgcacgaggacaccttggacatcctgcaaatggacgtgacggaccgacagtccattc
tggacgcccgggacgggctggaggagaagcgggtggacattctggtgtgcaacgccggcgtgggtttgatggggccgctggagctgcagtccctggactccatgagg
cagattctggaggtcaacctgctggggaccatccagaccatccaggccttcctgccgcagatgaaggccaggggccagggtcgcatcctggtcaccggcagcaccgg
agggcttcacggcctcccatttaatgaggtgtactgtgccagtaaatttgcaatagagggagcgtgtgagagtttggcaatcctcctgcaccacttcaacatccacg
```

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ctctacgaaaagtacctgcagcactgcggcactgttttccaaaacgcagcccaggacaccgaggacatcgtcaaggtatttctggaggccatccagtcgcccggccc
cgcattccgatacttcaccagcggcgtcgttccgcctctggcccaaatgaagatggccgaaccagatggctcgcggtgcatccgtgccatgagcaaaatcatattct
cgaccgaggggaagaatag

>TR11870_GND4A
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cgcattccgatacttcaccagcggcgtcgttccgcctctggcccaaatgaagatggccgaaccagatggctcgcggtgcatccgtgccatgagcaaaatcatattct
cgaccgaggggaagaatag

B) Alignment including all deduced nucleotide sequences for yellowbelly flounder *Hsd17b1*

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TR11870_GND4A      atggataagaaggtggtgctgatcacaggctgctcctcggggatcggcctgagcctggcc       60
TR27673_BR2A       ------------------------------------------------------------       0
TR26936_GND2A      Atggataagaaggtggtgctgatcacaggctgctcctcggggatcggcctcagcctggcc       60

TR36520_GND3A      gtccgattggcctccgaccccggcgaaacgttcaaagtttatgccacgatgaggaacctg       120
TR11870_GND4A      gtccgattggcctccgaccccggcgaaacgttcaaagtttatgccacgatgaggaacctg       120
TR27673_BR2A       ------------------------------------------------------------       0
TR26936_GND2A      gtccgattggcctccgaccccggcgaaacgttcaaagtttatgccacgatgaggaacctg       120

TR36520_GND3A      gccaagaaggagcgcctcctggagagcgtcaggggactgcacgaggacaccttggacatc       180
TR11870_GND4A      gccaagaaggagcgcctcctggagagcgtcaggggactgcacgaggacaccttggacatc       180
TR27673_BR2A       ----------------------------Caggggactgcacgaggacaccttggacatc       31
TR26936_GND2A      gccaagaaggagcgcctcctggagagcgtcaggggactgcacgaggacaccttggacatc       180
                                               ******************************

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TR11870_GND4A      ctgcaaatggacgtgacggaccgacagtccattctggacgcccgggacgggctggaggag       240
TR27673_BR2A       ctgcaaatggacgtgacggaccgacagtccattctggacgcccgggacgggctggaggag       91
TR26936_GND2A      ctgcaaatggacgtgacggaccgacagtccattctggacgcccgggacgggctggaggag       240
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TR27673_BR2A       aagcgggtggacattctggtgtgcaacgccggcgtgggtttgatggggccgctggagctg       151
TR26936_GND2A      aagcgggtggacattctggtgtgcaacgccggcgtgggtttgatggggccgctggagctg       300
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TR26936_GND2A      cagtccctggactccatgaggcagattctggatgtcaacctgctggggaccatccagacc       360
                   ******************************** ***************************

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TR26936_GND2A      atccaggccttcctgccccagatgaaggccaggggccagggtcgcatcctggtcaccggc       420
                   ***************** ******************************************

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```

```
TR36520_GND3A      atagagggagcgtgtgagagtttggcaatcctcctgcaccacttcaacatccacgtgagt       540
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TR27673_BR2A       atagagggagcgtgtgagagtttggcaatcctcctgcaccacttcaacatccacgtgagt       391
TR26936_GND2A      atagagggagcgtgtgagagtttggcaatcctcctgcaccacttcaacatccacgtgagt       540
                   ************************************************************

TR36520_GND3A      ctcatcgagtgcggaccggtcaacaccgacttcctggtcaacctgcggaaggcggagctc       600
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```

C) Yellowbelly flounder *Hsd17b1* consensus sequence and translation

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gtccgattggcctccgaccccggcgaaacgttcaaagtttatgccacgatgaggaacctg
 V  R  L  A  S  D  P  G  E  T  F  K  V  Y  A  T  M  R  N  L
gccaagaaggagcgcctcctggagagcgtcaggggactgcacgaggacaccttggacatc
 A  K  K  E  R  L  L  E  S  V  R  G  L  H  E  D  T  L  D  I
ctgcaaatggacgtgacggaccgacagtccattctggacgcccgggacgggctggaggag
 L  Q  M  D  V  T  D  R  Q  S  I  L  D  A  R  D  G  L  E  E
aagcgggtggacattctggtgtgcaaccgccggcgtgggtttgatggggccgctggagctg
 K  R  V  D  I  L  V  C  N  A  G  V  G  L  M  G  P  L  E  L
cagtccctggactccatgaggcagattctggatgtcaacctgctggggaccatccagacc
 Q  S  L  D  S  M  R  Q  I  L  D  V  N  L  L  G  T  I  Q  T
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 I  Q  A  F  L  P  Q  M  K  A  R  G  Q  G  R  I  L  V  T  G
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 Q  H  C  G  T  V  F  Q  N  A  A  Q  D  T  E  D  I  V  K  V
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 F  L  E  A  I  Q  S  P  G  P  A  F  R  Y  F  T  S  G  V  V
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 P  P  L  A  Q  M  K  M  A  E  P  D  G  S  R  C  I  R  A  M
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 S  K  I  I  F  S  T  E  G  E  E  -
```

Appendix 3: *Cyp19a1a*

A) Nucleotide sequences for yellowbelly flounder *Cyp19a1a* from each tissue where it was expressed

```
>TR39273_GND2A
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aggccaaaaatctcaccatgaggttcttacccagacagaggggcagctggcaaacgctcaccgacgccaataagctgcccatgtcagattga
```

B) Alignment including all deduced nucleotide sequences for yellowbelly flounder *Cyp19a1a*

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TR39273_GND2A      ------------------------------------------------------------  0
TR21297_GND4A      atgttgtggcttttgcattaccctgacctggctggtaaccagctccgcacgcttataagg  60


TR24941_GND3A      ------------------------------------------------------------  0
TR39273_GND2A      ------------------------------------------------------------  0
TR21297_GND4A      aggaagagaatgtggaggtccctttcattactccgtttggtcgctggatttgcgcggcgg  120
```

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TR24941_GND3A      ---------------------------------atggatctgatgcctgcgcgtgacctg  27
TR39273_GND2A      ------------------------------------------------------------  0
TR21297_GND4A      tggtgtgtgcacctttatttttctctctcgtcccatggatctgatgcctgcgcgtgacctg  180

TR24941_GND3A      ccaatgactccgatgagattcgatgccatagcggcagacctcgtggccatgtccccaaat  87
TR39273_GND2A      ------------------------------------------------------------  0
TR21297_GND4A      ccaatgactccgatgagattcgatgccatagcggcagacctcgtggccatgtccccaaat  240

TR24941_GND3A      gccaccacagtggggtcacgaggcatctcagtggcgagcagaaccttgctgcttctgatc  147
TR39273_GND2A      ------------------------------------------------------------  0
TR21297_GND4A      gccaccacagtggggtcacgaggcatctcagtggcgagcagaaccttgctgcttctgatc  300

TR24941_GND3A      tgccttctgctggtcgtctggagccacacggacaagcgacctgtgccaggtccttcgttc  207
TR39273_GND2A      ---------------------------acggacaagcgacctgtgccaggtccttcgttc  33
TR21297_GND4A      tgccttctgctggtcgtctggagccacacggacaagcgacctgtgccaggtccttcgttc  360
                                             **********************************

TR24941_GND3A      tgtctgggtttggggccacttttgtcctatctgaggttcatctggaccggtataggcaca  267
TR39273_GND2A      tgtctgggtttggggccacttttgtcctatctgaggttcatctggaccggtataggcaca  93
TR21297_GND4A      tgtctgggtttggggccacttttgtcctatctgaggttcatctggaccggtataggcaca  420
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TR24941_GND3A      gcgagcaactactacaacaacaaatacggagacattgtcagagtttggatcaacggagag  327
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TR21297_GND4A      gcgagcaactactacaacaacaaatacggagacattgtcagagtttggatcaacggagag  480
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TR24941_GND3A      gagaccctcgttatcagcagaccgtcagcagtgcagcacgttctcaagaatggacactac  387
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TR24941_GND3A      acctcacgctttgggagcaagcaggggctgagctgcatcggcatgcacgagagaggcatc  447
TR39273_GND2A      acctcacgctttgggagcaagcaggggctgagctgcatcggcatgcacgagagaggcatc  273
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TR24941_GND3A      atatttaacaacaacgtcaccctgtggaaaaagatacgtgcttatttcgccaaagctctg  507
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TR21297_GND4A      atatttaacaacaacgtcaccctgtggaaaaagatacgcgcttatttcgccaaagctctg  660
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TR24941_GND3A      acaggtccaggcttgcagaagacagtggaggtctgtgtcacctccacgcagagtcacttg  567
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TR21297_GND4A      acaggtccaggcttgcagaagacggtggaggtctgtgtcacctccacgcagagtcacttg  720
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TR24941_GND3A      gacgacttggacaccttgggttacgttgatttcctaattttgctgcgctgcacggtagtc  627
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TR24941_GND3A      gacatctccaacagactattcctggatgttccgatcaatgagaaagagctcatggtgaag  687
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TR21297_GND4A      attgagaaatatttcgatacatggcagagtgtgctgatcaaaccagacatctacttcaag  900
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```

