Pituitary mRNA expression of FSH and LH in yellowbelly flounder (*Rhombosolea leporina*)

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Abstract

Recent interest has been expressed in cultivating the native, yellowbelly flounder (*Rhombosolea leporina*). This is largely due to its high market and cultural value. Currently, there is little research on this species, both in an aquaculture setting and from an ecological perspective. This study focused on assessing pituitary mRNA expression of two gonadotropin-beta subunit genes, follicle-stimulating hormone (*fshβ*) and luteinising hormone (*lhβ*), in wild-caught yellowbelly flounder, to better understand captive reproduction for aquaculture.

The first study characterised the expression of both pituitary *fshβ* and *lhβ* in wild-caught yellowbelly flounder in different stages of ovarian development: 1) previtellogenic (PVO), 2) early vitellogenic (EVit), 3) vitellogenic (VIT), and 4) final oocyte maturation (FOM). Both genes showed significant increases in expression between stages 1 and 4. The expression patterns of both pituitary *fshβ* and *lhβ* were similar, with a trend of increasing levels across all ovarian stages to peak at stage 4. However, peak expression levels were much greater in *fshβ*. This is unexpected compared to typical fish models, where pituitary *lhβ* expression is often many-fold greater than *fshβ* during oocyte maturation. The reason for this apparent anomaly in peak expression levels is unknown. However, the general profile of increasing levels of co-expressed pituitary *fshβ* and *lhβ* throughout oogenesis likely reflects the multiple-batch group synchronous ovarian development found in yellowbelly flounder.

The second study focused on quantifying *fshβ* and *lhβ* expression over a five-day period following the injection of gonadotropin-releasing hormone analogue (GnRHa). Each female yellowbelly flounder was assigned to one of four treatment groups: 25 µg/kg, 50 µg/kg, 100 µg/kg, or a control group (0 µg/kg). Although there were no statistically significant increases in *fshβ* or *lhβ* expression from day 1 to day 5, an upward trend in both *lhβ* and *fshβ* expression was observed in fish treated with 25 µg/kg of GnRHa. Similarly, an increasing trend in *lhβ* expression was observed in fish treated with 50 µg/kg of GnRHa. GnRHa-treated fish accounted for the highest number of individuals entering FOM, comprising 87% of all fish that reached this stage. Additionally, 85% of all fish treated with GnRHa progressed to early maturation (EM) or further. Interestingly, the 100 µg/kg GnRHa treatment resulted in a downward trend of both *fshβ* and *lhβ* expression on both

days 1 and 5 post-treatment. Despite this, the 100 µg/kg GnRHa treatment exhibited the joint-highest proportion of oocytes reaching maturity (33.3%) and had 90% of individuals reaching the early migratory (EM) stage or beyond. However, none of the fish were observed to ovulate over the 5-day experimental period.

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Table of Contents

List of Figures

List of Tables

Chapter 1 – General Introduction

1.1 Finfish aquaculture

Aquaculture, the practice of cultivating marine and freshwater organisms such as fish, shellfish, and crustaceans, plays and will continue to play a pivotal role in addressing various global challenges. The industry has gained significant importance due to its contributions to food security, environmental sustainability, economic development, and the conservation of wild fisheries.

Aquaculture is a crucial food source for a rapidly expanding global population (Garlock et al., 2020) projected to reach 9.7 billion by 2050. The overexploitation of wild fisheries has stagnated their contribution to global food stocks (Little et al., 2016). Many capture fisheries are experiencing noticeable declines worldwide, estimates suggest that approximately 95% are either fully exploited or overfished (FAO, 2020). As capture fisheries stagnate, aquaculture has become the fastest-growing food sector, with an annual growth rate of 6.7% between 1990 and 2020 (**Figure 1.1**) (FAO, 2024). This surpasses the growth rates of both pigs (<2.5%) and poultry (<5%) farming (Little et al., 2016). Additionally, fish like Atlantic salmon (*Salmo salar*) have some of the most efficient feed conversion ratios (1.0-1.2), compared to cattle (6.0-10.0), pigs (2.7-5.0), and chickens (1.7-2.0) (Fry et al., 2018). It is predicted that by 2050, seafood yield may need to increase by approximately 75% compared to current levels, necessitating a significant expansion in aquaculture production. The growth of aquaculture is essential for ensuring global food security, given the urgent need for increased food production and the rising per capita demand for seafood.

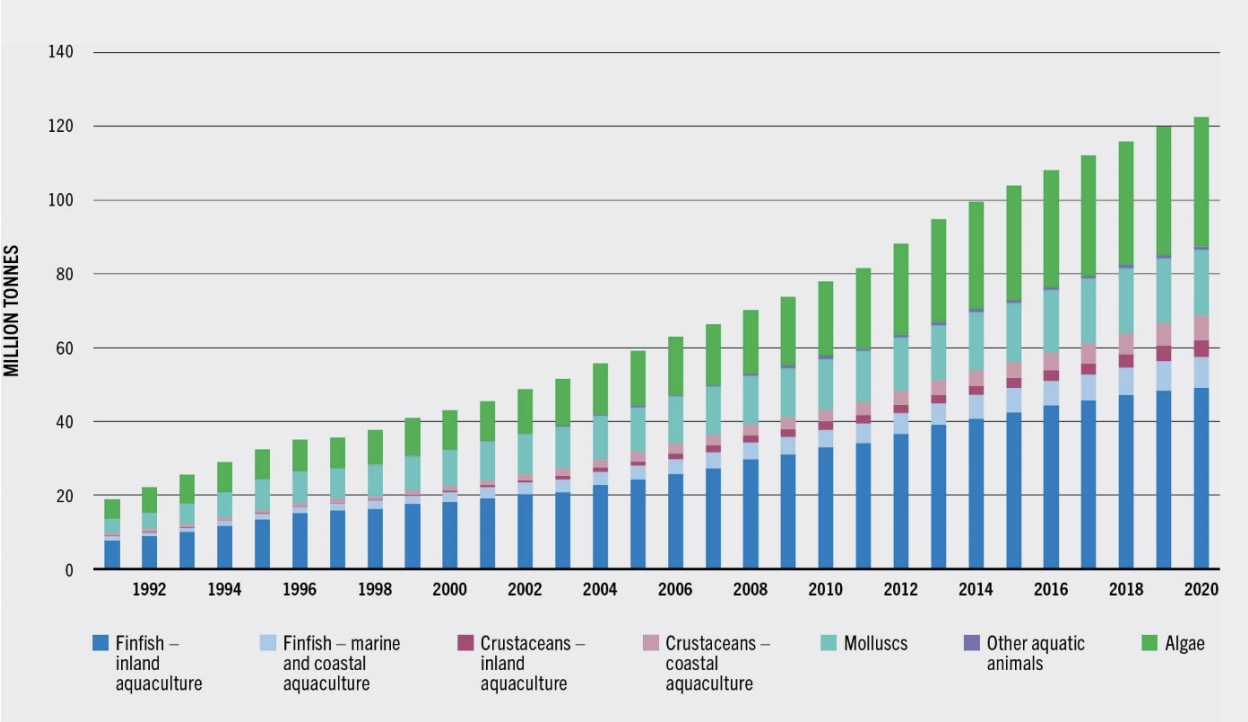

Figure 1.1. World aquaculture production from 1991 to 2020. Sourced from FAO (2022). Note: Data exclude shells and pearls. Data expressed in live weight equivalent.

1.2 Aquaculture in New Zealand

Aquaculture in New Zealand began in the 1960s with the farming of native rock oysters (*Saccostrea commercialis*), later replaced by the faster-growing Pacific oyster (*Crassostrea gigas*). The collapse of commercial mussel dredging led to the cultivation of the green-lipped mussel (*Perna canaliculus*), now one of the country's most successful species (Stenton-Dozey et al., 2021; Lloyd, 2003). Finfish farming started in freshwater locations with Chinook salmon (*Oncorhynchus tschawytscha*), and the first marine cage rearing began on Stewart Island in the 1980s. By 1989, salmon farming expanded to key areas like Stewart Island and Marlborough Sounds (Fløysand et al., 2016). In 2019, New Zealand's aquaculture industry generated NZ$673 million (Stenton-Dozey et al., 2021), with salmon, mussels, and oysters as key products, and is projected to reach NZ$3 billion in annual sales by 2035 (Ministry for Primary Industries (MPI), 2019).

Despite growth in some areas of New Zealand's aquaculture, finfish farming has seen little expansion or diversification beyond Chinook salmon over the past 50 years. However, recently new commercial operations such as Haku Kingfish and Manāki have started producing yellowtail kingfish (*Seriola lalandi*) and whitebait (*Galaxias* spp), respectively. These have resulted from

years of prior research (Symonds et al., 2014). Achieving industry expansion can be complicated due to complex legislation, social concerns, recreational and navigational uses, and culturally significant land (Banta & Gibbs, 2009). Despite New Zealand's 15,000 km of coastline, only 20,000 hectares are allocated to aquaculture, highlighting the limited space currently used and the potential for expansion. Diversifying the species farmed will help create new inclusive opportunities, broaden the market, and generate additional revenue streams while enhancing sustainability and resilience for the future of New Zealand's aquaculture industry.

1.3 Flatfish Aquaculture

Large international market demand exists for flatfish species. One of the key reasons for their popularity abroad is their healthy, high-quality white flesh, which is low in fat, rich in DHA and EPA (omega-3 fatty acids), and a good source of vitamins and minerals (Hibbeln et al., 2006). To further enhance the nutritional value of marine species, feed trials on flatfish have successfully increased the levels of several key lipids within the flesh (Choi et al., 2015; Xu et al., 2018). The commercial market for flatfish (Pleuronectiformes) in New Zealand is currently small, with commercial aquaculture production still undeveloped. Research has been conducted on both New Zealand turbot (*Colistium nudipinnis*) and brill (*Colistium guntheri*); however, significant mortalities in hatchery-reared juveniles hindered further progress, and no substantial developments have materialised (Diggles et al., 2000; Poortenaar et al., 2001).

Commercial flatfish production has been practised for decades in countries across Asia and Europe, using various cultivation methods. Since flatfish are benthic organisms, they perform relatively well at high densities in recirculating land-based raceways and tanks, without significantly affecting growth rates or compromising survivability (Merino et al., 2007). Studies show that winter flounder (*Pseudopleuronectes americanus*) can be stocked at densities as high as 200–300% without impeding growth, with 200% density appearing to be optimal compared to lower densities like 50% (Fairchild & Howell, 2001). Recirculating raceways, along with recirculating aquaculture systems (RAS), not only support higher fish densities and improved productivity but are also more environmentally friendly and cost-efficient. RAS are fully contained systems that prevent environmental pollution and the spread of diseases or parasites, issues often

associated with coastal and open-ocean net pens, by recycling up to 99% of the water volume daily (Tal et al., 2009).

Due to fishing pressures and overexploitation of wild flatfish stocks over several decades, global capture fisheries for flatfish have drastically declined. The industry peaked in the 1970s with around 3 million tonnes caught, compared to just 1.7 million tonnes in 2010 (Cheung & Oyinlola, 2018). For example, Japanese spotted halibut (*Verasper variegatus*) has declined by 80% over the past 40 years (Tomiyama et al., 2021). As wild populations have diminished, flatfish aquaculture has grown steadily, increasing by 50% since 2005 (Campbell & Pauly, 2013). Many species are now being commercially cultured, including European turbot (*Scophthalmus maximus*), Japanese flounder (*Paralichthys olivaceus*), tongue sole (*Cynoglossus semilaevis*), Atlantic halibut (*Hippoglossus hippoglossus*), common sole (*Solea solea*), and Senegalese sole (*Solea senegalensis*) (Robledo et al., 2017). However, one of the main challenges to the further growth and sustainability of flatfish aquaculture is the control of reproduction. Most, if not all, Pleuronectiformes, such as Senegalese sole, experience reproductive dysfunction and various life cycle issues in captivity, limiting commercial production.

1.4 Yellowbelly flounder

The yellowbelly flounder, known as Pātiki tōtara (*Rhombosolea leporina*), is an endemic species of flounder in New Zealand, belonging to the order Pleuronectiformes. Yellowbelly flounder inhabit a variety of coastal areas, including estuaries, river mouths, rivers, and mudflats. They are ambush predators, feeding on crabs, shellfish, brittle stars, and freshwater invertebrates. It is believed that juvenile flounder undergo sexual differentiation at a tail length of approximately 47 mm for females and 57 mm for males (Koverman, 2018). Peak spawning occurs from late August or early September through November under natural conditions, during which flounder migrate to depths of 12–30 meters to spawn (Colman, 1973). Younger females (around 30 cm) can produce approximately 250,000 eggs, while older, more mature females (around 45 cm) can produce up to 1.25 million eggs in a single spawning event (Colman, 1973). It is most likely that the reproductive process begins well before the spawning season.

1.4.1 Relevance of yellowbelly flounder

Like all endemic species in New Zealand, the yellowbelly flounder holds significant cultural, economic, and recreational importance. It is considered a tāonga species for Māori, meaning it is central to the identity and well-being of many Māori communities. For generations, tāonga species have sustained communities and helped pass on customary practices and knowledge from one generation to the next (NIWA, 2017).

Yellowbelly flounder has significant potential to become a successful aquaculture species. They have a relatively fast growth rate, reaching marketable size within two to three years (Colman, 1974). With a natural spawning season that spans from June to December (Colman, 1973; Koverman, 2018), there is the possibility to establish year-round. Both the domestic and international markets have strong demand for flatfish species. In New Zealand, the current 2024 retail value of yellowbelly flounder is $26 to $35 per kilogram gutted weight, exceeding the market value of both snapper (*Pagrus auratus*) and yellowtail kingfish (*Seriola lalandi*), two of the country's most popular finfish species (Seafood NZ, 2019). The economic importance of flatfish is also reflected in the 2022 annual report, which shows that 178,929 kg of flatfish were caught and exported to 11 different countries, generating a commercial value of $2,273,868 (inclusive of eight flatfish species) (Seafood NZ, 2022).

With no current commercial production of flatfish in New Zealand, ongoing research and experiments are well-timed, as wild fisheries face increasing challenges. Wild flatfish stocks are declining across New Zealand due to environmental factors and overfishing in some areas, driven by human activity (McKenzie et al., 2013).

1.5 Reproduction in Teleost species

Teleost reproduction is influenced by environmental stimuli such as temperature, photoperiod, and seasonal rainfall, which often coincide with favourable conditions like abundant food, longer daylight hours, and warmer temperatures (Bromage et al., 2001). For successful reproduction in cultured finfish, both environmental and internal stimuli must work together to determine the timing and success of reproduction.