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TR21297_GND4A      atgctgctgctcctcaaacaaaatccaaatgtggagctgcagctgctacaagagatagac  1200
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TR21297_GND4A      accgttttaggcgataggcggctgcagaggggggacctcccgaagctgcacgtactggag  1260
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TR21297_GND4A      accggtcgcatgcaccggatggacattttctgcaaacccaatgaattcagcctggacaac  1440
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TR21297_GND4A      ttccaaaaaagcactcctcgacgttatttccagccattcggttcaggcccccgggcctgt  1500
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TR21297_GND4A      gttggcaaacacatcgccatggtgatgatgaaatccatcctggtgacactgctctcccag  1560
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TR21297_GND4A      agacagaggggcagctggcaaacgctcaccgacgccaataagctgcccatgtcagattga  1740
                   ************************************************************
```

C) Yellowbelly flounder *Cyp19a1a* consensus sequence and translation

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gcagacctcgtggccatgtccccaaatgccaccacagtggggtcacgaggcatctcagtg
 A  D  L  V  A  M  S  P  N  A  T  T  V  G  S  R  G  I  S  V
gcgagcagaaccttgctgcttctgatctgccttctgctggtcgtctggagccacacggac
 A  S  R  T  L  L  L  L  I  C  L  L  L  V  V  W  S  H  T  D
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 K  R  P  V  P  G  P  S  F  C  L  G  L  G  P  L  L  S  Y  L
aggttcatctggaccggtataggcacagcgagcaactactacaacaacaaatacggagac
 R  F  I  W  T  G  I  G  T  A  S  N  Y  Y  N  N  K  Y  G  D
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 I  V  R  V  W  I  N  G  E  E  T  L  V  I  S  R  P  S  A  V
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 Q  H  V  L  K  N  G  H  Y  T  S  R  F  G  S  K  Q  G  L  S
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 C  I  G  M  H  E  R  G  I  I  F  N  N  N  V  T  L  W  K  K
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 I  R  A  Y  F  A  K  A  L  T  G  P  G  L  Q  K  T  V  E  V
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 C  V  T  S  T  Q  S  H  L  D  D  L  D  T  L  G  Y  V  D  F
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 I  N  E  K  E  L  M  V  K  I  E  K  Y  F  D  T  W  Q  S  V
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 L  I  K  P  D  I  Y  F  K  F  D  R  I  H  Q  R  H  K  T  A
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 Q  A  E  K  L  D  D  I  N  F  T  T  E  L  I  F  A  Q  G  H
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 D  T  L  S  V  S  L  F  F  M  L  L  L  L  K  Q  N  P  N  V
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Appendix 4: *Cyp19a1b*

A) Nucleotide sequences for yellowbelly flounder *Cyp19a1b* from each tissue where it was expressed

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B) Alignment including all deduced nucleotide sequences for yellowbelly flounder *Cyp19a1b*

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TR6156_PIT4A       atggacgctgtggtgcaggtcaccgtcctcctgcttcttcttcttctcctgctgctgctg  60
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TR60916_BRA4       ------------------------------------------------------------  0


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TR6156_PIT4A       gtgctggtcatggccgcccggagccgagcgaaccgctcacaaatgccgggtccttccttc  120
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TR61632_BRA2A      gatgatgacctcgactttgcaacggagctcatctttgcacagaaccatggggagctgtcg  733
TR60916_BRA4       gatgatgacctcgactttgcaacggagctcatctttgcacagaaccacggggagctgtcg  733
                   ************************************************ ************

TR36293_PIT2A      gcggacaacgtcaggcagtgcgtgctggagatggtgatcgcggcgcccgacacccttcc  900
TR26286_PIT3A      gcggacaacgtcaggcagtgcgtgctggagatggtgatcgcggcgcccgacaccctgtcc  900
TR6156_PIT4A       gcggacaacgtcaggcagtgcgtgctggagatggtgatcgcggcgcccgacaccctgtcc  900
TR61632_BRA2A      gcggacaacgtcaggcagtgcgtgctggagatggtgatcgcggcgcccgacacccttcc  793
TR60916_BRA4       gcggacaacgtcaggcagtgcgtgctggagatggtgatcgcggcgcccgacaccctgtcc  793
                   ************************************************************ ***

TR36293_PIT2A      gtcagcatcttcttcatgctcatgctgctgaaacaaaacccggatttggagctgagcatt  960
TR26286_PIT3A      gtcagcatcttcttcatgctcatgctgctgaaacaaaacccggatttggagctgagcatt  960
TR6156_PIT4A       gtcagcatcttcttcatgctcatgctgctgaaacaaaacccggatttggagctgagcatt  960
TR61632_BRA2A      gtcagcatcttcttcatgctcatgctgctgaaacaaaacccggatttggagctgagcatt  853
TR60916_BRA4       gtcagcatcttcttcatgctcatgctgctgaaacaaaacccggatttggagctgagcatt  853
                   ************************************************************

TR36293_PIT2A      gtggaggagatgaacgccgtctcggatgagcagggtgccgagaagtatcaaagccttaag  1020
TR26286_PIT3A      gtggaggagatgaacaccgtcacggatgagcagggtgccgagaagtatcaaagccttaag  1020
TR6156_PIT4A       gtggaggagatgaacaccgtcacggatgagcagggtgccgagaagtatcaaagccttaag  1020
TR61632_BRA2A      gtggaggagatgaacgccgtctcggatgagcagggtgccgagaagtatcaaagccttaag  913
TR60916_BRA4       gtggaggagatgaacaccgtcacggatgagcagggtgccgagaagtatcaaagccttaag  913
                   ***************.*****:************************************
```

```
TR36293_PIT2A      ctgctggagaatttcatcaaggagtccctgcgctttcaccccgtggtggatttcaccatg  1080
TR26286_PIT3A      ctgctggagaatttcatcaaggagtccctgcgctttcaccccgtggtggatttcaccatg  1080
TR6156_PIT4A       ctgctggagaatttcatcaaggagtccctgcgctttcaccccgtggtggatttcaccatg  1080
TR61632_BRA2A      ctgctggagaatttcatcaaggagtccctgcgctttcaccctgtggtggatttcaccatg  973
TR60916_BRA4       ctgctggagaatttcatcaaggagtccctgcgctttcaccccgtggtggatttcaccatg  973
                   **************************************** ******************

TR36293_PIT2A      cgcaaggccctggaggacgacgtgatcgaaggcacgcgcatcggcaagggaaccaacatc  1140
TR26286_PIT3A      cgcaaggccctggaggacgacgtgatcgaaggcacgcgcatcggcaagggaaccaacatc  1140
TR6156_PIT4A       cgcaaggccctggaggacgacgtgatcgaaggcacgcgcatcggcaagggaaccaacatc  1140
TR61632_BRA2A      cgcaaggccctggaggacgacgtgatcgaaggcacgcgcatcggcaagggaaccaacatc  1033
TR60916_BRA4       cgcaaggccctggaggacgacgtgatcgaaggcacgcgcatcggcaagggaaccaacatc  1033
                   ************************************************************

TR36293_PIT2A      atcctcaacattggcctgatgcacaagaccgaattcttccccaagccgatggagttcagc  1200
TR26286_PIT3A      atcctcaacattggcctgatgcacaagaccgaattcttccccaagccgatggagttcagc  1200
TR6156_PIT4A       atcctcaacattggcctgatgcacaagaccgaattcttccccaagccgatggagttcagc  1200
TR61632_BRA2A      atcctcaacattggcctgatgcacaagaccgaattcttccccaagccgatggagttcagc  1093
TR60916_BRA4       atcctcaacattggcctgatgcacaagaccgaattcttccccaagccgatggagttcagc  1093
                   ************************************************************

TR36293_PIT2A      ctgttgaacttcgacaaaaatgtgcccagtcgctacttccagccgttcggctgcgggccc  1260
TR26286_PIT3A      ctgttgaactttgacaaaaatgtgcccagtcgctacttccagccgttcggctgcgggccc  1260
TR6156_PIT4A       ctgttgaacttcgacaaaaatgtgcccagtcgctacttccagccgttcggctgcgggccc  1260
TR61632_BRA2A      ctgttgaacttcgacaaaaatgtgcccagtcgctacttccagccgttcggctgcgggccc  1153
TR60916_BRA4       ctgttgaacttcgacaaaaatgtgcccagtcgctacttccagccgttcggctgcgggccc  1153
                   *********** ************************************************

TR36293_PIT2A      cgctcctgcgtgggcaagcacatcgccatggtgatgatgaaggccatcctggtcaccctg  1320
TR26286_PIT3A      cgctcctgcgtgggcaagcacatcgccatggtgatgatgaaggccatcctggtcaccctg  1320
TR6156_PIT4A       cgctcctgcgtgggcaagcacatcgccatggtgatgatgaaggccatcctggtcaccctg  1320
TR61632_BRA2A      cgctcctgcgtgggcaagcacatcgccatggtgatgatgaaggccatcctggtcaccctg  1213
TR60916_BRA4       cgctcctgcgtgggcaagcacatcgccatggtgatgatgaaggccatcctggtcaccctg  1213
                   ************************************************************

TR36293_PIT2A      ctgtcgcggtacacggtgtgtcctcgccgaggctgcaacctggccagcatccggcagacc  1380
TR26286_PIT3A      ctgtcgcggtacacggtgtgtcctcgccgaggctgcaacctggccagcatccggcagacc  1380
TR6156_PIT4A       ctgtcgcggtacacggtgtgtcctcgccgaggctgcaacctggccagcatccggcagacc  1380
TR61632_BRA2A      ctgtcgcggtacacggtgtgtcctcgccgaggctgcaacctggccagcatccggcagacc  1273
TR60916_BRA4       ctgtcgcggtacacggtgtgtcctcgccgaggctgcaacctggccagcatccggcagacc  1273
                   ************************************************************

TR36293_PIT2A      aacaacctgtcgcagcagcccgtggaggacgaccacggcctggccatgcgcttcgtccct  1440
TR26286_PIT3A      aacaacctgtcgcagcagcccgtggaggacgaccacggcctggccatgcgcttcgtccct  1440
TR6156_PIT4A       aacaacctgtcgcagcagccggtggaggacgaccacggcctggccatgcgcttcgtccct  1440
TR61632_BRA2A      aacaacctgtcgcagcagcccgtggaggacgaccacggcctggccatgcgcttcgtccct  1333
TR60916_BRA4       aacaacctgtcgcagcagcccgtggaggacgaccacggcctggccatgcgcttcgtccct  1333
                   ******************** ****************************************

TR36293_PIT2A      cggggcgcgggcgccctgcgtcgcgacctctga    1473
TR26286_PIT3A      cggggcgcgggcgccctgcgtcgcgacctctga    1473
TR6156_PIT4A       cggggcgcgggcgccctgcgtcgcgacctctga    1473
TR61632_BRA2A      cggggcgcgggcgccctgcgtcgcgacctctga    1366
TR60916_BRA4       cggggcgcgggcgccctgcgtcgcgacctctga    1366
                   **********************************
```