1.5.1 Reproductive axis

The key system of structures regulating puberty and sexual reproduction in teleost species is the endocrine system, known as the hypothalamus–pituitary–gonad (HPG) axis (**Figure 1.2**). The HPG axis consists of three main components: the brain/hypothalamus, the pituitary gland, and the gonads. This endocrine pathway is activated during puberty and gains full physiological competence over a period of time (Weltzien et al., 2004). It functions like a cascade, where endocrine and neuroendocrine signals flow down from the brain, initiating various reproductive processes that culminate in the gonads (Nagahama, 1994). Through neurotransmitters and neurohormones, these environmental stimuli trigger a response in the hypothalamus, prompting the synthesis and release of gonadotropin-releasing hormone (GnRH). GnRH then binds to its receptors in the pituitary, where it stimulates the production of two gonadotropins: follicle-stimulating hormone (FSH) and luteinising hormone (LH) (Sébert et al., 2008; RI, 1988). These hormones are responsible for activating and regulating gametogenesis and steroidogenesis within the gonads. FSH and LH play crucial roles in reproduction, stimulating the synthesis of sex steroid hormones, such as androgens and estrogens, which directly regulate gamete development (Nagahama 1994).

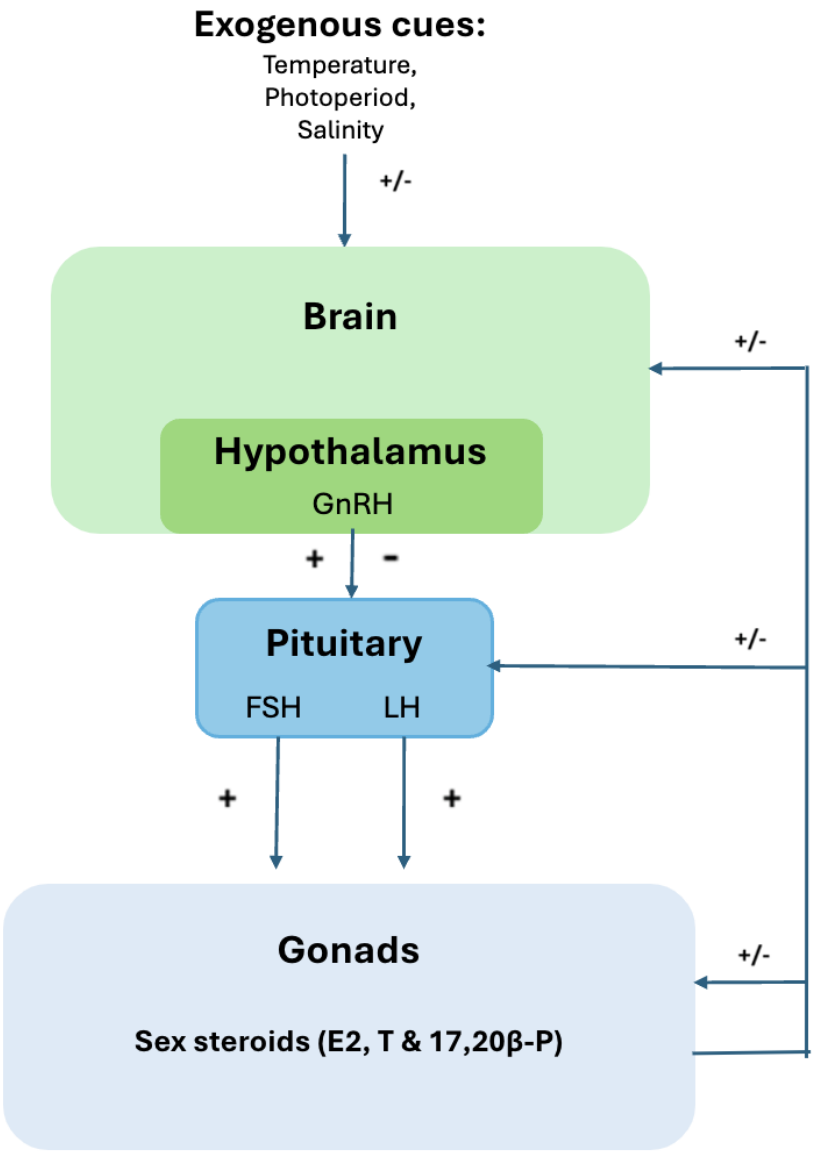

Figure 1.2. Schematic illustration of the hypothalamus–pituitary–gonad (HPG) axis in teleost species. This illustration simplifies and generalises the main pathways focused on within the study of yellowbelly flounder. Abbreviations: GnRH, gonadotropin releasing hormone; FSH, follicle-stimulating hormone; LH, luteinising hormone; E2, estradiol; T, testosterone; 17,20β-P, 17,20βdihydroxy-4-pregnen-3-one.

1.5.2 Oogenesis

Most finfish species reproduce by laying eggs (oviparous). Ovarian development falls into three categories: 1.) synchronous, where all oocytes mature and ovulate simultaneously; 2.) group-synchronous, where at least two distinct oocyte groups exist during development, but in the instance where multiple clutches of eggs are spawned within a season, fish may be considered multiple-batch group synchronous; and 3.) asynchronous, where oocytes at all stages are present without a dominant group (Wallace & Selman, 1981; Mylonas et al., 2010).

Oogenesis is a dynamic process in which oocytes develop through distinct stages within the ovaries, following a similar pattern across teleost species. It begins with primordial germ cells (PGCs) undergoing mitotic divisions during embryogenesis, migrating into the developing gonads (Yoshizaki et al., 2002). These divisions produce oogonia, which later enter the first meiotic division to form primary oocytes (Lubzens et al., 2010). This transition is regulated by estradiol (E2) and progestogen production in response to FSH expression (Lubzens et al., 2010; Yaron & Levavi-Sivan, 2011). Ovarian follicle development begins once meiosis is initiated.

Previtellogenesis consists of two meiotic arrests during the chromatin nucleolus and peri-nucleolus stages, where meiosis remains halted at prophase I through primary and secondary growth (Lubzens et al., 2017). The secondary growth stage marks the transition to cortical alveoli formation (Brown-Peterson et al., 2011; Patiño & Sullivan, 2002). Rising E2 and FSH levels stimulate follicle cells, triggering vitellogenesis (Nagahama, 1994). FSH-driven E2 production activates the liver to synthesise vitellogenin (yolk precursors) and choriogenins (Davail et al., 1998; Nagahama & Yamashita, 2008). The oocyte then absorbs these nutrients, such as proteins, lipids and vitamins, converting them into yolk proteins (vitellogenin sequestration) (Lubzens et al., 2010; Nagahama & Yamashita, 2008).

Following vitellogenesis, final oocyte maturation (FOM) and ovulation prepare the oocyte for fertilisation. This process is triggered by a maturation-inducing hormone (MIH), which resumes meiosis I, leading to the final maturation stage, germinal vesicle migration (GVM) (Lubzens et al., 2010; Yaron & Levavi-Sivan, 2011). During FOM, hormonal regulation of oogenesis classically shifts from FSH to LH (Lubzens et al., 2010; Nagahama, 1944; Nagahama & Yamashita, 2008). FOM is marked by lipid and vitellogenin granule coalescence, germinal vesicle migration, and germinal vesicle breakdown (GVBD), culminating in meiosis I (prophase I) completion and polar body extrusion (Lubzens et al., 2010; Planas et al., 2008). Oocyte hydration follows, driven by ion transport and increased osmotic pressure, causing a rapid increase in diameter (West, 1990). At metaphase II (meiosis II), ovulation occurs, where the oocyte separates from granulosa cells, ruptures the follicular layer, and is released into the ovarian or gut cavity, depending on the species (Nagahama & Yamashita, 2008). Upon fertilisation, meiosis II resumes, leading to second polar body extrusion, completing oocyte maturation (Lubzens et al., 2017; Mañanós et al., 2008).

After spawning, unovulated oocytes undergo atresia, where they are reabsorbed into the body (Lubzens et al., 2010). This involves granulosa cell enlargement, infiltration of the oocyte, and phagocytosis of its contents (Nagahama, 1983). Atresia can also be triggered by environmental stressors such as temperature fluctuations, stress, starvation, or captivity (Hunter & Macewicz, 1985; Clearwater & Pankhurst, 1997). While oocyte development is a relatively continuous process, the key events outlined above can be used as milestones to categorise ovarian development into discrete stages for the study of fish reproduction.

1.6 Reproductive failure and hormone therapy

Wild-caught broodstock fish often experience reproductive failure. This often relates to stress, either from capture and handling or the lack of appropriate environmental cues. Hormonal induction in cultured finfish species has become a crucial method for overcoming reproductive dysfunctions, such as failure to undergo final oocyte maturation (FOM) and reduced volume and quality of milt (sperm) (Mylonas & Zohar, 2000). Various hormone treatments such as pituitary extracts (Su et al., 2013) and human chorionic gonadotropin (hCG) (Zakęś & Demska-Zakęś, 2005) have been used to address reproductive dysfunctions related to ovulation, spawning, and milt production. However, both treatments have their disadvantages, such as eliciting an immune response, difficulty in titrating effective dosage and in the latter, risk of contamination from non-reproductive hormones and diseases (Lam, 1982; Zohar & Mylonas, 2001; Evans & Claiborne, 2005; Mylonas et al., 2017). As a result, synthetic GnRH agonists (GnRHa) are more common. This treatment provides several advantages. First, GnRHa is a small decapeptide and does not trigger immune responses. Second, GnRHa can help resolve endocrine disruptions in captive finfish that hinder ovulation and spawning, providing balanced stimulation of reproductive processes and indirectly or directly influencing the release of hormones like prolactin (Weber et al., 1995), growth hormone (Marchant et al., 1989) and thyroid hormones (Sullivan et al., 1989) during key reproductive stages (Zohar & Mylonas, 2001). Third, since GnRHa is synthesised in a pure form, there is no risk of disease transmission. Additionally, GnRHa has proven more effective than other hormonal treatments because it acts directly on the pituitary rather than on other organs, such as the gonads (Zohar & Mylonas, 2001). Due to these advantages, GnRHa, along with luteinising hormone-releasing hormone analogue (LHRHa), is rapidly replacing other hormones for spawning induction in aquaculture facilities.

1.7 Molecular tools within aquaculture research

Advancements in molecular technologies have significantly transformed aquaculture research, enabling a deeper understanding at the molecular level. The genome sequencing of various aquaculture species has provided valuable insights, supporting selective breeding, enhancing disease resistance, maintaining genetic diversity, and promoting sustainable fisheries management (Mechaly et al., 2024). To date, thirteen species within the Pleuronectiformes order have undergone genome sequencing using next-generation sequencing (NGS) technologies (Villarreal et al., 2024), including Japanese flounder (*Paralichthys olivaceus*) (Shao et al., 2017), turbot (*Scophthalmus maximus*) (Maroso et al., 2018), Senegalese sole (*Solea senegalensis*) (Guerrero-Cózar et al., 2021), and tongue sole (*Cynoglossidae*) (Chen et al., 2014). In aquaculture research, quantitative polymerase chain reaction (qPCR) is often used alongside NGS analysis. While NGS identifies potential biomarkers or candidate genes across the genome, qPCR provides high-precision quantification of their expression levels. This approach is widely employed in aquaculture studies (Zheng & Sun, 2011; Larsen et al., 2007; Williams et al., 2003), as it enables the assessment of gene expression to better understand the biological processes regulating aquatic organisms in their environments.

1.8 Research aims and objectives

Previous work in yellowbelly flounder indicates that they experience both acute and chronic stress following capture and introduction into captivity. This impacts reproduction in captivity. It is therefore desirable to produce a stock that is acclimated to captive conditions to accelerate further research toward the possible domestication of this species. Short-term solutions involve inducing reproduction in wild-caught broodstock. However, this requires an improved understanding of yellowbelly flounder reproductive physiology as well as the optimisation of a protocol for induced reproduction.

The primary aim of this study was to examine the activity of the pituitary gonadotropic system in both wild and captive yellowbelly flounder to help inform their induced reproduction for aquaculture. This was achieved through the following two key objectives using qPCR and light microscopy:

1.) Characterisation of pituitary *fshβ* and *lhβ* expression in wild *Rhombosolea leporina* at different stages of ovarian development (Chapter 2).

2.) Quantification of pituitary *fshβ* and *lhβ* expression at different time points over a five-day period following injection with a gonadotropin-releasing hormone analogue (GnRHa) (Chapter 3).

1.9 Ethics Statement

The work conducted during this study was approved by the University of Waikato Animal Ethics Committee (approval number 1149). The wild-caught flounder were obtained by a licensed fisherman, and standard approved measures and procedures were followed throughout the experiment.

Chapter 2 - Gonadotropin expression and oocyte development in wild-caught yellowbelly flounder

2.1 Introduction

Like many teleost species, yellowbelly flounder undergo distinct stages of oocyte maturation. However, the relationship between this process and pituitary gonadotropin expression remains largely unexplored. Gonadotropins, specifically luteinising hormone (LH) and follicle-stimulating hormone (FSH), play pivotal roles in regulating oogenesis by mediating the development and maturation of oocytes.

2.1.1 Gonadotropin system

The hypothalamus-pituitary-gonad (HPG) axis consists of three interconnected physiological components. Neuroendocrine neurons in the preoptic regions of the brain (hypothalamus) act as a bridge between the central nervous system and the endocrine system (Weltzien et al., 2004). Multiple inputs, both stimulatory and inhibitory, influence these neurons, which then produce gonadotropin-releasing hormone (GnRH) as an output. In teleosts, GnRH neurons directly connect to gonadotropin-producing cells (gonadotropes) within the pituitary, stimulating the production and release of two gonadotropins: follicle-stimulating hormone (FSH) and luteinising hormone (LH) (Weltzien et al., 2004; Golan et al., 2014). FSH and LH are released into the bloodstream, where they promote the transcription of steroidogenic enzymes involved in the synthesis of sex steroids, such as testosterone and 17β-estradiol via their specific receptors on the thecal and granulosa cells of the ovarian follicles (Nagahama, 1994; George et al., 2011). Therefore, environmental cues are effectively integrated via the HPG-axis to coordinate gonadal development and reproduction.

In female teleost species, FSH induces germ cell development and follicular growth. Whereas LH triggers ovulation (Hedger, 1998; Mroueh & Danforth, 1988). In most finfish species, FSH is present in the pituitary gland and plasma during the early stages of development, regulating the initial phases of gametogenesis (formation of ova). Oocytes develop from oogonia in the ovaries as oogonia undergo proliferative mitotic divisions and enter the meiotic transition phase (**Figure**

2.1). The oocyte follicle consists of two layers of steroidogenic cells: the inner granulosa cells and the outer thecal cells (**Figure 2.2**) (Patiño & Sullivan, 2002; Nagahama, 1994). FSH upregulates the expression of the enzyme STAR (Steroidogenic Acute Regulatory protein), which drives the uptake of cholesterol into the mitochondria of the thecal cells, where it is used as a substrate for testosterone (T) production. Testosterone diffuses out of the thecal cells and is converted into estradiol (E2) within the granulosa cells (Planas et al., 2008). In finfish ovaries, E2 production occurs during oocyte growth. Estradiol enters the bloodstream and stimulates the liver to produce vitellogenin proteins, which are then transported by the blood to the oocytes during vitellogenesis (Nagahama, 1994; Yaron et al., 2003). FSH primarily regulates oocyte development up to the end of vitellogenesis (Lubzens et al., 2010; Nagahama, 1994). As plasma testosterone levels increase, they negatively feedback on the pituitary, inhibiting FSH release and promoting LH production. LH is the key mediator in oocyte maturation, stimulating the follicular expression of genes encoding the enzymes responsible for the production of maturation-inducing hormone (MIH) and ultimately maturation-promoting factor (MPF) (Nagahama, 1994; Nagahama & Yamashita, 2008). In many fish, the primary MIH, regulated by LH, is 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-DP) (Aizen et al., 2012; Ogino et al., 2016).

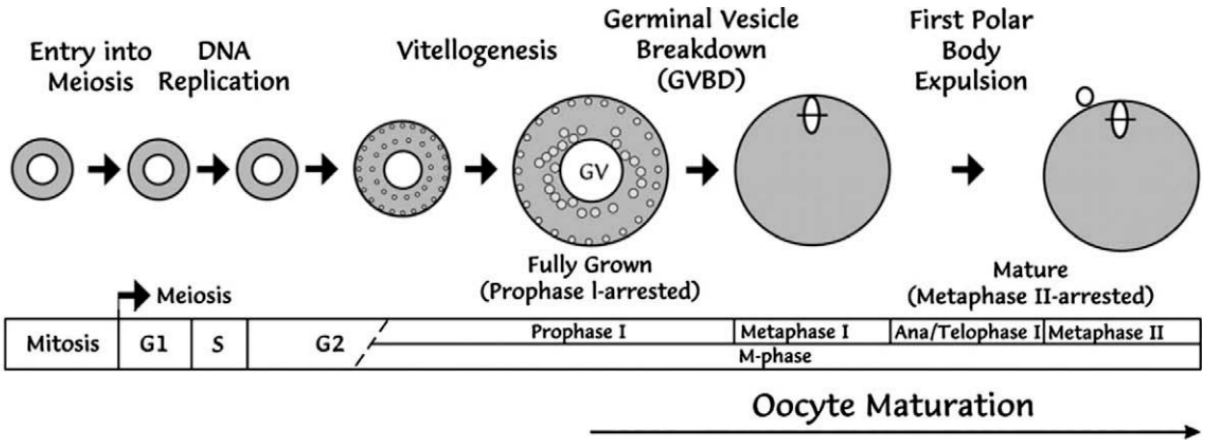

Figure 2.1. A simplified schematic representation of oogenesis in teleost fish. Sourced from Lubzens et al. (2010).

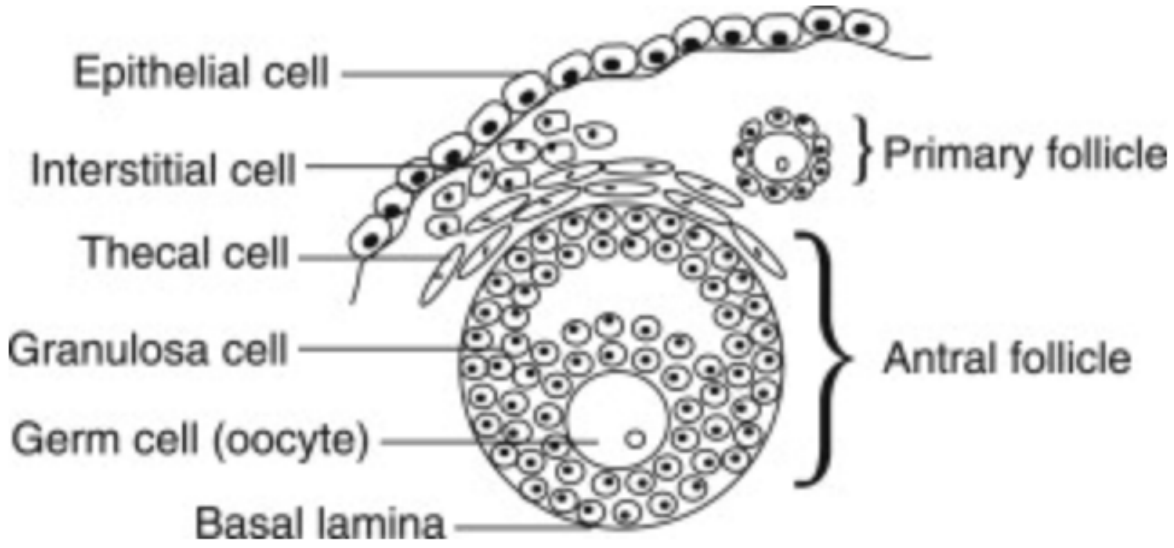

Figure 2.2. Cell types present within an ovary. Sourced from McQueen (2017).

The process by which MIH stimulates oocyte maturation involves a complex interaction between oocyte MIH receptors and signal transduction pathways. The production of maturation-promoting factor (MPF) facilitates the breakdown of the germinal vesicle and promotes oocyte maturation (Nagahama & Yamashita, 2008). Germinal vesicle breakdown (GVBD) occurs during the final stages of oocyte maturation, particularly during meiosis. It refers to the dissolution or breakdown of the nuclear membrane surrounding the germinal vesicle (nucleus) within the oocyte (Haider & Inbaraj, 1989). GVBD is a crucial event that marks the resumption of meiosis and the transition of the oocyte from prophase I to metaphase II, enabling the oocyte to become fertilisable.

Research has demonstrated that C21 steroids are powerful triggers of GVBD. Of these, 17α,20β-DP has proven to be the most effective C21 steroid at inducing GVBD in the majority of teleost species studied so far (Goetz, 1983; Nagahama et al., 1983). Testosterone and other C19 steroids can induce oocyte maturation/GVBD at high concentrations, whereas E2 and other C18 steroids have generally been ineffective at inducing oocyte maturation/GVBD in finfish oocytes (Nagahama & Yamashita, 2008). MIH plays a key role in promoting oocytes to undergo the first meiotic division from prophase I arrest, allowing them to mature fully and be ovulated as fertilisable eggs (Lubzens et al., 2010; Nagahama, 1944).

2.1.2 Background context and aim

While extensive research has been conducted on the gonadotropin system in many teleost species, very little attention has been given to understanding the HPG-axis in yellowbelly flounder. Most studies focus on general reproductive biology and ecology or on the effect of GnRHa treatments (Colman, 1973; Webb, 1972; Ellis-Smith, 2022). To date, there have been no studies to investigate the association between pituitary gonadotropin expression and oocyte development in this species. This study seeks to characterise pituitary gonadotropin expression in association with oocyte development in wild-caught yellowbelly flounder. Improving the current understanding of their reproductive physiology will help inform future efforts to cultivate this species.

This chapter aims to describe pituitary expression of follicle-stimulating hormone (*fshβ*) and luteinising hormone (*lhβ*) in association to the stage of ovarian development in wild-caught female yellowbelly flounder.

2.2 Methods

The tissue samples used in this work were collected as part of a previous study (Koverman, 2018) between 2015 and 2017. Adult yellowbelly flounder (*Rhombosolea leporina*) were caught at two locations (Miranda and Piako) in the southern part of the Firth of Thames, New Zealand. The flounder were captured by a commercial fisherman. The fish were dissected within 24 hours. All pituitary samples were removed and stored in RNAlater and then frozen at -80°C until further analysis.

2.2.1 Histology

The histological analysis was completed by Koverman (2018) from the guidelines in Mumford (2004). In brief, fresh ovarian tissue samples were fixed in neutral buffered formalin for 12-24 hours and then transferred to 70% ethanol prior to dehydration and paraffin embedding. Sections were cut at 4-5 µm and stained with haematoxylin and eosin. Histological details of the oocytes were reclassified into four groups—previtellogenic (PVO), early vitellogenic (EVit), vitellogenic (VIT), and final oocyte maturation (FOM)—based on their ovarian development (**Table 2.1**) and visually represented (**Figure 2.3**) by the leading cohort of oocytes.

Pituitary samples corresponding to a selection of individuals with gonadal histology from each of the ovarian stages described above were used to quantify pituitary gonadotropin expression.

Table 2.1. Stages of oocyte maturation in yellowbelly flounder, aligning with the histological observations of the oldest oocyte cohort. Oocyte diameters are the inclusive quartile minimum and maximum, rounded to the nearest 1 µm. Modified from Koverman (2018).

Ovarian stage		Histological description of the oocyte	*n*
I	Previtellogenic	The ooplasm is basophilic, and the zona radiata is either not visible or presents as a thin eosinophilic band surrounding the cytoplasm. Follicle cells form a thin layer surrounding the oocyte.	6
II	Early Vitellogenic	Oocytes increase significantly in size, cortical alveoli granules are present with lipid droplets. Nucleoli are located at the periphery of the germinal vesicle. The ooplasm is primarily basophilic as the first vitellogenin granules start to transition into the oocyte. The zona radiata is evident.	6
III	Vitellogenic	Further increase in oocyte diameter. Eosinophilic vitellogenin granules are visible within the ooplasm in addition to cortical alveoli. Lipid droplets increase in number. The zona radiata thickens visibly.	8
IV	Final Oocyte Maturation	Oocyte diameter increases. Lipid droplets coalesce, often contacting the germinal vesicle. The germinal starts to migrate from the centre. Vitellogenin granules may coalesce into larger masses of smooth ooplasm.	7

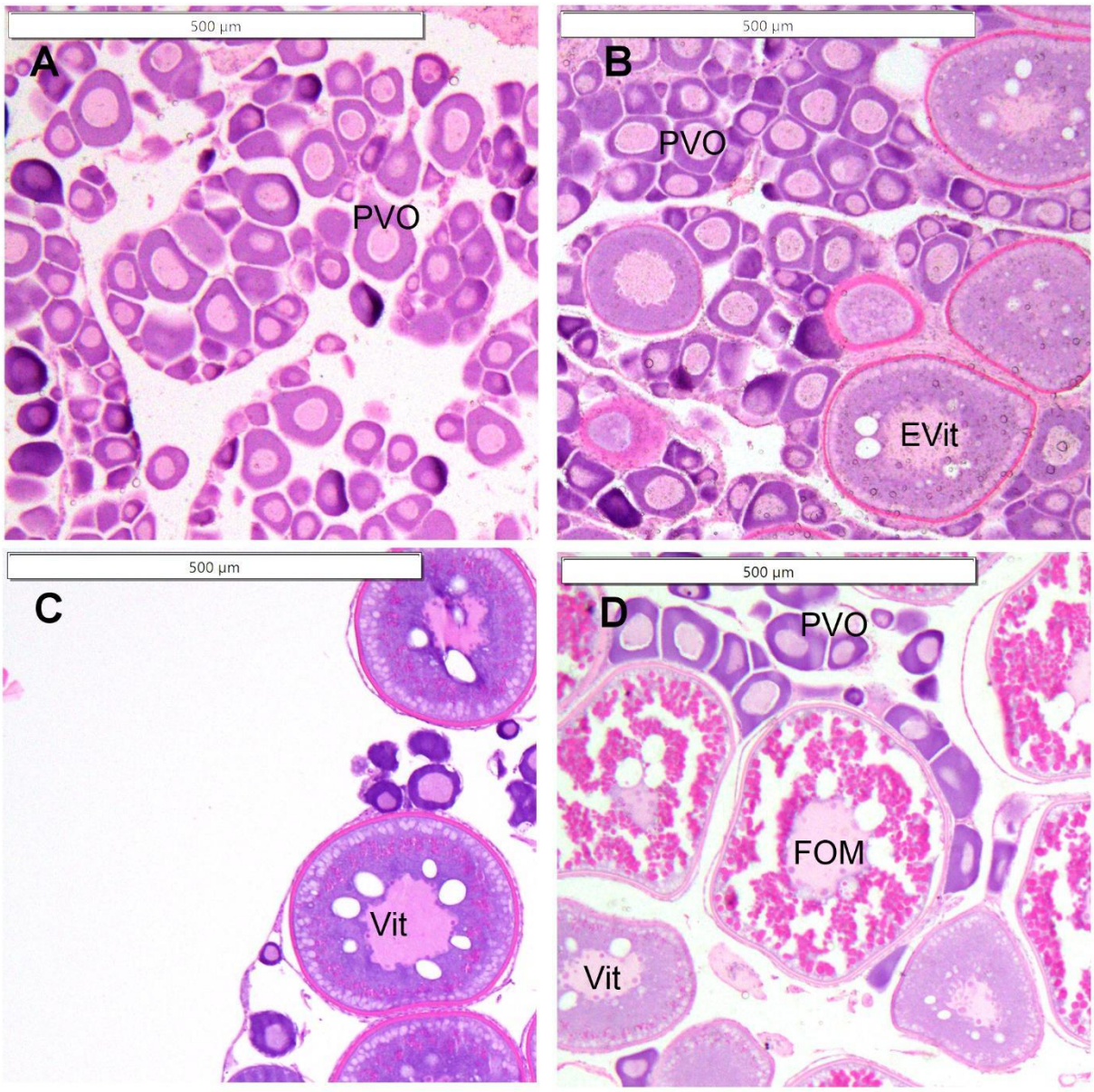

Figure 2.3. Photomicrographs of varying oocyte stages from female yellowbelly flounder. Previtellogenic, PVO; early vitellogenic, EVit; vitellogenic, VIT; final oocyte maturation, (FOM).

2.2.2 Primer design

Follicle-stimulating hormone beta (*fshβ*) and luteinising hormone beta (*lhβ*) are the primary genes of interest, while Ribosomal Protein L18 (*rp18*) and TATA-binding protein (*tbp*) serve as housekeeping genes, all based on transcriptome data. Housekeeping genes are used in qPCR as internal controls to normalise the expression of target genes of interest. The qPCR primers were developed using criteria including: i) melting temperatures (Tm) between 59-65°C, ii) primer

lengths of 20-60 nucleotides, and iii) a GC content of 40-60%. To ensure efficient amplification, the Tm difference between forward and reverse primers was kept within 3°C, and they were designed to span exon-exon boundaries to prevent genomic DNA amplification. For each gene, 2-4 sets of primers were designed and tested to achieve optimal performance. The final selected primers were chosen for their lack of nonspecific amplification and high efficiency in qPCR analysis. During testing, optimal annealing temperatures were determined. The final primer sequences and their specific properties are displayed in **Table 2.2**.

Table 2.2. Primer sequences were designed for qPCR amplification of target genes (*fshβ* and *lhβ*) and housekeeping genes (*rpl18* and *tbp*) in yellowbelly flounder. Each gene's average efficiency and its standard deviation (±SD) are presented for all qPCR runs. Abbreviations: (*fshβ*), Follicle-stimulating hormone beta; (*lhβ*), luteinising hormone beta; (*rpl18*), Ribosomal Protein L18; (*tbp*), TATA-binding protein; Tm, melting temperature; F, forward; R, reverse.