C) Yellowbelly flounder *Cyp19a1b* consensus sequence and translation

```
atggacgctgtggtgcaggtcaccgtcctcctgcttcttcttcttctcctgctgctgctg
 M  D  A  V  V  Q  V  T  V  L  L  L  L  L  L  L  L  L  L  L
gtgctggtcatggccgcccggagccgagcgaaccgctcacaaatgccgggtccttccttc
 V  L  V  M  A  A  R  S  R  A  N  R  S  Q  M  P  G  P  S  F
tgggcaggactgggcccggtcctctcctacgccaggttcctcttgactggcatcggcacg
 W  A  G  L  G  P  V  L  S  Y  A  R  F  L  L  T  G  I  G  T
gcgagcaactactacaaccacaggtacggcgacatggtgcgggtgtggattaacggcgag
 A  S  N  Y  Y  N  H  R  Y  G  D  M  V  R  V  W  I  N  G  E
gagaccctcatcgtgagcaggtcttcggtcgtgtatcacgttctgaggagcgcccactac
 E  T  L  I  V  S  R  S  S  V  V  Y  H  V  L  R  S  A  H  Y
accgccagattcgggagcaagcaaggcctgcagtgcatcgggatggaagggaggggatc
 T  A  R  F  G  S  K  Q  G  L  Q  C  I  G  M  E  G  R  G  I
atcttcaacagtgacgtcccactctggaaaaatatcagggagcgcttttcgaaagccttg
 I  F  N  S  D  V  P  L  W  K  N  I  R  E  R  F  S  K  A  L
accggccccaccctccagaggacggtggccatctgtgtgagctccactgccaaacacctg
```

```
 T  G  P  T  L  Q  R  T  V  A  I  C  V  S  S  T  A  K  H  L
gacagcctgcaggagatgaccgacgcttccggcaacgtggacgccctcaatctgctgagg
 D  S  L  Q  E  M  T  D  A  S  G  N  V  D  A  L  N  L  L  R
gccgtggtcgtggacatctccaacaggcttttcctccgggtgccactcaatgagaaagac
 A  V  V  V  D  I  S  N  R  L  F  L  R  V  P  L  N  E  K  D
ttgctgaagaaaatccacaactacttcgaaacctggcaaacggttctcctgaagcccgat
 L  L  K  K  I  H  N  Y  F  E  T  W  Q  T  V  L  L  K  P  D
gtgttcttcaagattggatggctgtgtaacaagcataacaaagcagcccaagagcttcaa
 V  F  F  K  I  G  W  L  C  N  K  H  N  K  A  A  Q  E  L  Q
gacgtgatggagagcctcctggaaatcaagagacaaatgataaatgagtccgagaagctg
 D  V  M  E  S  L  L  E  I  K  R  Q  M  I  N  E  S  E  K  L
gatgatgacctcgactttgcaacggagctcatctttgcacagaaccacggggagctgtcg
 D  D  D  L  D  F  A  T  E  L  I  F  A  Q  N  H  G  E  L  S
gcggacaacgtcaggcagtgcgtgctggagatggtgatcgcggcgcccgacaccctgtcc
 A  D  N  V  R  Q  C  V  L  E  M  V  I  A  A  P  D  T  L  S
gtcagcatcttcttcatgctcatgctgctgaaacaaaacccggatttggagctgagcatt
 V  S  I  F  F  M  L  M  L  L  K  Q  N  P  D  L  E  L  S  I
gtggaggagatgaacaccgtcacggatgagcagggtgccgagaagtatcaaagccttaag
 V  E  E  M  N  T  V  T  D  E  Q  G  A  E  K  Y  Q  S  L  K
ctgctggagaatttcatcaaggagtccctgcgctttcaccccgtggtggatttcaccatg
 L  L  E  N  F  I  K  E  S  L  R  F  H  P  V  V  D  F  T  M
cgcaaggccctggaggacgacgtgatcgaaggcacgcgcatcggcaagggaaccaacatc
 R  K  A  L  E  D  D  V  I  E  G  T  R  I  G  K  G  T  N  I
atcctcaacattggcctgatgcacaagaccgaattcttccccaagccgatggagttcagc
 I  L  N  I  G  L  M  H  K  T  E  F  F  P  K  P  M  E  F  S
ctgttgaacttcgacaaaaatgtgcccagtcgctacttccagccgttcggctgcgggccc
 L  L  N  F  D  K  N  V  P  S  R  Y  F  Q  P  F  G  C  G  P
cgctcctgcgtgggcaagcacatcgccatggtgatgatgaaggccatcctggtcaccctg
 R  S  C  V  G  K  H  I  A  M  V  M  M  K  A  I  L  V  T  L
ctgtcgcggtacacggtgtgtcctcgccgaggctgcaacctggccagcatccggcagacc
 L  S  R  Y  T  V  C  P  R  R  G  C  N  L  A  S  I  R  Q  T
aacaacctgtcgcagcagccggtggaggacgaccacggcctggccatgcgcttcgtccct
 N  N  L  S  Q  Q  P  V  E  D  D  H  G  L  A  M  R  F  V  P
Cggggcgcgggcgccctgcgtcgcgacctctga
 R  G  A  G  A  L  R  R  D  L  -
```

Appendix 5: *Gpr54*

A) Nucleotide sequences for yellowbelly flounder *Gpr54* from each tissue where it was expressed

```
>TR118_BR2A
Ccctggaactccaccgagttgccgtggatcaacggctcccggctcaacttgtctctgggaagactcggacccggcggcggcggcggcgacgacggcgaggagg
aaagagggcagcacccctcctcacggacgcctggctggtgccgctcttcttctcgctcatcatgctggtcgggctggtgggcaatgccctggttatctatgt
catctccaaacaccggcagatgaggaccgcgaccaacttctacatagcaaacctggccgcgaccgacatcattttcctggtgtgctgcgtcccatttaccgcc
accctgtatcccctgcccgggtggatcttcggcaacttcatgtgcaagttcgtcgccttcctacagcaggtgacggtccaggccacgtgcatcactctgactg
ccatgagtggagaccgctgctacgtaacggtgtaccccctgaagtctctccgccaccgcacgcccaggatagccatgatcgtcagcgtctgcatttggatcgg
ctccttcatcctgtccacgccgattctgatgtaccagcgtatagaggacggctactggtacggcccgagacaatactgcatggagcgattcccctcgaaaacc
caggagagggctttcatcctctaccagttcattgcggcctacctgctgcccgtgctcaccatatccttctgctacacgctgatggtgaagagggtcggccagc
ccaccgtggagcccgtggacaacaactaccaggtcaacatcctttcggaaagaacgataagcatcaggagcaaggtctccaggatggtggtggtgatcgtcct
cctcttcgccatctgctggggacccattcaaatctttgtcctcttccagtccttccacgccaactaccggcccaactacgtcacgtacaagatcaagacgtgg
gccaactgcatgtcctacgccaactcctccgtcaaccccatcgtctacggcttcatgggcgccagcttccagaagtcctttcgcaagaccttccccttcctgt
tcaggcacaaggtcagagatagcagcacggcctcgaggacggccaacgccgagatcaagtttgtggcggcggcggcggcagcggttgggggcaacaacaacaa
caaccacgaggacggcggcggcggcggcgggcattag

>TR61730_BR4A
gtacccccgaagtctctccgccaccgcacgcccaggatagccatgatcgtcagcgtctgcatttggatcggctccttcatcctgtccacgccgattctgatg
taccagcgtatagaggacggctactggtacggcccgagacaatattgcatggagcgattcccctcgaaaacccaggagagggctttcatcctctaccagttca
ttgcggcctacctgctgcccgtgctcaccatatccttctgctacacgctgatggtgaagagggtcggccagcccaccgtggagcccgtggacaacaactacca
ggtcaacatcctttcggaaagaacgataagcatcaggagcaaggtctccaggatggtggtcgtgatcgtcctcctcttcgccatctgctggggacccattcaa
atctttgtcctcttccagtccttccacgccaactaccggcccaactacgtcacgtacaagatcaagacgtgggccaactgcatgtcctacgccaactcctccg
tcaaccccatcgtctacggcttcatgggcgccagcttccagaagtcctttcgcaagaccttcccctttcctgttcaggcacaaggtcagagatagcagcacggc
ctcgaggacggccaacgccgagatcaagtttgtggcggcggcggcggcggcggttgggggcaacaacaacaacaaccacgaggacggcggcggcgg
```

B) Alignment including all deduced nucleotide sequences for yellowbelly flounder *Gpr54*

```
TR118_BR2A       Ccctggaactccaccgagttgccgtggatcaacggctcccggctcaacttgtctctggga   60
TR61730_BR4A     ------------------------------------------------------------   0

TR118_BR2A       agactcggacccggcggcggcggcggcgacgacggcgaggaggaaagagggcagcacccc   120
TR61730_BR4A     ------------------------------------------------------------   0

TR118_BR2A       ttcctcacggacgcctggctggtgccgctcttcttctcgctcatcatgctggtcgggctg   180
TR61730_BR4A     ------------------------------------------------------------   0

TR118_BR2A       gtgggcaatgccctggttatctatgtcatctccaaacaccggcagatgaggaccgcgacc   240
TR61730_BR4A     ------------------------------------------------------------   0

TR118_BR2A       aacttctacatagcaaacctggccgcgaccgacatcattttcctggtgtgctgcgtccca   300
TR61730_BR4A     ------------------------------------------------------------   0

TR118_BR2A       tttaccgccaccctgtatcccctgcccgggtggatcttcggcaacttcatgtgcaagttc   360
TR61730_BR4A     ------------------------------------------------------------   0

TR118_BR2A       gtcgccttcctacagcaggtgacggtccaggccacgtgcatcactctgactgccatgagt   420
TR61730_BR4A     ------------------------------------------------------------   0

TR118_BR2A       ggagaccgctgctacgtaacggtgtacccccctgaagtctctccgccaccgcacgcccagg   480
TR61730_BR4A     ------------------------gtacccccctgaagtctctccgccaccgcacgcccagg   37
                                         ************************************

TR118_BR2A       atagccatgatcgtcagcgtctgcatttggatcggctccttcatcctgtccacgccgatt    540
TR61730_BR4A     atagccatgatcgtcagcgtctgcatttggatcggctccttcatcctgtccacgccgatt    97
                 ************************************************************

TR118_BR2A       ctgatgtaccagcgtatagaggacggctactggtacggcccgagacaatactgcatggag   600
TR61730_BR4A     ctgatgtaccagcgtatagaggacggctactggtacggcccgagacaatattgcatggag   157
                 ************************************************* *********

TR118_BR2A       cgattcccctcgaaaacccaggagagggctttcatcctctaccagttcattgcggcctac   660
TR61730_BR4A     cgattcccctcgaaaacccaggagagggctttcatcctctaccagttcattgcggcctac   217
                 ************************************************************

TR118_BR2A       ctgctgcccgtgctcaccatatccttctgctacacgctgatggtgaagagggtcggccag   720
TR61730_BR4A     ctgctgcccgtgctcaccatatccttctgctacacgctgatggtgaagagggtcggccag   277
                 ************************************************************

TR118_BR2A       cccaccgtggagcccgtggacaacaactaccaggtcaacatcctttcggaaagaacgata   780
TR61730_BR4A     cccaccgtggagcccgtggacaacaactaccaggtcaacatcctttcggaaagaacgata   337
                 ************************************************************

TR118_BR2A       agcatcaggagcaaggtctccaggatggtggtggtgatcgtcctcctcttcgccatctgc   840
TR61730_BR4A     agcatcaggagcaaggtctccaggatggtggtcgtgatcgtcctcctcttcgccatctgc   397
                 ******************************** ***************************

TR118_BR2A       tggggacccattcaaatctttgtcctcttccagtccttccacgccaactaccggcccaac   900
TR61730_BR4A     tggggacccattcaaatctttgtcctcttccagtccttccacgccaactaccggcccaac   457
                 ************************************************************

TR118_BR2A       tacgtcacgtacaagatcaagacgtgggccaactgcatgtcctacgccaactcctccgtc   960
TR61730_BR4A     tacgtcacgtacaagatcaagacgtgggccaactgcatgtcctacgccaactcctccgtc   517
                 ************************************************************

TR118_BR2A       aaccccatcgtctacggcttcatgggcgccagcttccagaagtcctttcgcaagaccttc   1020
TR61730_BR4A     aaccccatcgtctacggcttcatgggcgccagcttccagaagtcctttcgcaagaccttc   577
                 ************************************************************

TR118_BR2A       cccttcctgttcaggcacaaggtcagagatagcagcacggcctcgaggacggccaacgcc   1080
TR61730_BR4A     cccttcctgttcaggcacaaggtcagagatagcagcacggcctcgaggacggccaacgcc   637
                 ************************************************************
```

```
TR118_BR2A        gagatcaagtttgtggcggcggcggcggcagcggttgggggcaacaacaacaacaaccac   1140
TR61730_BR4A      gagatcaagtttgtggcggcggcggcggcggttgggggcaacaacaacaacaaccac   697
                  ****************************.******************************

TR118_BR2A        gaggacggcggcggcggcggcgggcattag        1170
TR61730_BR4A      gaggacggcggcggcgg-------------        714
                  *****************
```