Gene	Primer sequence (5'-3')	Annealing temp (°C)	Tm (°C)	Amplicon size (bp)	Efficiency (Mean%± SD)	GC (%)
fshβ	F: GACAGTGCTACCATAAGGATCCG	60	61.8	178	0.94±0.03	52
	R: CAGGAAGTGTTAACTGAATTACACGC	60	59.5			42
lhβ	F: CACTGCAACACCAAGGACCC	60	64.9	235	0.87±0.09	60
	R: GTATGTCGTTCATGCAGAAGTCGG	60	61.8			50
rpl18	F: GCTCCTGGTCAAGTTGTACCG	64	63.2	176	0.92±0.03	57
	R: GACATCTTCATCTTACGGATCAGGC	64	61.6			48
tbp	F: TGGGCTGTAAACTAGACTTGAAGAC	59	60.5	170	0.91±0.01	40
	R: CGACTGATCCTCGCTCTTGG	59	63.3			60

2.2.3 RNA extraction and quality

Obtaining RNA of high purity, free from genomic DNA contamination, is crucial for optimal qPCR outcomes and reduces the risk of amplifying nonspecific products. RNA was extracted from 27 (n=27) female yellowbelly flounder pituitary samples using the Direct-zol™ RNA Miniprep kit (Zymo Research). The use of TRIzol® reagent enables the extraction of high-quality RNA from tissue lysate. Total RNA extraction from the pituitary tissue was performed according to the manufacturer's guidelines. All 27 pituitary samples were homogenised in 2 ml RNase/DNase-free tubes, each containing 800 µL of TRIzol® reagent and approximately 50 µL of silica beads with diameters of 0.1 mm and 0.5 mm. The samples were homogenised using a Precellys® Evolution homogeniser at 6800 rpm for three cycles of 20-second bursts, with 30-second intervals between

each cycle to allow for cooling. If the sample's lysate remained cloudy after homogenisation, it was subjected to a second cycle. Cloudiness indicates incomplete homogenisation. Once homogenisation was complete and the lysate was clear, the liquid portion (supernatant) was carefully pipetted out of the tube containing the beads and transferred into a new 1.5 ml RNase/DNase-free tube. Then, 800 µL of 100% ethanol was added to create a 1:1 ratio, and each tube was mixed by gentle inversion for 15 seconds.

The entire contents were then loaded into a ZymoSpin™ centre column within a 1.5 ml tube and centrifuged for 1 minute at 13,500 rpm. The flow-through was discarded, and each sample was subjected to DNase I treatment (Zymo Research) by first adding 400 µL of RNA Wash Buffer to the centre column, followed by centrifugation for 1 minute at 13,500 rpm. Again, the flow-through was discarded, and 75 µL of digestion buffer and 5 µL of DNase I were pipetted directly onto the centre column matrix. Each sample was incubated at room temperature (20-30°C) for 15 minutes. After incubation, 400 µL of Direct-zol Prewash (Zymo Research) was added to the centre column and centrifuged for 1 minute at 13,500 rpm. The flow-through was discarded. This step was repeated twice to ensure the complete removal of DNase from every sample. The final washing step involved adding 700 µL of RNA Wash Buffer and centrifuging for 2 minutes at 13,500 rpm to remove any residual ethanol. To elute the RNA from the centre column matrix, 50 µL of DNase/RNase-free water was added and centrifuged for 1 minute at 13,500 rpm. The concentration and purity of the RNA were assessed using the DeNovix® DS-11 spectrophotometer (DeNovix) shortly after elution. A portion of the mRNA was promptly used for cDNA synthesis, which was then stored at -20°C to prevent RNA degradation. The remaining mRNA was stored at -80°C.

Spectrophotometric Analysis

After RNA extraction, a small volume of each sample was reserved for spectrophotometric analysis. The concentration and purity of the RNA were assessed using a DeNovix® DS-11 spectrophotometer (DeNovix) (**Appendix 3, Table 1**), which measures absorbance at specific UV wavelengths. First, the instrument was calibrated with 1 µL of DEPC-treated water, followed by the analysis of each sample by loading 1 µL into the machine. After analysing each sample, the loading site was cleaned using Kimtech Science™ KimWipes™ Delicate Task Wipes (Mediray). The software determined the sample concentrations by comparing the absorbance readings at 260 nm between the blank and each sample. Purity was evaluated based on the A230/A260 and

A260/A280 absorbance ratios, with a ratio of approximately 2.0 indicating high RNA purity. RNA and DNA typically absorb light at 260 nm. A ratio of 1.8 is generally accepted as "pure" for DNA, and 2.0 is accepted as "pure" for RNA. If the ratio is considerably lower in either case, it may indicate the presence of proteins, phenol, or other contaminants that absorb strongly at or near 280 nm. The 260/230 ratio can reveal contaminants such as proteins and chaotropic salts. Pure nucleic acid samples typically have a 260/230 ratio of 2 or higher. Anything below 2 suggests the presence of other factors in the sample.

Gel electrophoresis

After RNA extraction, RNA quality was also assessed using gel electrophoresis. A 1% agarose gel was prepared by weighing out 0.25 g of agarose powder and placing it into a 50 ml conical flask. Then, 25 ml of 1x Tris-acetate-EDTA (TAE) solution was added to the flask. The solution was microwaved at maximum power until it reached boiling point, becoming clear, indicating the complete dissolution of the agarose. After cooling, 25 µL of sodium hypochlorite (bleach) and 2.5 µL of GelRed® Nucleic Acid Stain (Sigma) were added to the solution. Once cooled to room temperature, the solution was carefully poured into the electrophoresis gel mold, ensuring no air bubbles were present, and a well comb was placed into the mold. The gel was left to set for 15 minutes before removing the well combs. The gel was then placed into the electrophoresis tank (Life Sciences, Mini Horizontal), and 1x TAE buffer was added to immerse the gel completely. A mixture of 2 µL of loading buffer and 5 µL of RNA template was prepared using a pipette on Parafilm® (Sigma-Aldrich) before loading 6 µL into each well. The first well was loaded with 4 µL of the Solis BioDyne 1kb DNA ladder, which is used as a reference. After connecting both power cords, the gel was run for 20 minutes at 90 V. This allowed the RNA to migrate toward the positive electrode due to its negatively charged phosphate backbone and to separate by size. After electrophoresis, the gel was carefully transferred to the iBright™ CL750 Imaging System (ThermoFisher), where images were captured and recorded (**Appendix 2, Figure 1**).

2.2.4 Complementary DNA (cDNA) synthesis

The qScript™ XLT cDNA SuperMix kit (Quanta Biosciences) was used to synthesise RNA into cDNA. Frozen kit reagents and RNA samples were first thawed, thoroughly mixed, and then placed on ice. The volume of RNA sample used for cDNA synthesis depended on its concentration

(ng/μL), as determined by the DeNovix results obtained earlier (**Appendix 3, Table 1**). To synthesise cDNA from 1 μg of RNA, the following equation was used:

$$RNA\ Volume = (L) = \frac{2}{RNA\ Concentration\ (ng/L)/1000}$$

cDNA was synthesised from each RNA sample following the manufacturer's guidelines. Before analysis, various components were added to RNase/DNase-free PCR tubes with a total volume of 0.2 mL (**Table 2.3**), resulting in a final volume of 20 μL:

Table 2.3. Volumes and reaction constituents necessary for cDNA synthesis. Volumes obtained for each cDNA synthesis reaction are in **Appendix 4**, **Table 1**.

Reaction constituents:	**Volume per 20 μL:**
qScript XLT cDNA SuperMix (μL)	4
RNA template (μL)	RNA Concentration-dependent
RNase/DNase-free water (μL)	RNA Concentration-dependent

Every tube was thoroughly vortexed to mix the components before being briefly centrifuged to ensure all contents were at the bottom of the tube. The labelled 0.2 mL tubes were then placed into the SimpliAmp™ Thermal Cycler (Thermofisher) in a uniform layout to balance the pressure while the machine was active. The procedure began with an incubation stage at 25°C for 5 minutes, followed by 42°C for 60 minutes, 85°C for 5 minutes, and then held at 4°C until extracted. Each cDNA sample was then pipetted into individual 1.5 mL tubes and stored at -20°C until further use. The cDNA was diluted to a 1:100 ratio using molecular grade water and stored at -20°C before being analysed using quantitative PCR (qPCR) to assess gene expression.

2.2.5 Quantitative PCR (qPCR)

qPCR was conducted following the manufacturer's guidelines using a 48-well Magnetic Induction Cycler (MIC) qPCR (Bio Molecular Systems) with PerfeCTa® SYBR® Green FastMix® (Quanta Biosciences) as the reagent. Before analysis, all the required constituents were added into a 0.2 ml RNase/DNase-free MIC PCR tube (Dnature) (**Table 2.4**), ensuring a total volume of 20 μL in each reaction.

Table 2.4. Volumes of each constituent required for qPCR analysis.

Reaction constituents:	**Total volume per reaction (20 μL)**

Forward primer (µL)	1
Reverse primer (µL)	1
cDNA template (µL)	8
PerfeCTa® SYBR® Green FastMix® (µL)	10

All samples were duplicated, and No Template Controls (NTCs) were also conducted for each primer pair to ensure no contamination. NTCs contained all the constituents mentioned above, except the cDNA template, which was substituted with molecular-grade RNase/DNase-free water. A positive control duplicate was also included for each qPCR assay, aiming to verify the consistency of cq values across all assays. Amplification of cDNA was performed under the following conditions: one cycle at 95°C for 30 seconds, followed by 40 cycles at 95°C for 15 seconds and 40 cycles at the optimum pre-determined annealing temperature (**Table 2.2**) for each housekeeping gene of interest for 30 seconds. After each cycle, the fluorescence signal output was measured at 60°C for each sample. The melting curve (Melt on Green) analysis for each qPCR sample was established by recording fluorescence at every degree from 65°C to 95°C to verify the amplification of a single product, displaying the results on a graph.

2.2.6 Statistical analysis

Gonadotropin expressions across oocyte stages were compared using a one-way ANOVA (Analysis of Variance). Post-hoc analysis with Tukey's HSD (Honestly Significant Difference) test was conducted to identify variations in gonadotropin expression among different oocyte stages. Statistical significance was set at $p < 0.05$ for all analyses.

2.3 Results

2.3.1 Follicle-stimulating hormone (*fshβ*) beta expression in relation to oocyte growth

Follicle-stimulating hormone beta expression was significantly different between oocyte developmental stages, with the normalised fold expression differing ($F(3, 26) = 3.57$, $p = 0.03$, ANOVA) across the stages (**Appendix 1**, **Table 1**; **Figure 2.4**). Analysis using a post-hoc test concluded that there is a significant difference between stage 1 (Previtellogenic (PV)) and stage 4 (Final Oocyte maturation (FOM)) ($Q = 4.2473$, $p = 0.030$).

2.3.2 Luteinising hormone (*lhβ*) beta expression in relation to oocyte growth

Luteinising hormone beta expression was significantly different between oocyte developmental stages, with the normalised fold expression differing ($F(3,26) = 3.11$, $p = 0.046$, ANOVA) across the stages (**Appendix 1**, **Table 2**; **Figure 2.4**). Analysis using a post hoc Tukey HSD test concluded that there is a significant difference between stage 1 (Previtellogenic (PV)) and stage 4 (Final Oocyte Maturation (FOM)) ($Q = 3.9210$, $p = 0.049$).

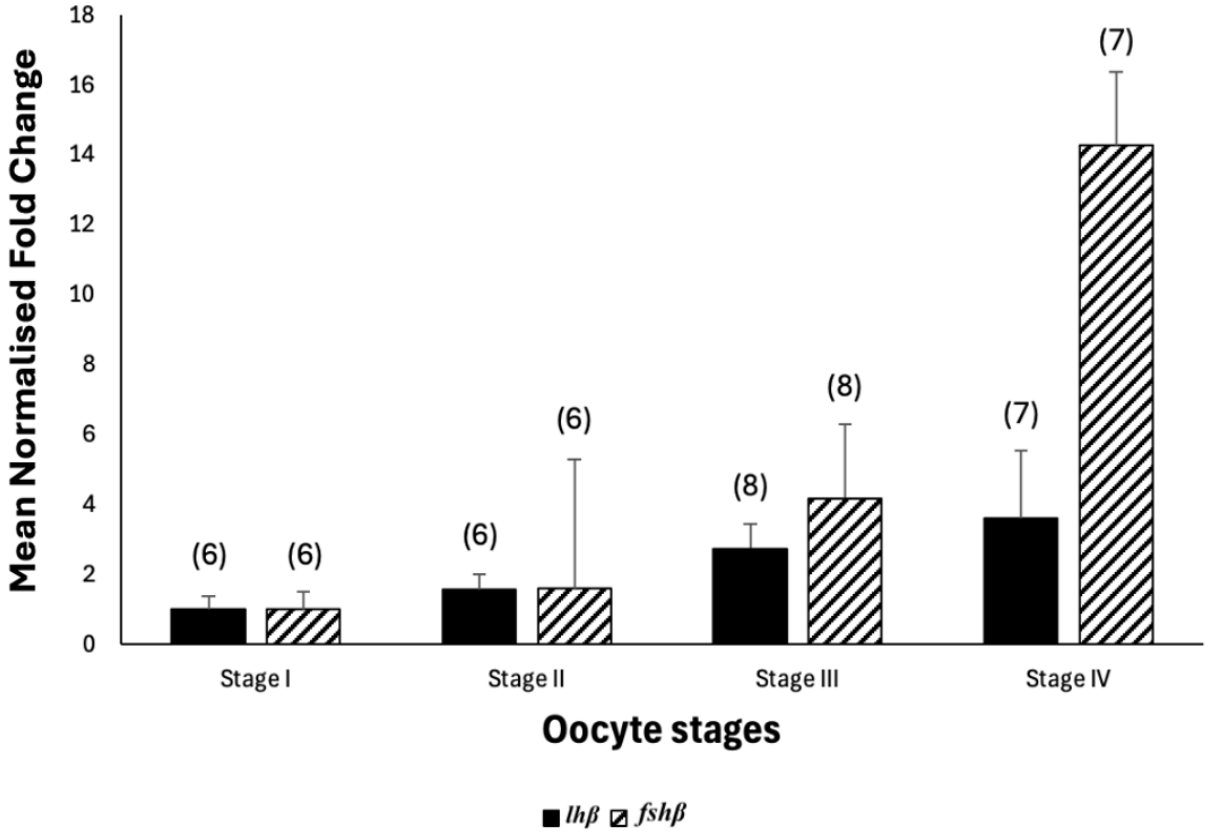

Figure 2.4. Mean normalised fold change of both luteinising hormone (*lhβ*) and follicle-stimulating hormone (*fshβ*) within the pituitary against each oocyte stage (Stage I: Previtellogenic (PV), Stage II: early vitellogenic (EVit), Stage III: Vitellogenic (VIT), Stage IV: Final Oocyte Maturation (FOM)). Error bars show standard error. The sample size (n) for each treatment is shown in parentheses above each bar.

2.4 Discussion

This study aimed to understand how oocyte maturation influences the expression levels of luteinising hormone (*lhβ*) and follicle-stimulating hormone (*fshβ*) in the pituitaries of female yellowbelly flounder during their natural spawning season. It provides valuable insight into the natural hormonal patterns associated with oocyte maturation in wild yellowbelly flounder, establishing a crucial baseline for further research. Understanding how gonadotropins are naturally

expressed within the pituitary helps to determine whether observed changes in gonadotropin levels are a result of GnRHa administration or the effects of stress and/or captivity.

2.4.1 Gonadotropin mRNA subunit expression (*lhβ* & *fshβ*) in relation to oocyte development

There is evidence that the yellowbelly flounder gonadotropic system broadly aligns with other teleosts and vertebrates in facilitating oogenesis. The results from the analysis showed that pituitary *fshβ* and *lhβ* expression increased as the oocytes matured and progressed through the later stages of oogenesis. However, in contrast to salmonid models, the fish in the current study showed notably higher peak levels of pituitary *fshβ* expression when oocytes were maturing compared to the equivalent peak in *lhβ* expression. This is unexpected as both peak pituitary *lhβ* expression and plasma LH concentrations are typically associated with final oocyte maturation and ovulation (Gomez et al., 1999; Nagahama & Yamashita, 2008; Levavi-Sivan et al., 2010; Taranger et al., 2015). For example, in rainbow trout (*Oncorhynchus mykiss*), pituitary *lhβ* expression increases sharply during final oocyte maturation and peaks post-ovulation (Gomez et al., 1999). Taranger et al. (2015) report that peak pituitary *lhβ* expression in Atlantic salmon (*Salmo salar*) is approximately 20-fold greater than that of *fshβ.* Similarly, peak pituitary *lhβ* expression occurred at the end of ovulation and exceeded peak *fshβ* expression in Malaysian river catfish (*Hemibagrus nemurus*) (Zulperi et al., 2017). These studies exhibit a more classical pattern of gonadotropin expression, where increasing pituitary *fshβ* expression occurs during vitellogenic oocyte stages, followed by a dramatic upregulation of pituitary *lhβ* expression during FOM. It should be noted that these fish have group synchronous ovarian development, producing a single batch of eggs per season. In contrast, yellowbelly flounder have a multiple-batch group synchronous pattern of ovarian development. Therefore, several cohorts of oocytes can enter and/or progress through vitellogenesis at the same time as others are in FOM. This would likely require both active FSH and LH signalling in the ovary which, may also result from the co-expression of *fshβ* and *lhβ* in the pituitary. It remains unknown whether the markedly lower peak pituitary *lhβ* expression levels were an artefact of the study or if they actually reflect a true pattern in wild yellowbelly flounder. To this extent, elevated levels of FSH have been recorded in post-spawned rainbow trout and is thought to be important for initiating gametogenesis (Gomez et al. 1999).

2.4.2 Gonadotropin (*lhβ* and *fshβ*) mRNA expression in multiple-group synchronous batch spawners

Other fish with multiple-batch group synchronous ovarian development show gonadotropin profiles that differ from the classical group synchronous model. Pham et al. (2008) concluded that patterns of FSH and LH immunostaining in the pituitary of Japanese flounder (*Paralichthys olivaceus*) indicate that both gonadotropins are associated with oocyte maturation in this batch spawning species. Moreover, Kajimura et al. (2001) found increasing levels of pituitary *fshβ* and *lhβ* expression throughout oogenesis in Japanese flounder. These results resemble the ones found in the current study with yellowbelly flounder. In female greater amberjack (*Seriola dumerili*), pituitary *fshβ* expression increased as expected during ovarian development but also remained significantly elevated during the post-spawning period (Nyuji et al., 2016). These studies raise the possibility that pituitary *fshβ* expression may persist throughout oogenesis to support the development of multiple oocyte cohorts. Moreover, pituitary *fshβ* expression may remain elevated even into post-spawning. Other studies show that expression of pituitary *fshβ* and *lhβ* is dynamic and can fluctuate over a 24-hour period. In gilthead seabream (*Sparus aurata*), pituitary *fshβ* and *lhβ* were co-expressed, increasing 8-4 and 12-8 hours prior to spawning, respectively (Gothilf et al., 1997). Peak expression levels occurred 4 hours apart prior to spawning. Taken collectively, these studies demonstrate that pituitary gonadotropin expression profiles in fish with multiple-batch group synchronous ovarian development are often characterised by the co-expression of both pituitary *fshβ* and *lhβ*, and these may increase throughout oogenesis.

It is also important to note that this study has focused on quantifying pituitary mRNA transcripts, not the native hormones themselves. The data reported are only correlative in terms of their association with activity in the lower HPG axis and cannot, therefore, be extrapolated to represent the actual hormonal dynamics within the organs. The precise roles of both gonadotropin hormones during oogenesis and spawning are complex and remain challenging to fully elucidate. As the yellowbelly flounder is a newly emerging aquaculture candidate, comprehensive knowledge of its reproductive biology is still lacking. This study represents an initial investigation into the pituitary expression of gonadotropins. However, a more complete characterisation of the yellowbelly flounder's gonadotropic system would require an assessment of their respective gonadal receptors.

This would further improve the current understanding of the endocrine regulation and spawning dynamics of this species.

Chapter 3 - Effects of GnRHa on the gonadotropic system of captive yellowbelly flounder

3.1 Introduction

To establish yellowbelly flounder as a viable aquaculture species, effective control of reproduction in captivity is crucial. However, reproductive dysfunction and stress in wild-caught broodstock must be addressed, particularly in a captive setting. Reproductive dysfunction is typically caused by disruptions in the hormonal cascade of the hypothalamus-pituitary-gonad (HPG) axis, which regulates reproduction. A common approach to overcoming this issue is the administration of an exogenous gonadotropin-releasing hormone agonist (GnRHa). GnRH is naturally produced in the hypothalamus, while gonadotropin-releasing hormone agonist GnRHa is a synthetic form of the GnRH peptide. The reproductive system is controlled by the HPG axis. Naturally, the hypothalamus is stimulated by endogenous rhythms, environmental cues, and various hormones. These stimuli trigger the production of GnRH from the hypothalamus, where GnRH neurons directly innervate the gonadotrope cells in the pituitary. The stimulation of gonadotrope cells in the pituitary results in the production and secretion of two gonadotropin hormones, FSH and LH. The treatment of fish with GnRHa activates GnRH receptors on the pituitary gonadotrope cells to elicit an endogenous release of gonadotropins and thereby reinitiate reproduction.

There are multiple different forms of delivery methods for GnRH administration, including injections, sustained-release cholesterol-based pellets, Ethylene-Vinyl Acetate (EVAc) and biodegradable microspheres (Mylonas & Zohar, 2009; Rainis & Ballestrazzi, 2005). GnRHa treatment has been successful in multiple species across the teleost order, including greater amberjack (*Seriola dumerili*) (Mylonas et al., 2004), European sea bass (*Dicentrarchus labrax*) (Rainis et al., 2003), and meagre (*Argyrosomus regius*) (Mylonas et al., 2016). It has also proven effective in flatfish species, such as Senegalese sole (*Solea senegalensis*) (Agulleiro et al., 2006), turbot (*Scophthalmus maximus*) (Mugnier et al., 2000), summer flounder (*Paralichthys dentatus*) (Berlinsky et al., 1997), and yellowtail flounder (*Pleuronectes ferrugineus*) (Clearwater & Crim, 1998).

3.1.1 Background context and aim

To understand the effect of GnRHa administration on luteinising hormone (*lhβ*) and follicle-stimulating hormone (*fshβ*) expression in captive female yellowbelly flounder, it is essential to study how the exogenous hormone affects their transcriptional regulation in the pituitary and its downstream influence on reproductive physiology. Very little research has been conducted on the effects of GnRHa administration in yellowbelly flounder, except for Ellis-Smith's (2022) study on the influence of GnRHa treatment on ovarian development. Understanding the expression of both gonadotropin genes following GnRHa administration is an additional step toward achieving control over captive reproduction. This knowledge can ultimately help inform a suitable hormonal induction protocol to overcome capture-related reproductive failure in wild-caught yellowbelly flounder, and thereby accelerate the production of a F1 generation of hatchery-reared fish.

This chapter aims to better understand the quantification of pituitary *lhβ* and *fshβ* expression over a five-day period following an injection with different concentrations of GnRHa.

3.2 Methods

All the fish were held at Sulphur Point (Coastal Marine Field Station) in Tauranga, within 1000 L of recirculating aquaculture systems. All qPCR assays and laboratory work were conducted at the University of Waikato Laboratories on Durham Street, Tauranga, New Zealand.

3.2.1 Fish collection and general husbandry

Adult yellowbelly flounder were caught using set nets in Kawhia Harbour (38° 03 '56.9"S 174° 51' 54.8"E), New Zealand. After capture, the fish were kept in an outdoor holding tank until relocation. They were transported by road in a large, insulated container using double-wrapped polythene bags containing clean seawater and pressurised with pure oxygen gas. Upon arrival at the Sulphur Point Coastal Marine Field Station in Tauranga, the fish were bathed in a 100 L aerated seawater container with 10% formalin (250 mg L^{-1}) for 30 minutes as a prophylaxis against microbial infection and ectopic parasites. Following this treatment, the fish were distributed evenly among three aerated recirculating tanks, with two tanks containing 13 fish each and one tank containing 14 fish. The fish were housed in three separate 1000 L recirculating aquaculture

systems. Water quality parameters (ammonia, nitrate & nitrite levels) were monitored daily, and fresh seawater exchanges were performed as needed. Water changes were conducted when ammonia, nitrate, or nitrite levels exceeded their recommended thresholds of 0.5 ppm, 1 ppm, and 40 ppm, respectively. Any faecal or biological matter was siphoned out to maintain water quality. The photoperiod was controlled using a downlight (DETA 9W Tri-colour Dimmable LED Downlight) set at 970 lm (4000K) and operated on a timer to approximate the natural ambient photoperiod.

3.2.2 Experimental set-up

The following day (day 0, experiment start), 40 yellowbelly flounders were individually placed into an aerated 10 L saltwater bath containing 2-phenoxyethanol (Sigma-Aldrich) (0.6 ml L^{-1}) for heavy sedation. Sedation was identified by a slowing of gill ventilation, no response to touch, and an inability to right themselves after being turned upside down. Once sedated, the fish were sexed by backlighting the gonads, a reliable method previously used to determine the sex of yellowbelly flounder (Koverman, 2018; Ellis-Smith, 2022). Male testes have a short triangular shape, whereas female gonads are elongated triangles (**Figure 3.1**). A photograph of the gonads was taken, and the fish was weighed (g), measured (cm), tagged, and had an ovarian biopsy performed using a catheter (external diameter 20 mm) inserted into the genital pore and up into the upper ovarian lobe. Each fish then received a single intraperitoneal injection of one of three GnRHa treatments (Sigma Aldrich) at doses of 25 µg/kg, 50 µg/kg, or 100 µg/kg, or a Ringer's solution treatment (25 µg/kg). The control treatment was a sham injection, consisting of Ringer's solution used for marine teleost species. Medical-grade glue was applied to seal the needle puncture, preventing any leakage of GnRHa or the Ringer's solution. After the procedure, the fish were transferred to an aerated holding tank. Once they had recovered, with normal gill ventilation rate and equilibrium, each fish was randomly assigned to one of the four treatment groups. Each GnRHa treatment group consisted of 10 female fish distributed across three tanks (3-4 individuals per tank). Two tanks had 13 fish, and one had 14. This ensured that the total biomass was similar throughout all tanks. The fish were biopsied on days 1 and 5, during which tissue was extracted. On day 1, 16 flounder were biopsied, and on day 5, 20 flounder were biopsied. Due to time constraints, the fish were not biopsied on day 0.

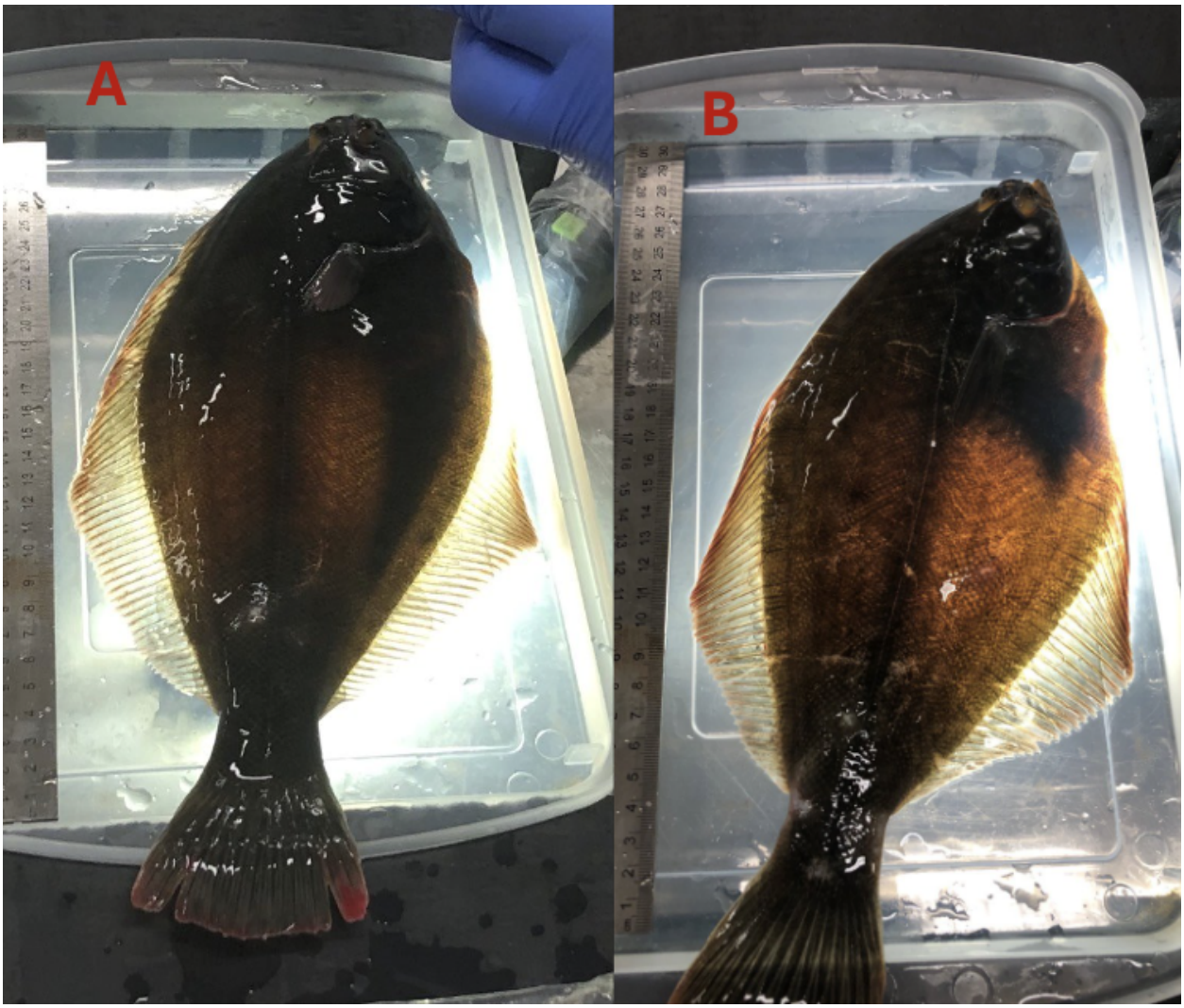

Figure 3.1. Illuminated view of the female (A) and male (B) gonads shape with backlighting from a torch.