C) Yellowbelly flounder *Gpr54* consensus sequence and translation

```
cagcacccctttcctcacggacgcctggctggtgccgctcttcttctcgctcatcatgctg
 Q  H  P  F  L  T  D  A  W  L  V  P  L  F  F  S  L  I  M  L
gtcgggctggtgggcaatgccctggttatctatgtcatctccaaacaccggcagatgagg
 V  G  L  V  G  N  A  L  V  I  Y  V  I  S  K  H  R  Q  M  R
accgcgaccaacttctacatagcaaacctggccgcgaccgacatcattttcctggtgtgc
 T  A  T  N  F  Y  I  A  N  L  A  A  T  D  I  I  F  L  V  C
tgcgtcccatttaccgccaccctgtatcccctgcccgggtggatcttcggcaacttcatg
 C  V  P  F  T  A  T  L  Y  P  L  P  G  W  I  F  G  N  F  M
tgcaagttcgtcgccttcctacagcaggtgacggtccaggccacgtgcatcactctgact
 C  K  F  V  A  F  L  Q  Q  V  T  V  Q  A  T  C  I  T  L  T
gccatgagtggagaccgctgctacgtaacggtgtacccccctgaagtctctccgccaccgc
 A  M  S  G  D  R  C  Y  V  T  V  Y  P  L  K  S  L  R  H  R
acgcccaggatagccatgatcgtcagcgtctgcatttggatcggctccttcatcctgtcc
 T  P  R  I  A  M  I  V  S  V  C  I  W  I  G  S  F  I  L  S
acgccgattctgatgtaccagcgtatagaggacggctactggtacggcccgagacaatac
 T  P  I  L  M  Y  Q  R  I  E  D  G  Y  W  Y  G  P  R  Q  Y
tgcatggagcgattcccctcgaaaacccaggagagggctttcatcctctaccagttcatt
 C  M  E  R  F  P  S  K  T  Q  E  R  A  F  I  L  Y  Q  F  I
gcggcctacctgctgcccgtgctcaccatatccttctgctacacgctgatggtgaagagg
 A  A  Y  L  L  P  V  L  T  I  S  F  C  Y  T  L  M  V  K  R
gtcggccagcccaccgtggagcccgtggacaacaactaccaggtcaacatcctttcggaa
 V  G  Q  P  T  V  E  P  V  D  N  N  Y  Q  V  N  I  L  S  E
agaacgataagcatcaggagcaaggtctccaggatggtggtcgtgatcgtcctcctcttc
 R  T  I  S  I  R  S  K  V  S  R  M  V  V  V  I  V  L  L  F
gccatctgctggggacccattcaaatctttgtcctcttccagtccttccacgccaactac
 A  I  C  W  G  P  I  Q  I  F  V  L  F  Q  S  F  H  A  N  Y
cggcccaactacgtcacgtacaagatcaagacgtgggccaactgcatgtcctacgccaac
 R  P  N  Y  V  T  Y  K  I  K  T  W  A  N  C  M  S  Y  A  N
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 S  S  V  N  P  I  V  Y  G  F  M  G  A  S  F  Q  K  S  F  R
aagaccttccccttcctgttcaggcacaaggtcagagatagcagcacggcctcgaggacg
 K  T  F  P  F  L  F  R  H  K  V  R  D  S  S  T  A  S  R  T
gccaacgccgagatcaagtttgtggcg
 A  N  A  E  I  K  F  V  A
```

Appendix 6: *Fshβ*

A) Nucleotide sequences for yellowbelly flounder *Fshβ* from each tissue where it was expressed

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B) Alignment including all deduced nucleotide sequences for yellowbelly flounder *Fshβ*

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GND4A_TR15933      tacggcggtttgaagagtaccagaaggagcaaaacttgcaaccgaggttcaacacagctc    66
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PIT2A_TR4724       atcaattgggttgattttgacatgcatttgtgttttgactcttttcttgcaccacgcaaa    628
BRA4A_TR67027      ------------------------------------------------------------    521
GND4A_TR15933      ------------------------------------------------------------    535
PIT3A_TR44621      atcaattgggttgattttgacatgcctttgtgttttgactcttttcttgcaccacgcaaa    630
GND2A_TR1143       ------------------------------------------------------------    515
GND3A_TR11453      ------------------------------------------------------------    525

PIT4A_TR12247      cacaaagacacgataccgtctacccttttcaaagcaaaaaataaaaaaatgtgacaaata    835
BRA3A_TR12178      ------------------------------------------------------------    508
PIT2A_TR4724       ------------------------------------------------------------    628
BRA4A_TR67027      ------------------------------------------------------------    521
GND4A_TR15933      ------------------------------------------------------------    535
PIT3A_TR44621      ------------------------------------------------------------    630
GND2A_TR1143       ------------------------------------------------------------    515
GND3A_TR11453      ------------------------------------------------------------    525
```

C) Yellowbelly flounder *Fshβ* consensus sequence and translation