3.2.3 Tissue extraction

The fish were placed one by one into a 10 L aerated saltwater bath of 2-phenoxyethanol (0.6 ml L^{-1}) and heavily sedated. The fish were then euthanised by rapid decapitation using a sharp knife. The brain, hypothalamus, pituitary, and a section of gonadal tissues were dissected from each fish. Each tissue sample was placed into separate 1.5 mL centrifuge tubes with RNAlater® (Thermo Fisher, USA). The tissues were stored in the refrigerator at 4°C until transported on dry ice (-80°C) to the -80°C freezer, ready for RNA extraction and qPCR analysis.

3.2.4 Oocyte staging

The oocytes used in this analysis were staged based on fresh and histologically fixed oocyte data from Ellis-Smith (2022), using oocyte size and key features such as opacity/translucency, presence

of yolk granules, occurrence/size of lipid droplets, as well as the position of the germinal vesicle. The ovarian developmental stages were determined according to these described characteristics of the leading oocyte cohort. This allowed ovarian development to be classified into the following categories: previtellogenic (PVO), vitellogenic (Vit), early maturation (EM), and germinal vesicle migration (GVM) (**Figure 3.4**). The leading cohort of oocytes represented the most developmentally advanced group present in the ovaries.

3.2.5 Oocyte measurement

After each ovarian biopsy, a sample of each female's ovaries was dispersed in a Petri dish, and Serra's clearing solution was added. Oocytes were assessed using an Olympus SZ61 stereo microscope, and images of multiple oocytes were captured from each sample with the aim of identifying 50 oocytes per sample. Each sample was paired with an image of a 2.0 mm micrometre slide at the same magnification. Oocyte diameters were measured using ImageJ software. The image of the micrometre slide was used to calibrate the measurements on ImageJ, where two perpendicular measurements of each oocyte were taken. The average of these two measurements determined the diameter of each oocyte. The majority of fish had between 32 and 68 individual oocytes, except for three individuals that had fewer than 20. All oocytes were measured to ensure a representative sample of oocyte diameters was established.

3.2.6 RNA analysis

All pituitaries used for the analysis in this chapter underwent identical procedures for preparation, extraction, synthesis, and qPCR as outlined in Chapter 2. For more detailed information, refer to Chapter 2, Methods 2.2.2: RNA extraction and quality to 2.2.5: Quantitative PCR (qPCR).

3.2.7 Statistical analysis

Gonadotropin beta expression (*lhβ* & *fshβ*) was compared to the controls following GnRHa treatment using two-sample t-tests. Statistical significance was set at $p < 0.05$ for all analyses.

3.3 Results

3.3.1 Fish survival and condition

All female yellowbelly flounders exhibited macroscopic signs of active oogenesis. This conclusion was based on the visible outline of the upper ovary, which was at least slightly raised above the abdominal cavity (**Figure 3.2**).

Each fish was weighed on day 0 before being randomly assigned to a treatment group. There were no significant weight differences between groups (ANOVA, $p = 0.56$). The largest individual weighed 580 grams, while the smallest weighed 245 grams. The highest mean weight was found in the 100 µg/kg GnRHa treatment group (400.5 g), and the lowest mean weight was observed in the 50 µg/kg GnRHa treatment group (317.5 g) (**Table 3.1**).

Four flounders died during the experiment. One pituitary sample (sample 35, control) was excluded from analysis due to insufficient RNA quality, leaving 35 pituitary samples for analysis.

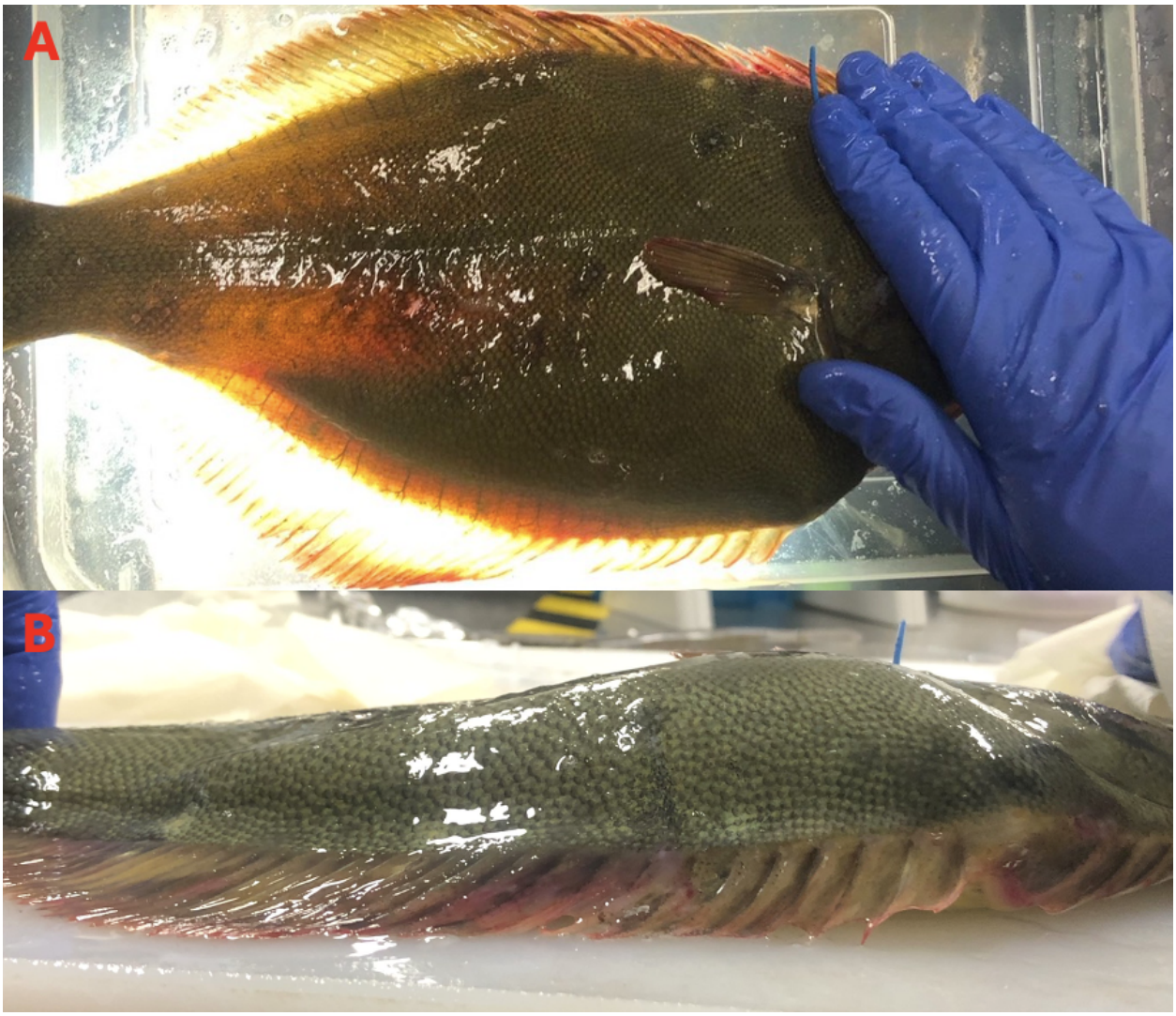

Figure 3.2. The dorsal (A) and lateral (B) views of the female oocyte shape with backlighting from a torch.

Table 3.1. Average body weight of female yellowbelly flounder on day 0, prior to treatment.

Treatment	Number of female flounder	Body weight (g)
Control	10	389.5 ± 63.1
25 µg/kg of GnRHa	10	322 ± 54.8
50 µg/kg of GnRHa	10	317.5 ± 73.3
100 µg/kg of GnRHa	10	400.5 ± 83.7

3.3.2 Oocyte staging against treatment

Of the 35 female yellowbelly flounder, 15 (42.86%) reached oocyte maturity, defined as final oocyte maturation (FOM) or beyond. Among the 15 individuals that reached oocyte maturity, two were controls, five were from the 25 µg/kg treatment group, three were from the 50 µg/kg

treatment group, and five were from the 100 µg/kg treatment group (**Figure 3.3**). Additionally, 14 flounders reached early maturation (C, **Figure 3.4**). This indicates that 80.6% of female flounder reached either maturity or early maturation five days after GnRHa administration.

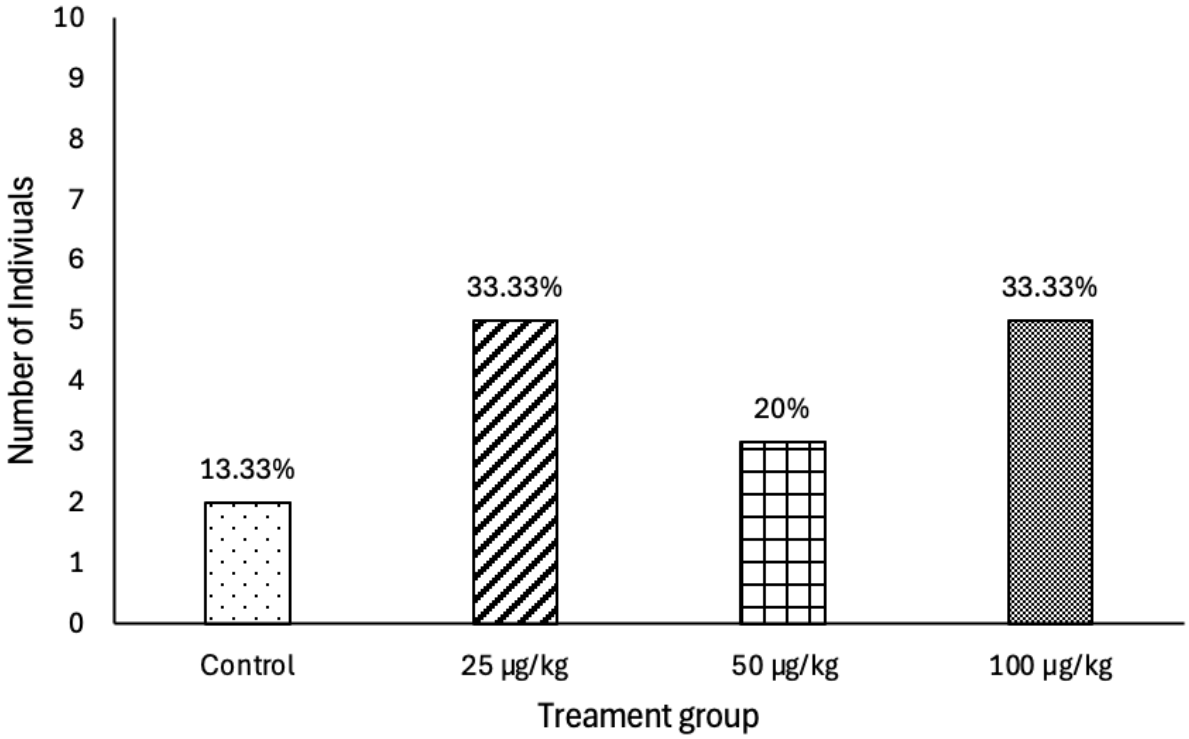

Figure 3.3. Number of female yellowbelly flounder (n = 15) reaching oocyte maturity in each treatment group. Percentages are shown above each bar.

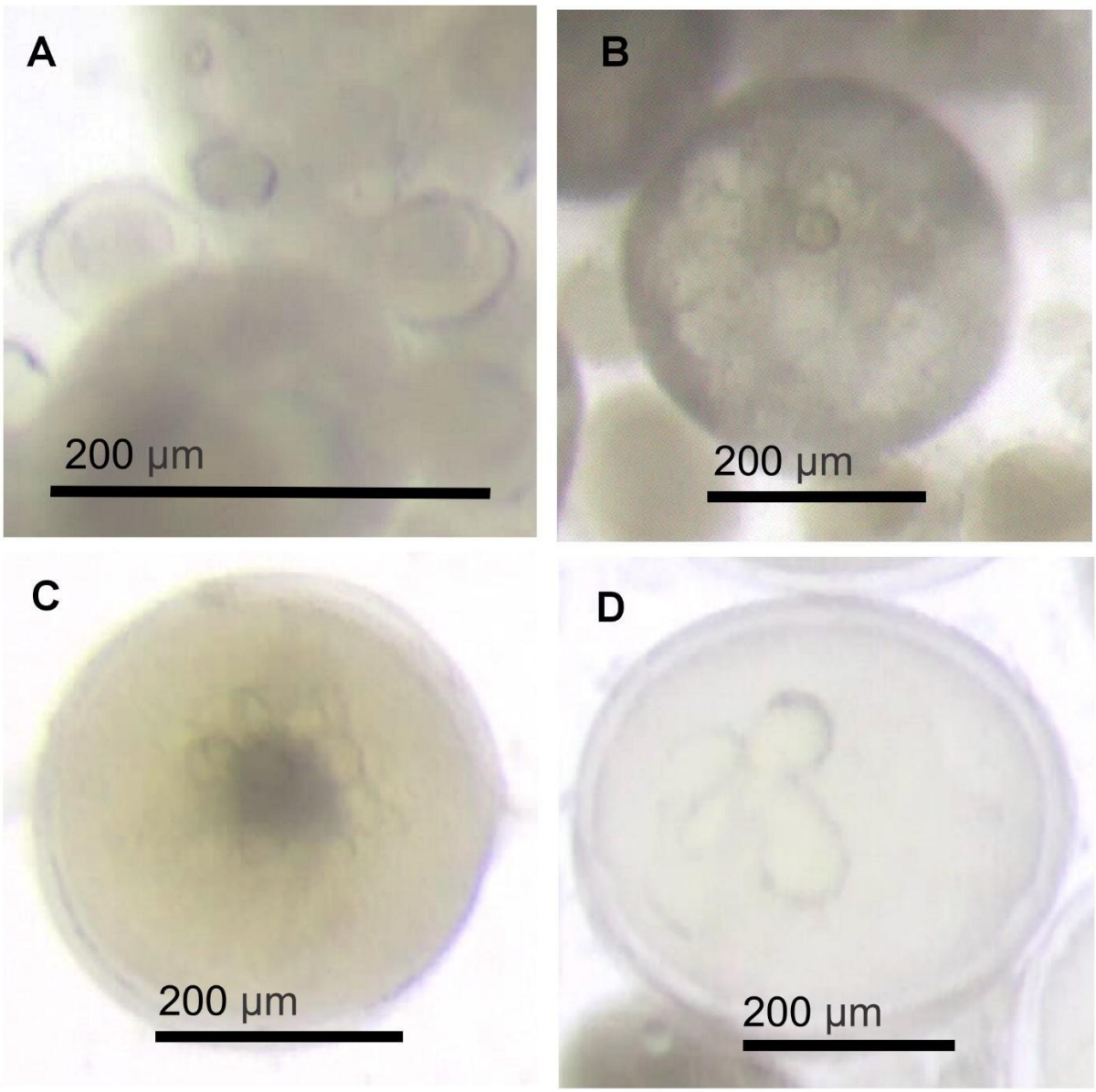

Figure 3.4. Developmental stages of fresh oocytes from adult yellowbelly flounder. (A) previtellogenic (PVO), (B) vitellogenic (Vit), (C) early maturation (EM), and (D) germinal vesicle migration (GVM).

3.3.3 Follicle-stimulating hormone beta (*fshβ*) expression

There were no significant differences in follicle-stimulating hormone beta (*fshβ*) expression on day 1 or day 5 in any of the treatments compared to the controls. Although no significant results were observed, a trend of increased expression was observed in the 25 µg/kg treatment, with a 0.5-fold increase relative to the control on day 5. When comparing *fshβ* expression between day 1 and day 5 within the 25 µg/kg treatment, there was a 0.75-fold increase (**Figure 3.5**). Interestingly, *fshβ* expression levels in the 50 µg/kg treatment were the only treatment higher than the control,

showing a 0.45-fold greater expression on day 1 (**Figure 3.5)**. However, by day 5, *fshβ* expression had decreased to 1.125, returning to levels similar to the control (1). Notably, a trend of decreased pituitary *fshβ* expression was observed in the 100 µg/kg treatment compared to the controls on both day 1 (0.75-fold) and day 5 (0.45-fold).

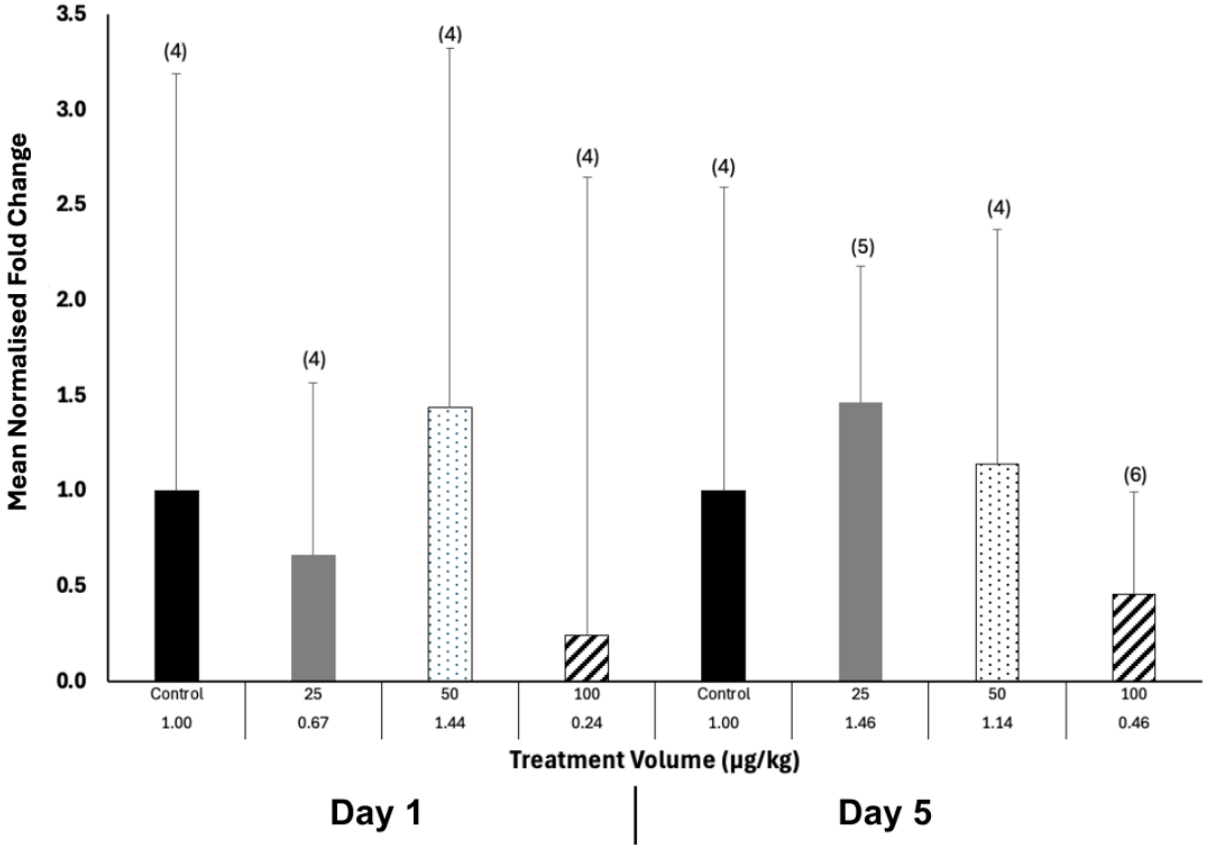

Figure 3.5. Mean normalised fold change of follicle-stimulating hormone (*fshβ*) for each GnRHa treatment concentration (Control, 25 µg/kg, 50 µg/kg, 100 µg/kg) and days post-injection (D1; Day 1, D5; Day 5). Error bars indicate standard error. The sample size (n) for each treatment is shown in parentheses above each bar.

3.3.4 Luteinising hormone beta (*lhβ*) expression

There were no significant differences in luteinising hormone beta (*lhβ*) expression on day 1 or day 5 in any of the treatments when compared to the controls. Although no significant results were observed, a trend of increasing expression was noted in the 25 µg/kg (0.65-fold) and 50 µg/kg (0.5-fold) treatments compared to the control on day 5 (**Figure 3.6**). The expression of *lhβ* from day 1 to day 5 showed an increasing trend in the 25 µg/kg (0.9-fold) and 50 µg/kg (0.5-fold) treatments (**Figure 3.6**). On day 1 post-injection, *lhβ* expression in the 25 µg/kg and 50 µg/kg treatments was similar to that in the controls. Interestingly, a trend of decreased pituitary *lhβ*

expression was observed in the 100 µg/kg treatment group compared to the control on both day 1 (0.85-fold) and day 5 (0.5-fold). However, *lhβ* expression in the 100 µg/kg treatment group increased on day 5 compared to day 1, although it remained desensitised relative to the control.

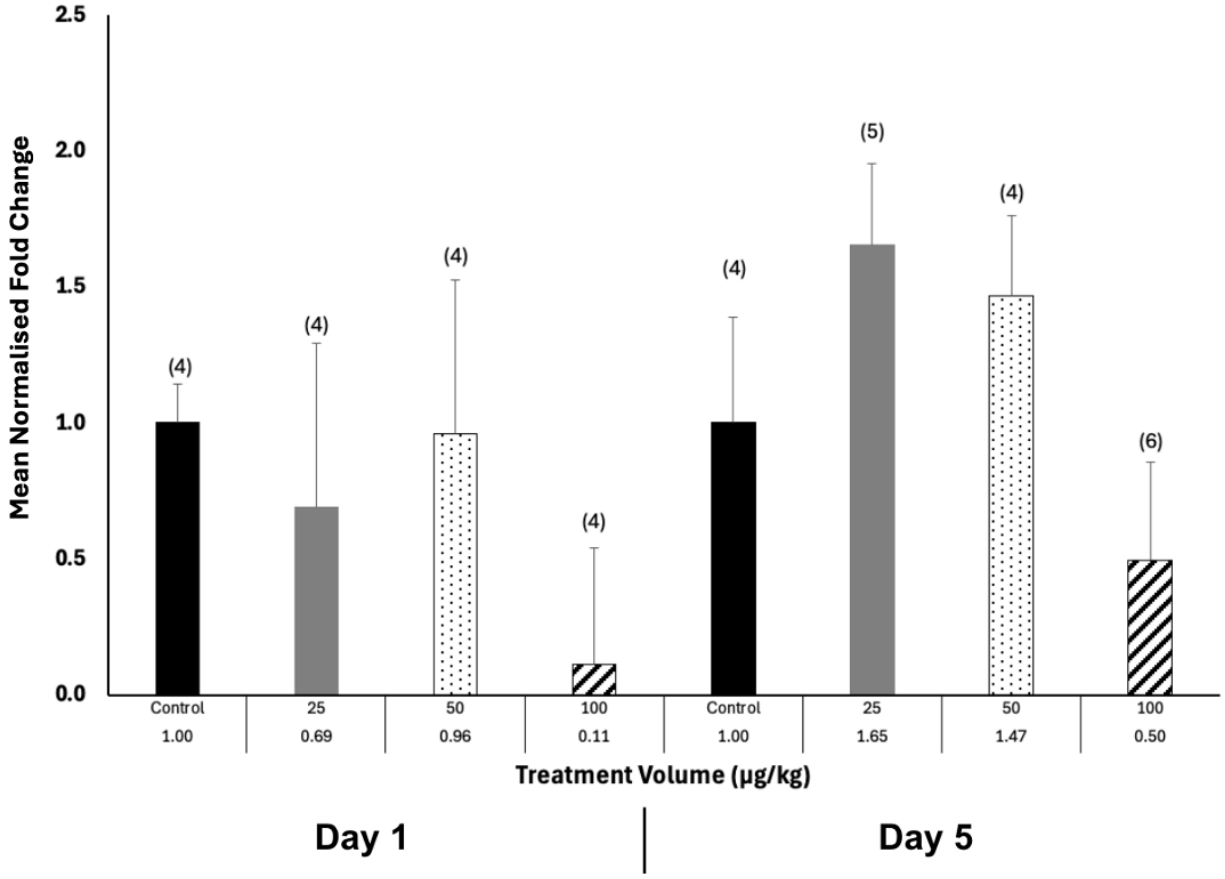

Figure 3.6. Mean normalised fold change of luteinising hormone (*lhβ*) for each GnRHa treatment concentration (Control, 25 µg/kg, 50 µg/kg, 100 µg/kg) and days post-injection (D1; Day 1, D5; Day 5) is presented. Error bars indicate standard error. The sample size (n) for each treatment is shown in parentheses above each bar.

3.4 Discussion

3.4.1 Introduction

This study aimed to examine how GnRHa influences pituitary gonadotropin beta expression (*fshβ* and *lhβ*) and oocyte maturation in female yellowbelly flounder (*Rhombosolea leporina*). With the view to informing optimal protocols for inducing final oocyte maturation (FOM) and ovulation in this potential aquaculture species. Currently, a substantial amount of research has been conducted on GnRHa administration in multiple flatfish species globally; however, very little research has been conducted on yellowbelly flounder with a view to their aquaculture potential.

3.4.2 Pituitary luteinising hormone beta (*lhβ*) expression in response to GnRHa treatment

Treatment with GnRHa was associated with the highest number of fish entering final oocyte maturation, alongside elevated pituitary gonadotropin beta expression levels compared to controls, although these trends were not statistically significant. Despite individual variability and the lack of statistical significance, *lhβ* expression levels in both the 25 µg/kg and 50 µg/kg GnRHa treatment groups exhibited an upward trend between day 1 and day 5. These two treatment groups together accounted for 53.3% of the mature oocyte cohorts. Considering GnRHa treatments alone, GnRHa was shown to promote significant oocyte growth, with 48% of oocytes reaching maturity and 85% reaching the early migratory (EM) stage. Similarly, various exogenous hormonal treatments in greenback flounder (*Rhombosolea tapirina*) led to substantial oocyte development, advancing vitellogenic oocytes through to the hydration stage (Poortenaar & Pankhurst, 2000). The observed rise in *lhβ*, and consequently the production of E2, T, and 17α,20β-DP, is critical for driving oocyte growth and plays a key role in enabling oocytes to reach full maturity. The observed trend of increasing gonadotropin expression aligns with findings from other studies, which show that the rise in both total gonadotropin and gonadotropin beta expression following GnRHa administration can be rapid and pronounced, depending on the species. For instance, female gilthead seabream (*Sparus aurata*), which exhibit asynchronous ovarian development, showed increased *lhβ* expression levels between 12 and 8 hours before spawning, with levels remaining high up to 8 hours before and persisting until 4 hours after spawning (Gothilf et al., 1997). Similarly, Mateos et al. (2002) found that in Mediterranean sea bass (*Dicentrarchus labrax*), administration of GnRHa significantly elevated LH expression within 3 hours after injection, and *lhβ* expression increased twofold after GnRHa injection compared to sham-injected fish. Furthermore, Mylonas et al. (1997e) demonstrated that LH expression rose soon after GnRHa injection and remained elevated until GnRHa was cleared from the system within striped bass (*Morone saxatilis*). Although *lhβ* expression data in the current study are limited to day 5, due to all fish being euthanised at that time point, the observed upward trend suggests that *lhβ* expression may have continued to rise beyond day 5, potentially reaching a higher, yet undetermined, peak, as some oocyte cohorts had not yet reached FOM. However, it cannot be ruled out that peak *lhβ* expression may have already occurred during FOM between day 1 and day 5. Fish were also visually assessed daily for any obvious abdominal swelling throughout this period. Studies in

teleost species have shown that peak *lhβ* expression can occur between 6 and 24 hours following peak GnRH stimulation within the HPG axis (Mylonas & Zohar, 2000; Chi et al., 2015; Ma et al., 2020). Despite the variability, these findings support the idea that exogenous GnRHa promotes oocyte maturation through the upregulation of gonadotropins in yellowbelly flounder.