```
atgcggctggttgtcagggcagcagtgctggcaatggtgtgggcaggtcagtgctgcggc
 M  R  L  V  V  R  A  A  V  L  A  M  V  W  A  G  Q  C  C  G
ttcggctgccatccaacaaacatcagcatccccgtggagagctgtggcaggaccgtctgc
 F  G  C  H  P  T  N  I  S  I  P  V  E  S  C  G  R  T  V  C
atctacaccaccgtgtgcgcgggacagtgctaccataaggatccggtccacatcggcaac
 I  Y  T  T  V  C  A  G  Q  C  Y  H  K  D  P  V  H  I  G  N
gatgactggcctaaacagaaagtctgtaatggaaactggtcctatgagatcaagtacttt
 D  D  W  P  K  Q  K  V  C  N  G  N  W  S  Y  E  I  K  Y  F
aatggatgtccagtgggtgtcacgtaccccgtggccacaaactgtgaatgcactgcgtgt
 N  G  C  P  V  G  V  T  Y  P  V  A  T  N  C  E  C  T  A  C
aattcagttaacacttcctgcgggcgtttttacggagacattgtcagctgtctgcccctg
 N  S  V  N  T  S  C  G  R  F  Y  G  D  I  V  S  C  L  P  L
taa
 -
```

Appendix 7: *Lhβ*

A) Nucleotide sequences for yellowbelly flounder *Lhβ* from each tissue where it was expressed

>BRA2A_TR10278
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>BRA4A_TR45155
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B) Alignment including all deduced nucleotide sequences for yellowbelly flounder *Lhβ*

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PIT3A_TR29338      gccaaaagtacaggtaatctgggtggaagcacggtgacgtccgggcaggcgggagagagc   60
GND3A_TR8550       ------------------------------------------------------------   0
BRA3A_TR36775      ------------------------------------------------------------   0
PIT4A_TR25817      ----------------------------------------ccgggcaggcgggagagagc   20
GND4A_TR8952       ------------------------------------------------------------   0
BRA4A_TR45155      ------------------------------------------------------------   0
PIT2A_TR37567      ------------------------------------------------------------   0
BRA2A_TR10278      ------------------------------------------------------------   0
GND2A_TR21641      ------------------------------------------------------------   0

PIT3A_TR29338      gttaatcctggatacggggccccaacgggggacgtataaaggcccgagaggctgcgccca   120
GND3A_TR8550       ------------------------------------------------------------   0
BRA3A_TR36775      ------------------------------------------------------------   0
PIT4A_TR25817      gttaatcctggatacggggccccaacgggggacgtataaaggcccgagaggctgcgccca   80
GND4A_TR8952       ------------------------------------------------------------   0
BRA4A_TR45155      ------------------------------------------------------------   0
PIT2A_TR37567      ----------------gggccccaacgggggccgtataaaggcccgagaggctgcgccca   44
BRA2A_TR10278      ------------------------------------------------------------   0
GND2A_TR21641      ------------------------------------------------------------   0

PIT3A_TR29338      gaagccagagcgaccatggcggccacgcgggtccaggtggtgtcgctgcttctgggactt   180
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BRA3A_TR36775      ----------------ggcggccacgcgggtccaggtggtgtcgctgcttctgggactt   43
PIT4A_TR25817      gaagccagagcgaccatggcggccacgcgggtccaggtggtgtcgctgcttctgggactt   140
GND4A_TR8952       ---------------atggcggccacgcgggtccaggtggtgtcgctgcttctgggactt   45
BRA4A_TR45155      ----------cgaccatggcggccacgcgggtccaggtggtgtcgctgcttctgggactt   50
PIT2A_TR37567      gaagccagagcgaccatggcggccacgcgggtccaggtggtgtcgctgcttctggggctt   104
BRA2A_TR10278      ----------------ggcggccacgcgggtccaggtggtgtcgctgcttctggggctt   43
GND2A_TR21641      -------------------------------------gtggtgtcgctgcttctggggctt   24
                                                       ********************.***

PIT3A_TR29338      tgcctcctggccttggcaggggcctttcagctgccgccctgtcagctcgtcaatcagacg   240
GND3A_TR8550       tgcctcctggccttggcaggggcctttcagctgccgccctgtcagctcgtcaatcagacg   110
BRA3A_TR36775      tgcctcctggccttggcaggggcctttcagctgccgccctgtcagctcgtcaatcagacg   103
PIT4A_TR25817      tgtctcctggccttggcaggggcctttcagctgccgccctgtcagctcgtcaatcagacg   200
GND4A_TR8952       tgtctcctggccttggcaggggcctttcagctgccgccctgtcagctcgtcaatcagacg   105
BRA4A_TR45155      tgtctcctggccttggcaggggcctttcagctgccgccctgtcagctcgtcaatcagacg   110
PIT2A_TR37567      tgtctcctggccttggcaggggcctttcagctgccgccctgtcagctcgtcaatcagacg   164
BRA2A_TR10278      tgtctcctggccttggcaggggcctttcagctgccgccctgtcagctcgtcaatcagacg   103
GND2A_TR21641      tgtctcctggccttggcaggggcctttcagctgccgccctgtcagctcgtcaatcagacg   84
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PIT3A_TR29338      gtgtccctggagaaggacggctgtcccaggtgtcacccggtggagaccaccatctgcagc   300
GND3A_TR8550       gtgtccctggagaaggacggctgtcccaggtgtcacccggtggagaccaccatctgcagc   170
BRA3A_TR36775      gtgtccctggagaaggacggctgtcccaggtgtcacccggtggagaccaccatctgcagc   163
PIT4A_TR25817      gtgtccctggagaaggacggctgtcccaggtgtcacccggtggagaccaccatctgcagc   260
GND4A_TR8952       gtgtccctggagaaggacggctgtcccaggtgtcacccggtggagaccaccatctgcagc   165
BRA4A_TR45155      gtgtccctggagaaggacggctgtcccaggtgtcacccggtggagaccaccatctgcagc   170
PIT2A_TR37567      gtgtccctggagaaggacggctgtcccaggtgtcacccggtggagaccaccatctgcagc   224
BRA2A_TR10278      gtgtccctggagaaggacggctgtcccaggtgtcacccggtggagaccaccatctgcagc   163
GND2A_TR21641      gtgtccctggagaaggacggctgtcccaggtgtcacccggtggagaccaccatctgcagc   144
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PIT3A_TR29338      ggccactgcaacaccaaggaccccgtggtgaagctgcccttccagaacgtgttccagcac   360
GND3A_TR8550       ggccactgcaacaccaaggaccccgtggtgaagctgcccttccagaacgtgttccagcac   230
BRA3A_TR36775      ggccactgcaacaccaaggaccccgtggtgaagctgcccttccagaacgtgttccagcac   223
PIT4A_TR25817      ggccactgcaacaccaaggaccccgtggtgaagctgcccttccagaacgtgttccagcac   320
GND4A_TR8952       ggccactgcaacaccaaggaccccgtggtgaagctgcccttccagaacgtgttccagcac   225
BRA4A_TR45155      ggccactgcaacaccaaggaccccgtggtgaagctgcccttccagaacgtgttccagcac   230
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BRA2A_TR10278      ggccactgcaacaccaaggaccccgtggtgaagctgcccttccagaacgtgttccagcac   223
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GND4A_TR8952       gtgtgcacctaccgggacgtccactaccggagcttcgagctgcccgactgtcgtgcgggc   285
BRA4A_TR45155      gtgtgcacctaccgggacgtccactaccggagcttcgagctgcccgactgtcgtgcgggc   290
PIT2A_TR37567      gtgtgcacctaccgggacgtccactaccggagcttcgagctgcccgactgtcgggcgggc   344
BRA2A_TR10278      gtgtgcacctaccgggacgtccactaccggagcttcgagctgcccgactgtcgggcgggc   283
GND2A_TR21641      gtgtgcacctaccgggacgtccactaccggagcttcgagctgcccgactgtcgggcgggc   264
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BRA3A_TR36775      gtcgaccccatcgtcacgtaccccgtggcgtccagctgcaactgcggccgctgcgccatg  343
PIT4A_TR25817      gtcgaccccatcgtcacgtaccccgtggcgtccagctgcaactgcggccgctgcgccatg  440
GND4A_TR8952       gtcgaccccatcgtcacgtaccccgtggcgtccagctgcaactgcggccgctgcgccatg  345
BRA4A_TR45155      gtcgaccccatcgtcacgtaccccgtggcgtccagctgcaactgcggccgctgcgccatg  350
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BRA2A_TR10278      gtcgaccccatcgtcacgtaccccgtggcgtccagctgcaactgcggccgctgcgccatg  343
GND2A_TR21641      gtcgacc-------------------------------------------------------  271
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BRA3A_TR36775      gacacgtccgactgcaccttcgagagcctgcagcccgacttctgcatgaacgacatacct  403
PIT4A_TR25817      gacacgtccgactgcaccttcgagagcctgcagcccgacttctgcatgaacgacatacct  500
GND4A_TR8952       gacacgtccgactgcaccttcgagagcctgcagcccgacttctgcatgaacgacatacct  405
BRA4A_TR45155      gacacgtccgactgcaccttcgagagcctgcagcccgacttctgcatgaacgacatacct  410
PIT2A_TR37567      gacacgtccgactgcaccttcgagagcctgcagcccgacttctgcatgaacgacatacct  464
BRA2A_TR10278      gacacgtccgactgcaccttcgagagcctgcagcccgacttctgcatgaacgacatacct  403
GND2A_TR21641      ------------------------------------------------------------  271

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BRA3A_TR36775      ttctactactaatggaaaaacattggcaaatatgaatgcgtgctttaaaaagaaaaaccc  463
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GND4A_TR8952       ttctactactaatggaaaaacattggcaaacatgaatgcgtgctttaaaaagaaaaaccc  465
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BRA2A_TR10278      ttctactactaatggaaaaacattggcaaatatgaatgcgtgctttaaaaagaaaaaccc  463
GND2A_TR21641      ------------------------------------------------------------  271

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BRA3A_TR36775      c-------------------------------------------------------------  464
PIT4A_TR25817      ccc---cccacacacagagaaagttaataaaacgagtaaaa-----tcacccagtgcaca  612
GND4A_TR8952       ctcccacacacacacagagaaagttaataaaactagtaaaaccacatcaaccagtgcata  525
BRA4A_TR45155      ctcccacacacacacagagaaagttaataaaactagtaaaaccacatcaaccagtgcata  530
PIT2A_TR37567      ctcccacacacacacacagaaggttaataaaactagtaaaaccacatcaaccagtgcata  584
BRA2A_TR10278      ctcccacacacacacacagaagg----------------------------------------  486
GND2A_TR21641      ------------------------------------------------------------  271

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BRA3A_TR36775      ------------------------------------------------------------  464
PIT4A_TR25817      tttatattctgactattatggagaaaattctaatttttttgatttgaaagt---cactgga  669
GND4A_TR8952       tttatattctgactattatggagaaaactcaaatttttttgatttgaaagt---cactgga  582
BRA4A_TR45155      tttatattctgactattatggagaaaactcaaatttttttgatttgaaagt---cactgga  587
PIT2A_TR37567      tttatattctgactattatggagaaaaatctaatttttttgatttgaaagt---attcagt  641
BRA2A_TR10278      ------------------------------------------------------------  486
GND2A_TR21641      ------------------------------------------------------------  271

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BRA3A_TR36775      ------------------------------------------------------------  464
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GND4A_TR8952       aaatattaaaatggataatattaatacatttctactgcacaattaatgtatatcttatta  642
BRA4A_TR45155      aaatattaaaatggataatattaatacatttctactgcacaattaatgtatatcttatta  647
PIT2A_TR37567      g--gaaaaaaatggataatattaatacatttctactgcacaattaatgtatatcttatta  699
BRA2A_TR10278      ------------------------------------------------------------  486
GND2A_TR21641      ------------------------------------------------------------  271

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GND3A_TR8550       tgtacaaaataaatgtgtggcactggagtaataacatctaactgacactatatgcaaaga  698
BRA3A_TR36775      ------------------------------------------------------------  464
PIT4A_TR25817      tgtacaaaataaatgtgtggcactggagtaataacatc----tgacactatatgcaaaga  785
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BRA4A_TR45155      tgtacaaaataaatgtgtggcactggagtaataacatctaaccgacactatatgcaaaga  707
PIT2A_TR37567      tgtacaaaataaatgtgtggcactggagtaataacatctaactgacactatatgcaaaga  759
BRA2A_TR10278      ------------------------------------------------------------  486
GND2A_TR21641      ------------------------------------------------------------  271

PIT3A_TR29338      ccatagtatatatatatatat---------      861
GND3A_TR8550       cc----------------------------      700
BRA3A_TR36775      ----------------------------      464
PIT4A_TR25817      ccataggatatatatatatatatatata      815
GND4A_TR8952       ccat-------------------------      706
BRA4A_TR45155      cc----------------------------      709
PIT2A_TR37567      ccatagtatatatatatatatatatat-      788
BRA2A_TR10278      ----------------------------      486
GND2A_TR21641      ----------------------------      271
```

C) Yellowbelly flounder *Lhβ* consensus sequence and translation

```
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 A  G  A  F  Q  L  P  P  C  Q  L  V  N  Q  T  V  S  L  E  K
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 D  G  C  P  R  C  H  P  V  E  T  T  I  C  S  G  H  C  N  T
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 K  D  P  V  V  K  L  P  F  Q  N  V  F  Q  H  V  C  T  Y  R
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 D  V  H  Y  R  S  F  E  L  P  D  C  R  A  G  V  D  P  I  V
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 T  Y  P  V  A  S  S  C  N  C  G  R  C  A  M  D  T  S  D  C
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 T  F  E  S  L  Q  P  D  F  C  M  N  D  I  P  F  Y  Y  -
```

Appendix 8: *Fshr*

A) Nucleotide sequences for yellowbelly flounder *Fshr* from each tissue where it was expressed

```
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B) Alignment including all deduced nucleotide sequences for yellowbelly flounder *Fshr*

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TR27790_PIT2A      ------------------------------------------------------------  0
TR12530_PIT3A      atgatgaatgccacaatgtttctcgcgtgcgcggcgataaacgcggcggcggcggcctcg  60


TR24674_PIT4A      gtccctggccccgggagggaagcggagggcttattcgaggccggcttcaactaccggaac  120
TR27790_PIT2A      ------------------------------------------------------------  0
TR12530_PIT3A      gtccctggccccgggagggaagcggagggcttattcgaggccggcttcaactaccggaac  120


TR24674_PIT4A      tggccctcctgccgccacctgggagcaaaggtcacggagattccggccaatatctcccgc  180
TR27790_PIT2A      ------------------------------------------------------------  0
TR12530_PIT3A      tggccctcctgccgccacctgggaccaaaggtcacggagattccggccaatatctcccgc  180


TR24674_PIT4A      gacacccgctgcctgcatgtaaagcagacgcaagtcagcgtgattcccaggggtgccctt  240
TR27790_PIT2A      ------------------------------------------------------------  0
TR12530_PIT3A      gacacccgctgcctgcatgtaaagcagacgcaagtcagcgtgattcccaggggtgccctt  240


TR24674_PIT4A      aacagcctgcagcttctcagggaactcttcatattgaagaatgaaatgctggagagtatc  300
TR27790_PIT2A      ------------------------------------------------------------  0
TR12530_PIT3A      aacagcctgcagcttctcagggaactcttcatattgaagaatgaaatgctggagagtatc  300


TR24674_PIT4A      ggcccgtttgctttcgctgacctccttcagctggcagacgtcgtcatctctgaaaacgtt  360
TR27790_PIT2A      ------------------------------------------------------------  0
TR12530_PIT3A      ggcccgtttgctttcgctgacctccttcagctggcagacgtcgtcatctctgaaaacgtt  360
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TR27790_PIT2A      ------------------------------------------------------------  0
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TR24674_PIT4A      ataacaatgtccaaacacctcagttacatccacccagatgcgttcaggaacatcctaaaa  480
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TR12530_PIT3A      ataacaatgtccaaacacctcagttacatccacccagatgcgttcaggaacatcctaaaa  480


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TR12530_PIT3A      ctacagtatctgaccatctccaacaccgggctccgaagcttcccagacctcaccaagatc  540


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TR27790_PIT2A      ------------------------------------------------------------  0
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TR24674_PIT4A      cctgccaatgctttcagaggcatgtacacccaaactatctctgagatacggctcaccaga  660
TR27790_PIT2A      ----------------------------------------------acggctcaccaga  13
TR12530_PIT3A      cctgccaatgctttcagaggcatgtacacccaaactatctctgagatacggctcaccaga  660
                                                                 ************


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TR12530_PIT3A      ctgagaggcaaccaactgctcactcacatcaatcccaacgcctttgcgggttccagtgag  780
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TR27790_PIT2A      ttggtggtcctggacatctctcagacggccatcagctccctgccgcactccatcctcggc  193
TR12530_PIT3A      ttggtggtcctggacatctctcagacggccatcagctccctgccgcactccatcctcggc  840
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TR24674_PIT4A      ggactcaagcaactgatcgccgagtcggcgttccacctcaaagagcttccgcctccgcag  900
TR27790_PIT2A      ggactcaagcaactgatcgccgagtcggcgttccacctcaaagagcttccgcctccgcag  253
TR12530_PIT3A      ggactcaagcaactgatcgccgagtcggcgttccacctcaaagagcttccgcctccgcag  900
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TR24674_PIT4A      ctcttcctgaagctgcagcaggcaaacctgacatatccatcccactgctgcgccctgaac  960
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TR12530_PIT3A      ctcttcctgaagctgcagcaggcaaacctgacatatccatcccactgctgcgccctgaac  960
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TR12530_PIT3A      aggaacataagcaggaacaagtcgtggtgggacccctggtgctttgaccccagggctccg  1020
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TR12530_PIT3A      gattaccaatatctaccctggaactcctgcaccaacttcacgtccgtcacctgcagaccc  1080
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TR24674_PIT4A      ctctgcatgggcgtttacctggcggtcatagccaccgtggacgcgctcactcgaggccag  1320
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TR12530_PIT3A      ctctgcatgggcgtttacctggcggtcatagcgaccgtggacgcgctcactcgaggccag  1320
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TR24674_PIT4A      tattacaaccacgccttggactggcagatgggcctgggctgcagtgccgccggcttcttt  1380
TR27790_PIT2A      tattacaaccacgccttggactggcagatgggcctgggctgcagtgccgccggcttcttt  733
TR12530_PIT3A      tattacaaccacgccttggactggcagatgggcctgggctgcagtgccgccggcttcttt  1380
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TR24674_PIT4A      acagtgttcgccagcgagctgtcggtgttcacgctgacggccatcaccctggagcgctgg  1440
TR27790_PIT2A      acagtgttcgccagcgagctgtcggtgttcacgctgacggccatcaccctggagcgctgg  793
TR12530_PIT3A      acagtgttcgccagcgagctgtcggtgttcacgctgacggccatcaccctggagcgctgg  1440
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TR24674_PIT4A      cacaccatcacgtacgccatgcggctggaccgcaagctgcgtctgagacacgcgtgcgtc  1500
TR27790_PIT2A      cacaccatcacgtacgccatgcggctggaccgcaagctgcgcctgagacacgcgtgcgtc  853
TR12530_PIT3A      cacaccatcacgtacgccatgcggctggaccgcaagctgcgtctgagacacgcgtgcgtc  1500
                   ****************************************** ******************

TR24674_PIT4A      gtcatggcggccggctggatcttctcgtccctcgccgctctgctgcccacgctgggggtc  1560
TR27790_PIT2A      gtcatggcggccggctggatcttctcgtccctcgccgctctgctgcccacgctgggggtc  913
TR12530_PIT3A      gtcatggcggccggctggatcttctcgtccctcgccgctctgctgcccacgctgggggtc  1560
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TR24674_PIT4A      agcagctacagcaaggtcagtatatgcctgccaatggacgtggagtccctggttgcgcag  1620
TR27790_PIT2A      agcagctacagcaaggtcagtatatgcctgccaatggacgtggagtccctggttgcgcag  973
TR12530_PIT3A      agcagctacagcaaggtcagtatatgcctgccaatggacgtggagtccctggttgcgcag  1620
                   ************************************************************

TR24674_PIT4A      gtctacgtggtgtccgtcctcctcttcaacatcctggccttcttctgcgtgtgcggctgc  1680
TR27790_PIT2A      gtctacgtggtgtccgtcctcctcttcaacatcctggccttcttctgcgtgtgcggctgc  1033
TR12530_PIT3A      gtctacgtggtgtccgtcctcctcttcaacatcctggccttcttctgcgtgtgcggctgc  1680
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TR24674_PIT4A      tacctcagcatctacctgaccgtccgcaacccctcgtcggtgccggccaacgccgacacc  1740
TR27790_PIT2A      tacctcagcatctacctgaccgtccgcaacccctcgtcggtgccggccaacgccgacacc  1093
TR12530_PIT3A      tacctcagcatctacctgaccgtccgcaacccctcgtcggtgccggccaacgccgacacc  1740
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TR24674_PIT4A      cacgtggcccagcgcatggccatcctcatcttcaccgacttcgtctgcgtggcgcccatc  1800
TR27790_PIT2A      cacgtggcccagcgcatggccatcctcatcttcaccgacttcgtctgcgtggcgcccatc  1153
TR12530_PIT3A      cacgtggcccagcgcatggccatcctcatcttcaccgacttcgtctgcgtggcgcccatc  1800
                   ************************************************************

TR24674_PIT4A      tccttcttcgccgtgtccgccgccctcaagctccccctcatcaccgtctcggagtccaag  1860
TR27790_PIT2A      tccttcttcgccgtgtccgccgccctcaagctccccctcatcaccgtctcggagtccaag  1213
TR12530_PIT3A      tccttcttcgccgtgtccgccgccctcaagctccccctcatcaccgtctcggagtccaag  1860
                   ************************************************************

TR24674_PIT4A      ctcctgctggtcctcttctacccgatcaactcctgctccaaccccttcctgtacgccttc  1920
TR27790_PIT2A      ctcctgctggtcctcttctacccgatcaactcctgctccaaccccttcctgtacgccttc  1273
TR12530_PIT3A      ctcctgctggtcctcttctacccgatcaactcctgctccaaccccttcctgtacgccttc  1920
                   ************************************************************

TR24674_PIT4A      ttcacgcgcaccttccgccgggacttcttcctgctcgccgcccgcgtaggccttttcaag  1980
TR27790_PIT2A      ttcacgcgcaccttccgccgggacttcttcctgctcgccgcccgcgtaggccttttcaag  1333
TR12530_PIT3A      ttcacgcgcaccttccgccgggacttcttcctgctcgctgcccgcgtaggccttttcaag  1980
                   ************************************ ***********************

TR24674_PIT4A      gcccgcgcgcagatctaccgcacggagagctcatcctgccagcagccggcgtggacgtcc  2040
TR27790_PIT2A      gcccgcgcgcagatctaccgcacggagagctcatcctgccagcagccggcgtggacgtcc  1393
TR12530_PIT3A      gcccgcgcgcagatctaccgcacggagagctcatcctgccagcagccggcgtggacgtcc  2040
                   ************************************************************

TR24674_PIT4A      cccaagagcggccgcgtggcgctctgcgccctggccaacgggcggcgcctggacgcgaag  2100
TR27790_PIT2A      cccaagagcggccgcgtggcgctctgcgccctggccaacgggcggcgcctggacgcgaag  1453
TR12530_PIT3A      cccaagagcggccgcgtggcgctctgcgccctggccaacgggcggcgcctggacgcgaag  2100
                   ************************************************************

TR24674_PIT4A      cacgagtgctga        2112
TR27790_PIT2A      cacgagtgctga        1465
TR12530_PIT3A      cacgagtgctga        2112
                   ************
```

C) Yellowbelly flounder *Fshr* consensus sequence and translation

```
atgatgaatgccacaatgttcctcgcgtgcgcggcgataaacgcggcggcggcggcctcg
 M  M  N  A  T  M  F  L  A  C  A  A  I  N  A  A  A  A  A  S
gtccctggccccgggagggaagcggagggcttattcgaggccggcttcaactaccggaac
 V  P  G  P  G  R  E  A  E  G  L  F  E  A  G  F  N  Y  R  N
tggccctcctgccgccacctgggagcaaaggtcacggagattccggccaatatctcccgc
 W  P  S  C  R  H  L  G  A  K  V  T  E  I  P  A  N  I  S  R
gacacccgctgcctgcatgtaaagcagacgcaagtcagcgtgattcccaggggtgccctt
 D  T  R  C  L  H  V  K  Q  T  Q  V  S  V  I  P  R  G  A  L
aacagcctgcagcttctcagggaactcttcatattgaagaatgaaatgctggagagtatc
 N  S  L  Q  L  L  R  E  L  F  I  L  K  N  E  M  L  E  S  I
ggcccgtttgctttcgctgacctccttcagctggcagacgtcgtcatctctgaaaacgtt
 G  P  F  A  F  A  D  L  L  Q  L  A  D  V  V  I  S  E  N  V
gctctgcagagtatcggcgcttttgcgttctacaacctccctgaactcactgagataacc
 A  L  Q  S  I  G  A  F  A  F  Y  N  L  P  E  L  T  E  I  T
ataacaatgtccaaacacctcagttacatccacccagatgcgttcaggaacatcctaaaa
 I  T  M  S  K  H  L  S  Y  I  H  P  D  A  F  R  N  I  L  K
ctacagtatctgaccatctccaacaccgggctccgaagcttcccagacctcaccaagatc
 L  Q  Y  L  T  I  S  N  T  G  L  R  S  F  P  D  L  T  K  I
cactccgcgcccacaggttcctgtttgacctggaggagaacagccacatcgtgagagtc
 H  S  A  A  H  R  F  L  F  D  L  E  E  N  S  H  I  V  R  V
cctgccaatgctttcagaggcatgtacacccaaactatctctgagatacggctcaccaga
 P  A  N  A  F  R  G  M  Y  T  Q  T  I  S  E  I  R  L  T  R
aatggcatcaaggaggtggcgagcgacgccttcaacggcacaaagatgcaccgattgttc
 N  G  I  K  E  V  A  S  D  A  F  N  G  T  K  M  H  R  L  F
ctgagaggcaaccaactgctcactcacatcaatcccaacgcctttgcgggttccagtgag
 L  R  G  N  Q  L  L  T  H  I  N  P  N  A  F  A  G  S  S  E
ttggtggtcctggacatctctcagacggccatcagctccctgccgcactccatcctcggc
 L  V  V  L  D  I  S  Q  T  A  I  S  S  L  P  H  S  I  L  G
ggactcaagcaactgatcgccgagtcggcgttccacctcaaagagcttccgcctccgcag
 G  L  K  Q  L  I  A  E  S  A  F  H  L  K  E  L  P  P  P  Q
ctcttcctgaagctgcagcaggcaaacctgacatatccatcccactgctgcgccctgaac
 L  F  L  K  L  Q  Q  A  N  L  T  Y  P  S  H  C  C  A  L  N
aggaacataagcaggaacaagtcgtggtgggacccctggtgctttgaccccagggctccg
 R  N  I  S  R  N  K  S  W  W  D  P  W  C  F  D  P  R  A  P
gattaccaatatctaccctggaactcctgcaccaacttcacgtccgtcacctgcagaccc
 D  Y  Q  Y  L  P  W  N  S  C  T  N  F  T  S  V  T  C  R  P
atgacggatttgttcaacccctgcgaagacgtcatggccgccgtgcccctccgcgtcctc
 M  T  D  L  F  N  P  C  E  D  V  M  A  A  V  P  L  R  V  L
atctggatcatctccatactcgctctgctggggaacggcgtggtgctgctcgtgttgtta
 I  W  I  I  S  I  L  A  L  L  G  N  G  V  V  L  L  V  L  L
ggcagccgctccaagctgaccgtcccccgcttcctcatgagccacctggccttcgccgac
 G  S  R  S  K  L  T  V  P  R  F  L  M  S  H  L  A  F  A  D
ctctgcatgggcgtttacctggcggtcatagccaccgtggacgcgctcactcgaggccag
 L  C  M  G  V  Y  L  A  V  I  A  T  V  D  A  L  T  R  G  Q
tattacaaccacgccttggactggcagatgggcctgggctgcagtgccgccggcttcttt
 Y  Y  N  H  A  L  D  W  Q  M  G  L  G  C  S  A  A  G  F  F
acagtgttcgccagcgagctgtcggtgttcacgctgacggccatcaccctggagcgctgg
 T  V  F  A  S  E  L  S  V  F  T  L  T  A  I  T  L  E  R  W
cacaccatcacgtacgccatgcggctggaccgcaagctgcgtctgagacacgcgtgcgtc
 H  T  I  T  Y  A  M  R  L  D  R  K  L  R  L  R  H  A  C  V
gtcatggcggccggctggatcttctcgtccctcgccgctctgctgcccacgctgggggtc
 V  M  A  A  G  W  I  F  S  S  L  A  A  L  L  P  T  L  G  V
agcagctacagcaaggtcagtatatgcctgccaatggacgtggagtccctggttgcgcag
 S  S  Y  S  K  V  S  I  C  L  P  M  D  V  E  S  L  V  A  Q
gtctacgtggtgtccgtcctcctcttcaacatcctggccttcttctgcgtgtgcggctgc
 V  Y  V  V  S  V  L  L  F  N  I  L  A  F  F  C  V  C  G  C
tacctcagcatctacctgaccgtccgcaaccctcgtcggtgccggccaacgccgacacc
 Y  L  S  I  Y  L  T  V  R  N  P  S  S  V  P  A  N  A  D  T
cacgtggcccagcgcatggccatcctcatcttcaccgacttcgtctgcgtggcgcccatc
 H  V  A  Q  R  M  A  I  L  I  F  T  D  F  V  C  V  A  P  I
tccttcttcgccgtgtccgccgccctcaagctcccctcatcaccgtctcggagtccaag
 S  F  F  A  V  S  A  A  L  K  L  P  L  I  T  V  S  E  S  K
ctcctgctggtcctcttctacccgatcaactcctgctccaaccccttcctgtacgccttc
 L  L  L  V  L  F  Y  P  I  N  S  C  S  N  P  F  L  Y  A  F
ttcacgcgcaccttccgccgggacttcttcctgctcgccgcccgcgtaggccttttcaag
 F  T  R  T  F  R  R  D  F  F  L  L  A  A  R  V  G  L  F  K
gcccgcgcgcagatctaccgcacggagagctcatcctgccagcagccggcgtggacgtcc
 A  R  A  Q  I  Y  R  T  E  S  S  S  C  Q  Q  P  A  W  T  S
cccaagagcggccgcgtggcgctctgcgccctggccaacgggcggcgcctggacgcgaag
 P  K  S  G  R  V  A  L  C  A  L  A  N  G  R  R  L  D  A  K
cacgagtgctga
 H  E  C  -
```

Appendix 9: *Lhr*

A) Nucleotide sequences for yellowbelly flounder *Lhr* from each tissue where it was expressed

>TR45888_PIT2Aa
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ctgggacgtccacagcgtgtcttgcttcggggcccagctcttcccgcaattccactccagcacgcaggaagtgtggatggtgaggaccagactgtcctctgtccctc
gggacgccttctccaacctgtccaacgtctcccacatatacatctctgatgacgactctttgacaaatctggagaagcattccttccgcaacctttccagcctcaca
cacatacaactcaccggcctcaaaacactgacctacatcgaccaagaggcgtttaaagctctgcccaatttaaagtaccttgggatcaccaacacgggcctcacctc
cttccctgtgctccgatatgttcaatccatccaggaggatttcattttggagatagtggagaacgcctacgtacgagtgataccggccaattccttcgccggtatct
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cagcgtggagacgctccaggcgcgccacacctggtccctgagggcccttccggcccccggcgccttccggcacctgcagagcgccgagctgaccttccccagccact
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ggtgtgggcggtcggcctcctggccgtgctggccaacctgttggcgatggtcacgctgctgagcgggcggcgccggctgtcggtcacccgcttcctgatggcccacc
tggccctggccgacttctgcatgggcgcctacctgctgctcatcgcggcggtggacctgtacacgcactcgcagtactaccgctacgccgtggcctggcagacggga
ggcggctgcaacctggccggggcgctgtcggtcttcgccagcgagctgtccgtctacacgctgagcctggtcagcctgcagcgctggcgcgccatcttctacgccat
gcggcccgagcgcaagatgcgtctgcgccacgccgccgccctgatgctggccggctggacgctgtgcgccggcgccgccctgctgccgctgctgggcgtcagcagct
accagcgggtcagcatctgcctgcccatggaggcgggcacgccggccgcccgggcctacctggtgtgcgtgctgctggccaacgtgctggccatggccgtggtcagc
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cctgtgcctggcgcccatctgcttctacggcctgtcggcggcgctgcaccagccgctgatgaccgtcaccgactccaaggtgctgctggtgatcttctacccgctca
actcgtgcgcccaccccttcctctacgtcatcctgaccaaggccttccggagggacatcgcggcgctgctgagccggacgggcctgcggcggcaccaggtcccgctc
tgctga

>TR26069_PIT3Ab
atgacgaccacgcccgttctcctcctggtgctggtcctgcccccgctgtgctcctctgtggacgggacgcctcccccgggacgctgtccccctccctgcttctgcga
ctgggacgtccacagcgtgtcttgcttcggggcccagctcttcccgcaattccactccagcacgcaggaagtgtggatggtggggaccagactgtcctctgtccctc
gggacgccttctccaacctgtccaacgtctcccacatatacatctctgatgacgactctttgacaaatctggaggagcattccttccgcaacctttccagcctcaca
cacatacaactcaccggcctcaaaacactgacctacatcgaccaagaggcgtttaaagctctgcccaatttaaagtaccttgggatcaccaacacgggcctcacctc
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ctgacaaagctttgacagtcctgttgaacagtaacggtgtgagagaaatccagagtcacgccttcaatgggagccggctggaggaagtgttccttcacagaaacgtg
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cagcgtggagacgctccaggcgcgccacacctggtccctgagggcccttccggcccccggcgccttccggcacctgcagagcgccgagctgaccttccccagccact
gctgcggcctcaagatgctgaagcggtggacaggccgctccgaggaagccctctgcaacctgacggcgtggacggccccggagccgccgccggacggcgccccccg
cccgctccggagcgccggccgtgcggcggcgagctgcgctgcagcccgatgccggacgccctgaaccccctgcgaggacgtgatgagccgcggcttcctccgggtgct
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tggccctggccgacttctgcatgggcgcctacctgctgctcatcgcggcggtggacctgtacacgcactcgcagtactaccgctacgccgtggcctggcagacggga
ggcggctgcaacctggccggggcgctgtcggtcttcgccagcgagctgtccgtctacacgctgagcctggtcagcctgcagcgctggcgcgccatcttctacgccat
gcggcccgagcgcaagatgcgcctgcgccacgccgccgccctgatgctggccggctggacgctgtgcgccggcgccgccctgctgccgctgctgggcgtcagcagct
accagcgggtcagcatctgcctgcccatggaggcgggcacgccggccgcccgggcctacctggtgtgcgtgctgctggccaacgtgctggccatggccgtggtcagc
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actcgtgcgcccaccccttcctctacgtcatcctgaccaaggccttccggagggacatcgcggcgctgctgagccggacgggcctgcggcggcgccaggtcccgctc
tgctga

B) Alignment including all deduced nucleotide sequences for yellowbelly flounder *Lhr*

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TR45888_PIT2Aa      atgacgaccacgcccgtgctcctcctggtgctggtcctgcccccgctgtgctcctctgtg      60
TR26069_PIT3Ab      atgacgaccacgcccgttctcctcctggtgctggtcctgcccccgctgtgctcctctgtg      60
                    ***************** ****************************************

TR45888_PIT2Aa      gacgggacgccgcccccgggacgctgtcccctccctgcttctgcgactgggacgtccac      120
TR26069_PIT3Ab      gacgggacgcctcccccgggacgctgtcccctccctgcttctgcgactgggacgtccac      120
                    *********** ************************************************

TR45888_PIT2Aa      agcgtgtcttgcttcggggcccagctcttcccgcaattccactccagcacgcaggaagtg      180
TR26069_PIT3Ab      agcgtgtcttgcttcggggcccagctcttcccgcaattccactccagcacgcaggaagtg      180
                    ************************************************************

TR45888_PIT2Aa      tggatggtgaggaccagactgtcctctgtccctcgggacgccttctccaacctgtccaac      240
TR26069_PIT3Ab      tggatggtggggaccagactgtcctctgtccctcgggacgccttctccaacctgtccaac      240
                    *********.**************************************************
```

```
TR45888_PIT2Aa      gtctcccacatatacatctctgatgacgactctttgacaaatctggagaagcattccttc      300
TR26069_PIT3Ab      gtctcccacatatacatctctgatgacgactctttgacaaatctggaggagcattccttc      300
                    ************************************************.***********

TR45888_PIT2Aa      cgcaacctttccagcctcacacacatacaactcaccggcctcaaaacactgacctacatc      360
TR26069_PIT3Ab      cgcaacctttccagcctcacacacatacaactcaccggcctcaaaacactgacctacatc      360
                    ************************************************************

TR45888_PIT2Aa      gaccaagaggcgtttaaagctctgcccaatttaaagtaccttgggatcaccaacacgggc      420
TR26069_PIT3Ab      gaccaagaggcgtttaaagctctgcccaatttaaagtaccttgggatcaccaacacgggc      420
                    ************************************************************

TR45888_PIT2Aa      ctcacctccttccctgtgctccgatatgttcaatccatccaggaggatttcattttggag      480
TR26069_PIT3Ab      ctcacctccttccctgtgcttcgatatgttcaatccatccaggaggatttcattttggag      480
                    ******************* ****************************************

TR45888_PIT2Aa      atagtggagaacgcctacgtacgagtgataccggccaattccttcgccggtatctctgac      540
TR26069_PIT3Ab      atagtggagaacgcctacgtacgagtgataccggccaattccttcgccggtatctctgac      540
                    ************************************************************

TR45888_PIT2Aa      aaagctttgacagtcctgttgaacagtaacggtgtgagagaaatccagagtcacgccttc      600
TR26069_PIT3Ab      aaagctttgacagtcctgttgaacagtaacggtgtgagagaaatccagagtcacgccttc      600
                    ************************************************************

TR45888_PIT2Aa      aatgggagccggctggaggaagtgttccttcacagaaacgtggatttggaacacattgac      660
TR26069_PIT3Ab      aatgggagccggctggaggaagtgttccttcacagaaacgtggatttggaacacattgac      660
                    ************************************************************

TR45888_PIT2Aa      gaatgtgcgtttgacggcgcgatccaaggcccaactcacctggacctctcggacaccggc      720
TR26069_PIT3Ab      gaatgtgcgtttgacggcgcgatccaaggcccaactcacctggacctctcggacaccggc      720
                    ************************************************************

TR45888_PIT2Aa      gtgcgcgccctgccttccaggggcctgggcagcgtggagacgctccaggcgcgccacacc      780
TR26069_PIT3Ab      gtgcgcgccctgccttccaggggcctgggcagcgtggagacgctccaggcgcgccacacc      780
                    ************************************************************

TR45888_PIT2Aa      tggtccctgagggcccttccggcccccggcgccttccggcacctgcagagcgccgagctg      840
TR26069_PIT3Ab      tggtccctgagggcccttccggcccccggcgccttccggcacctgcagagcgccgagctg      840
                    ************************************************************

TR45888_PIT2Aa      accttccccagccactgctgcggcctcaagatgctgaagcggtggacaggccgctccgag      900
TR26069_PIT3Ab      accttccccagccactgctgcggcctcaagatgctgaagcggtggacaggccgctccgag      900
                    ************************************************************

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TR26069_PIT3Ab      gaagccctctgcaacctgacggcgtggacggccccggagccgccgccggacggcgccccc      960
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TR26069_PIT3Ab      ccgcccgctccggagcgccggccgtgcggcggcgagctgcgctgcagcccgatgccggac     1020
                    ************************************************************

TR45888_PIT2Aa      gccctgaaccccctgcgaggacgtgatgagccgcggcttcctccgggtgctggtgtgggcg     1080
TR26069_PIT3Ab      gccctgaaccccctgcgaggacgtgatgagccgcggcttcctccgggtgctggtgtgggcg     1080
                    ************************************************************

TR45888_PIT2Aa      gtcggcctcctggccgtgctggccaacctgttggcgatggtcacgctgctgagcgggcgg     1140
TR26069_PIT3Ab      gtcggcctcctggccgtgctggccaacctgctggcgatggtcacgctgctgagcgggcgg     1140
                    ****************************** ***************************

TR45888_PIT2Aa      cgccggctgtcggtcacccgcttcctgatggcccacctggccctggccgacttctgcatg     1200
TR26069_PIT3Ab      cgccggctgtcggtcacccgcttcctgatggcccacctggccctggccgacttctgcatg     1200
                    ************************************************************

TR45888_PIT2Aa      ggcgcctacctgctgctcatcgcggcggtggacctgtacacgcactcgcagtactaccgc     1260
TR26069_PIT3Ab      ggcgcctacctgctgctcatcgcggcggtggacctgtacacgcactcgcagtactaccgc     1260
                    ************************************************************

TR45888_PIT2Aa      tacgccgtggcctggcagacgggaggcggctgcaacctggccggggcgctgtcggtcttc     1320
TR26069_PIT3Ab      tacgccgtggcctggcagacgggaggcggctgcaacctggccggggcgctgtcggtcttc     1320
                    ************************************************************

TR45888_PIT2Aa      gccagcgagctgtccgtctacacgctgagcctggtcagcctgcagcgctggcgcgccatc     1380
TR26069_PIT3Ab      gccagcgagctgtccgtctacacgctgagcctggtcagcctgcagcgctggcgcgccatc     1380
                    ************************************************************
```

```
TR45888_PIT2Aa      ttctacgccatgcggcccgagcgcaagatgcgtctgcgccacgccgccgccctgatgctg      1440
TR26069_PIT3Ab      ttctacgccatgcggcccgagcgcaagatgcgcctgcgccacgccgccgccctgatgctg      1440
                    ******************************** *************************

TR45888_PIT2Aa      gccggctggacgctgtgcgccggcgccgccctgctgccgctgctgggcgtcagcagctac      1500
TR26069_PIT3Ab      gccggctggacgctgtgcgccggcgccgccctgctgccgctgctgggcgtcagcagctac      1500
                    ************************************************************

TR45888_PIT2Aa      cagcgggtcagcatctgcctgcccatggaggcgggcacgccggccgcccgggcctacctg      1560
TR26069_PIT3Ab      cagcgggtcagcatctgcctgcccatggaggcgggcacgccggccgcccgggcctacctg      1560
                    ************************************************************

TR45888_PIT2Aa      gtgtgcgtgctgctggccaacgtgctggccatggccgtggtcagcctgtgctacctgcac      1620
TR26069_PIT3Ab      gtgtgcgtgctgctggccaacgtgctggccatggccgtggtcagcctgtgctacctgcac      1620
                    ************************************************************

TR45888_PIT2Aa      atctactgcatggtgcacaacccgcggcacctgtccagccgccgcgacgccagcatggcc      1680
TR26069_PIT3Ab      atctactgcatggtgcacaacccgcggcacctgtccagccgccgcgacgccagcatggcc      1680
                    ************************************************************

TR45888_PIT2Aa      aagcgcatggccgtgctggtcttcaccagcttcctgtgcctggcgcccatctgcttctac      1740
TR26069_PIT3Ab      aagcgcatggccgtgctggtcttcaccagcttcctgtgcctggcgcccatctgcttctac      1740
                    ************************************************************

TR45888_PIT2Aa      ggcctgtcggcggcgctgcaccagccgctgatgaccgtcaccgactccaaggtgctgctg      1800
TR26069_PIT3Ab      ggcctgtcggcggcgctgcaccagccgctgatgaccgtcaccgactccaaggtgctgctg      1800
                    ************************************************************

TR45888_PIT2Aa      gtgatcttctacccgctcaactcgtgcgcccacccccttcctctacgtcatcctgaccaag      1860
TR26069_PIT3Ab      gtgatcttctacccgctcaactcgtgcgcccacccccttcctctacgtcatcctgaccaag      1860
                    ************************************************************

TR45888_PIT2Aa      gccttccggagggacatcgcggcgctgctgagccggacgggcctgcggcggcaccaggtc      1920
TR26069_PIT3Ab      gccttccggagggacatcgcggcgctgctgagccggacgggcctgcggcggcgccaggtc      1920
                    ************************************************************.*******

TR45888_PIT2Aa      ccgctctgctga  1932
TR26069_PIT3Ab      ccgctctgctga  1932
                    ************
```

C) Yellowbelly flounder *Lhr* consensus sequence and translation

```
atgacgaccacgcccgtgctcctcctggtgctggtcctgcccccgctgtgctcctctgtg
M  T  T  T  P  V  L  L  L  V  L  V  L  P  P  L  C  S  S  V
gacgggacgccgccccccgggacgctgtccccctccctgcttctgcgactgggacgtccac
D  G  T  P  P  P  G  R  C  P  P  P  C  F  C  D  W  D  V  H
agcgtgtcttgcttcggggcccagctcttcccgcaattccactccagcacgcaggaagtg
S  V  S  C  F  G  A  Q  L  F  P  Q  F  H  S  S  T  Q  E  V
tggatggtgaggaccagactgtcctctgtccctcgggacgccttctccaacctgtccaac
W  M  V  R  T  R  L  S  S  V  P  R  D  A  F  S  N  L  S  N
gtctcccacatatacatctctgatgacgactctttgacaaatctggagaagcattccttc
V  S  H  I  Y  I  S  D  D  D  S  L  T  N  L  E  K  H  S  F
cgcaacctttccagcctcacacacatacaactcaccggcctcaaaacactgacctacatc
R  N  L  S  S  L  T  H  I  Q  L  T  G  L  K  T  L  T  Y  I
gaccaagaggcgtttaaagctctgcccaatttaaagtaccttgggatcaccaacacgggc
D  Q  E  A  F  K  A  L  P  N  L  K  Y  L  G  I  T  N  T  G
ctcacctccttccctgtgctccgatatgttcaatccatccaggaggatttcattttggag
L  T  S  F  P  V  L  R  Y  V  Q  S  I  Q  E  D  F  I  L  E
atagtggagaacgcctacgtacgagtgataccggccaattccttcgccggtatctctgac
I  V  E  N  A  Y  V  R  V  I  P  A  N  S  F  A  G  I  S  D
aaagctttgacagtcctgttgaacagtaacggtgtgagagaaatccagagtcacgccttc
K  A  L  T  V  L  L  N  S  N  G  V  R  E  I  Q  S  H  A  F
aatgggagccggctggaggaagtgttccttcacagaaacgtggatttggaacacattgac
N  G  S  R  L  E  E  V  F  L  H  R  N  V  D  L  E  H  I  D
gaatgtgcgtttgacggcgcgatccaaggcccaactcacctggacctctcggacaccggc
E  C  A  F  D  G  A  I  Q  G  P  T  H  L  D  L  S  D  T  G
gtgcgcgccctgccttccaggggcctgggcagcgtggagacgctccaggcgcgccacacc
V  R  A  L  P  S  R  G  L  G  S  V  E  T  L  Q  A  R  H  T
tggtccctgagggcccttccggcccccggcgccttccggcacctgcagagcgccgagctg
W  S  L  R  A  L  P  A  P  G  A  F  R  H  L  Q  S  A  E  L
accttccccagccactgctgcggcctcaagatgctgaagcggtggacaggccgctccgag
T  F  P  S  H  C  C  G  L  K  M  L  K  R  W  T  G  R  S  E
gaagccctctgcaacctgacggcgtggacggccccggagccgccgccggacggcgccccc
```

```
E  A  L  C  N  L  T  A  W  T  A  P  E  P  P  P  D  G  A  P
ccgcccgctccggagcgccggccgtgcggcggcgagctgcgctgcagcccgatgccggac
P  P  A  P  E  R  R  P  C  G  G  E  L  R  C  S  P  M  P  D
gccctgaacccctgcgaggacgtgatgagccgcggcttcctccgggtgctggtgtgggcg
A  L  N  P  C  E  D  V  M  S  R  G  F  L  R  V  L  V  W  A
gtcggcctcctggccgtgctggccaacctgctggcgatggtcacgctgctgagcgggcgg
V  G  L  L  A  V  L  A  N  L  L  A  M  V  T  L  L  S  G  R
cgccggctgtcggtcacccgcttcctgatggcccacctggccctggccgacttctgcatg
R  R  L  S  V  T  R  F  L  M  A  H  L  A  L  A  D  F  C  M
ggcgcctacctgctgctcatcgcggcggtggacctgtacacgcactcgcagtactaccgc
G  A  Y  L  L  L  I  A  A  V  D  L  Y  T  H  S  Q  Y  Y  R
tacgccgtggcctggcagacgggaggcggctgcaacctggccggggcgctgtcggtcttc
Y  A  V  A  W  Q  T  G  G  G  C  N  L  A  G  A  L  S  V  F
gccagcgagctgtccgtctacacgctgagcctggtcagcctgcagcgctggcgcgccatc
A  S  E  L  S  V  Y  T  L  S  L  V  S  L  Q  R  W  R  A  I
ttctacgccatgcggcccgagcgcaagatgcgcctgcgccacgccgccgccctgatgctg
F  Y  A  M  R  P  E  R  K  M  R  L  R  H  A  A  A  L  M  L
gccggctggacgctgtgcgccggcgccgccctgctgccgctgctgggcgtcagcagctac
A  G  W  T  L  C  A  G  A  A  L  L  P  L  L  G  V  S  S  Y
cagcgggtcagcatctgcctgcccatggaggcgggcacgccggccgcccgggcctacctg
Q  R  V  S  I  C  L  P  M  E  A  G  T  P  A  A  R  A  Y  L
gtgtgcgtgctgctggccaacgtgctggccatggccgtggtcagcctgtgctacctgcac
V  C  V  L  L  A  N  V  L  A  M  A  V  V  S  L  C  Y  L  H
atctactgcatggtgcacaacccgcggcacctgtccagccgccgcgacgccagcatggcc
I  Y  C  M  V  H  N  P  R  H  L  S  S  R  R  D  A  S  M  A
aagcgcatggccgtgctggtcttcaccagcttcctgtgcctggcgcccatctgcttctac
K  R  M  A  V  L  V  F  T  S  F  L  C  L  A  P  I  C  F  Y
ggcctgtcggcggcgctgcaccagccgctgatgaccgtcaccgactccaaggtgctgctg
G  L  S  A  A  L  H  Q  P  L  M  T  V  T  D  S  K  V  L  L
gtgatcttctacccgctcaactcgtgcgcccacccctcctctacgtcatcctgaccaag
V  I  F  Y  P  L  N  S  C  A  H  P  F  L  Y  V  I  L  T  K
gccttccggagggacatcgcggcgctgctgagccggacgggcctgcggcggcaccaggtc
A  F  R  R  D  I  A  A  L  L  S  R  T  G  L  R  R  H  Q  V
ccgctctgctga
P  L  C  -
```