Yellowbelly flounder treated with 100 µg/kg of GnRHa indicated an unexpected and possible novel downregulation of pituitary gonadotropin beta expression. Specifically, levels of *lhβ* and *fshβ* expression decreased by an average of 0.9-fold and 0.7-fold, respectively, compared to the control group one day post-injection. Interestingly, this apparent downregulation of gonadotropin expression did not appear to inhibit oocyte maturation, with 90% of individuals in this treatment reaching FOM. Nonetheless, this result suggests that higher concentrations (e.g. 100 µg/kg in yellowbelly flounder), GnRHa may exert an inhibitory effect on gonadotropin expression in some species. Similar results have been documented in goldfish (*Carassius auratus*) (Habibi, 1991) and Mediterranean sea bass (*Dicentrarchus labrax*) (Mateos et al., 2002), where the administration of high GnRHa doses led to desensitisation of gonadotropins and suppression of pituitary activity. This highlights the importance of optimising GnRHa dosage for each species to ensure treatment efficacy (Barnett & Pankhurst, 1999). For instance, the recommended dosage for North Sea plaice (*Pleuronectes platessa*) is 50 µg/kg (Scott et al., 1999), whereas for turbot (*Scophthalmus maximus*), the dosage is 25 µg/kg (Mugnier et al., 2000). In addition to dosage, the delivery method for administering GnRH is also crucial for achieving the optimal effect in a study. Various delivery methods are available, including intramuscular injection, implants, microspheres, and monolithic implants (Zohar & Mylonas, 2001). Each method is chosen based on the study's objectives. For instance, while injections provide an immediate but short-lived release of GnRHa, sustained-release implants can extend hormone delivery for up to eight weeks (Zohar & Mylonas, 2001). Studies have shown that plasma GnRHa levels vary in duration across species and administration methods. In winter flounder (*Pseudopleuronectes americanus*), levels peaked at 4 hours and declined by 24 hours post-injection (Harmin & Crim, 1993), while in Senegalese sole, GnRHa remained detectable up to 3 days post-injection and up to 21 days with implants (Guzmán et al., 2009). In Atlantic halibut (*Hippoglossus hippoglossus*), implant administration led to peak levels by day 6, which remained elevated for 30 days (Vermeirssen et al., 2000). It is crucial to determine

the optimal dosage and method of hormone administration based on the specific research objective and species.

At five days post-injection, the 100 µg/kg treatment group had the lowest *lhβ* and *fshβ* expression levels. While these values were not statistically different to those of the other treatments, the observable trend reflects observations in Ellis-Smith's (2022) study. Her work found that yellowbelly flounder treated with 100 µg/kg GnRHa resulted in delayed ovulation latency compared to 50 µg/kg (28 days post-injection compared to 3-5 days, respectively). The results from both the present study and that of Ellis-Smith (2022) suggest that the 100 µg/kg dose would not be optimal for inducing FOM in this species within a short time frame. This could relate to the possible desensitisation of the gonadotrope cells in response to GnRHa overstimulation. It should be noted, however, that this is a correlative observation, and other explanations cannot be ruled out. For example, it is possible that elevated GnRHa dosage may inhibit pituitary release of the native LH hormone. Regardless, based on the current results and those of Ellis-Smith (2022), lower GnRHa doses in the range of 25 µg/kg to 50 µg/kg may be more physiologically suitable for inducing reproduction in this species.

3.4.3 Pituitary follicle-stimulating hormone beta (*fshβ*) expression in response to GnRHa

GnRHa treatment was associated with pituitary *fshβ* expression being highly variable throughout the treatment groups, with only fish being treated with 25 µg/kg showing an observable, but not significant increase. The administration of GnRHa primarily stimulates the production and release of LH from the pituitary gland in teleost species. Although it also induces FSH production, the effects of GnRHa are typically more pronounced on LH secretion, reflecting the hormone's stronger influence on LH synthesis and release (Mylonas et al., 2010). Studies on the endocrine system indicate that high concentrations of GnRH within the system predominantly favour *lhβ* expression (Stamatiades & Kaiser, 2018), whereas lower concentrations tend to favour *fshβ* expression (Haisenleder et al., 1991). Nonetheless, the production of FSH is regulated by hypothalamic GnRH. In salmonids, both FSH and LH are present at different stages throughout oogenesis. FSH is active during vitellogenesis, stimulating E2 production before declining at the end of vitellogenesis, and LH levels begin to increase (Swanson, 1991). Multiple studies on gilthead seabream (*Sparus aurata*) have shown that both FSH and LH were found within the

pituitary during the spawning season, and that both may act concurrently in processes like E2 production (Gothilf et al., 1997; Elizur et al., 1996). Yellowbelly flounder, like gilthead seabream, are batch spawners, releasing eggs over successive periods throughout the reproductive season. It is plausible that the variable pituitary *fshβ* and *lhβ* expression observed in all treatment groups following GnRHa treatment reflects that concurrent gonadotropin action is required to coordinate oocyte growth in this species. This presumably reflects the complexity of producing multiple cohorts of oocytes at different developmental stages, as seen in fish with multiple-batch group synchronous ovarian development. However, it should be noted that variability within the data could at least in part relate to limited sample size, where a single high value from one fish can skew results.

Each teleost species exhibits a different relationship between gonadotropin levels and GnRHa administration. It must also be considered that, even when changes in gonadotropin expression are observed, these results cannot be directly interpreted as reflecting actual hormone concentrations in the system. There is limited data on *fshβ* expression and plasma FSH concentrations in response to GnRHa administration. Typically, these studies indicate that GnRHa elicits a lower response in both *fshβ* expression and plasma FSH compared to *lhβ* expression and plasma LH. This has been observed in teleosts such as Mediterranean Sea bass (*Dicentrarchus labrax*) (Mateos et al., 2002), where LH expression significantly increased after GnRHa administration, but FSH showed no response to the exogenous GnRHa treatment. Similarly, in female greater amberjack (*Seriola dumerili*), FSH levels remained unchanged during the experiment, while LH significantly rose (Nyuji et al., 2019). Likewise, in red seabream (Okuzawa et al., 2016), no significant change in FSH expression was observed, whereas LH levels increased in both the pituitary and plasma. GnRH/a treatment elicits a similar response across species, though species-specific differences exist. In tilapia (*Oreochromis*), plasma FSH levels increased significantly following exogenous treatment, exceeding control levels. FSH expression closely mirrored LH expression, differing only marginally (Aizen et al., 2007). This variation highlights the complexity of reproductive regulation. In contrast, zebrafish (*Danio rerio*) primarily secrete LH in response to GnRH, while FSH activation occurs via a separate pathway involving cholecystokinin (CCK) rather than GnRH (Cohen et al., 2024). Although these studies highlight that GnRHa treatment tends to reliably stimulate plasma LH concentrations across teleost species, plasma FSH

concentrations are far more variable, illustrating the need to better understand species-specific gonadotropin expression during GnRHa administration and reproductive induction protocols.

3.4.4 Environmental influences on yellowbelly flounder reproduction

Temperature plays a crucial role throughout the spawning process, acting as an environmental cue that regulates reproduction. Before spawning, it stimulates hormone production, driving oocyte maturation (Watanabe et al., 1998). During spawning, optimal temperatures enhance fertilisation and synchronise spawning events for larval survival. After spawning, temperature influences embryo development, hatching success, and larval sex determination (Munro et al., 1990; Goto et al., 1999). Ovulated wild yellowbelly flounder can be found as early as June, and typically peak spawning September through to December (Colman, 1973), when average water temperatures (14°C) are at their coolest. In the current study, unseasonal temperature spikes in the experimental facility exposed the flounder to 19–20°C throughout the September study, about 4–5°C higher than the average coastal sea temperature of 14.5°C. Anomalous water temperatures can hinder ovulation in captive teleosts. Studies have shown elevated temperatures reduce gonad size in species like Atlantic salmon (*Salmo salar*), red seabream, and blue gourami (*Trichopodus trichopterus*) (Pankhurst et al., 2011; Okuzawa & Gen, 2013; David & Degani, 2011). Higher temperatures have been associated with temperature shock and stress, which can lead to atresia within the gonads (Corriero et al., 2021). A study on captive grey mullet (*Mugil cephalus L.*) demonstrated that elevated temperatures from 21°C to 26°C were linked to increased rates of oocyte atresia (Kuo et al., 1974). Similarly, a study on captive white sturgeon (*Acipenser transmontanus*) exposed to temperatures ≥ 18°C, compared to the typical seasonal temperature range of 10–16°C, observed ovarian atresia following the arrest of GVM (Linares-Casenave, 2001). Yellowbelly flounder naturally spawn during cooler water temperatures. The increase in temperature (4–5°C) within the experimental tanks is likely to have elevated stress and could have impacted the results. This may explain the 22% of ovarian samples showing evidence of atresia and the lack of ovulation observed during the study.

Despite several fish undergoing FOM, there was no evidence of ovulation. Previous work has indicated that female yellowbelly flounder are particularly prone to capture and disturbance-related stress, showing slow recovery periods which result in a failure to complete FOM and spawn (Ellis-

Smith 2022). Typically, after GnRHa administration, gonadotropin levels increase as GnRHa binds to receptors on the gonadotrope cells in the pituitary, to stimulate their release into the bloodstream (Mylonas & Zohar, 2009). This surge in gonadotropins promotes oocyte maturation and ovulation, ultimately leading to spawning. However, achieving successful spawning in captivity remains one of the primary challenges in aquaculture. Spawning success in captive broodstock is generally higher in subsequent generations, as they are better adapted to the controlled environment and artificial conditions (Pankhurst & Fitzgibbon, 2006). Wild-caught greenback flounder have shown significant increases in cortisol levels following capture, confinement, and transport. In contrast, cultured fish exposed to routine husbandry conditions exhibited low cortisol levels. However, fish held at medium to high stocking densities and subjected to five minutes of simulated grading displayed significantly elevated cortisol levels for up to 48 hours (Barnett & Pankhurst, 1998). This highlights the relevance to the current study, as it underscores the importance of managing stress, particularly in a short-duration study where elevated stress levels can influence the outcome.

Yellowbelly flounder are known to migrate to depths of 12–30 m for successful courtship and spawning (Colman, 1973), which may contribute to difficulties in replicating optimal conditions in captivity. Courtship and spawning behaviours have been observed in captive greenback flounder and southern flounder (*Paralichthys lethostigma*), where the male aligns his genital pore with the female's while swimming alongside her. The male then guides the female to the surface, where both release sperm and eggs simultaneously (Pankhurst & Fitzgibbon, 2006; Smith et al., 1999). Similarly, species such as the mangrove red snapper (*Lutjanus argentimaculatus*) (Emata, 2003) and gilthead seabream (Morretti, 1999) require a 2:1 sex ratio (two males to one female) to initiate successful spawning, even with luteinising hormone-releasing hormone analogue (LHRHa) administration. Managing the tank environment to encourage natural courtship and spawning is a challenging task. Future research should focus on replicating a more natural spawning environment in tank conditions, as GnRHa administration alone may not be sufficient to induce spawning in all species, including yellowbelly flounder.

The latency, or time to ovulation and ultimately spawning following GnRHa administration, varies among flatfish species but generally occurs within a week post-injection. For instance, a similar

experiment conducted on female yellowbelly flounder by Ellis-Smith (2022) found that fish injected with 50 µg/kg of GnRHa ovulated within 3 to 5 days post-injection. Comparing these results to other species within the Rhombosolea genus, greenback flounder administered LHRHa (50 µg/kg) ovulated between 1 to 7 days after administration (Poortenaar & Pankhurst, 2000; Poortenaar, 1998). Similarly, flatfish species within the Pleuronectiformes order, such as Senegalese sole, spawned 3 to 4 days post-injection when administered GnRHa (1 or 5 µg/kg) (Agulleiro et al., 2006). Similarly, yellowtail flounder treated with either a GnRHa pellet (100 µg/kg) or microspheres (75 µg/kg) spawned 4 to 15 days post-injection (Larsson et al., 1997). These findings suggest that the response to GnRHa treatment is broadly consistent across flatfish species, inducing spawning within a week post-injection. However, slight variations exist between species, dosage, and method of administration.

Chapter 4 - General discussion

4.1 Introduction

The primary objectives of this study were to better understand the characterisation of pituitary gonadotropin expression through their beta subunit in relation to oocyte development, as well as the effect of GnRHa on this system in wild-caught yellowbelly flounder (*Rhombosolea leporina*). While research has been conducted on gonadotropin expression in aquaculture within the Pleuronectiformes order, little attention has been given to yellowbelly flounder, particularly regarding the reproductive stages and the effects of GnRHa administration. An aquaculture industry for yellowbelly flounder would heavily depend on wild broodstock initially, and future selective breeding programs would also be required to add fresh genes from the wild gene pool. For a truly successful and sustainable industry, the development of subsequent generations beyond wild-caught individuals is necessary to establish a successful industry in New Zealand.

4.1.1 Gonadotropin expression in multiple-batch group synchronous ovarian development

Both *lhβ* and *fshβ* expression in female yellowbelly flounder followed an expected increasing trend as oocytes matured through oogenesis, aligning with patterns reported in the literature. However, an unexpected observation was that peak *fshβ* expression occurred in the maturational stage of fish, and relative expression levels exceeded those of *lhβ*. Classical salmonid models suggest that GnRH secretion from the pituitary stimulates FSH expression during the early stages of oogenesis (previtellogenesis and vitellogenesis), while LH dominates during final maturation and spawning. This is also reflected in pituitary gonadotropin expression data (Taranger et al. 2015). However, studies in fish with multiple-batch group synchronous ovarian development suggest that co-expression of pituitary *fshβ* and *lhβ* may be necessary to support the spawning of multiple egg batches (Gothilf et al. 1997; Kajimura et al. 2001). Yellowbelly flounder also exhibit multiple-batch group synchronous ovarian development, suggesting that the pituitary gonadotropin expression patterns observed in this study largely reflect that of other similar species, including flatfish (Shi et al., 2015; Pham et al., 2008; Kajimura et al., 2001). Further research is required to fully characterise the gonadotropic system within this species, to better understand how it regulates ovarian development and spawning.

4.1.2 Optimising GnRHa treatments in yellowbelly flounder

Following GnRHa administration, expression of both *fshβ* and *lhβ* increased as expected in both the 25 µg/kg and 50 µg/kg treatment groups between days 1 and 5. However, fish treated with 100 µg/kg of GnRHa showed much lower expression of both *lhβ* and *fshβ* from days 1 to 5 compared with the controls. This may reflect a possible desensitisation of the pituitary gonadotrope cells; a sudden high dose or continuous exposure to GnRHa can lead to desensitisation of the GnRH receptors (Neill, 2002). This phenomenon has also been documented in the pituitaries of both goldfish (*Carassius auratus*) and Mediterranean sea bass (*Dicentrarchus labrax*) (Habibi, 1991; Mateos et al., 2002). Previous work by Ellis-Smith (2022) on yellowbelly flounder using a sustained-release delivery vehicle concluded that multiple fish treated with 50 µg/kg of GnRHa ovulated within 3-5 days, whereas there was a delayed response in those treated with 100 µg/kg, which did not ovulate until 28 days post-injection. Moreover, in that study, only 22% of the 100 µg/kg treated fish had ovaries with hydrated oocytes compared to 55% of the fish in the 50 µg/kg treatment group. This further supports the hypothesis that there may be a threshold above which GnRHa concentrations impact oocyte maturation in this species. Further exploration of the underlying mechanisms of this apparent effect is warranted.

Optimising treatment, such as GnRHa dosage and delivery method, is a critical requirement in any induced spawning protocol. In this study, results showed that both 25 µg/kg and 50 µg/kg GnRHa treatments had a trend of increased *lhβ* and *fshβ* expression above control levels, whereas 100 µg/kg of GnRHa showed a trend for decreased pituitary gonadotropin expression below that of the control. When taken into consideration with the results of Ellis-Smith (2022), this study hints at lower GnRHa doses being more suitable for inducing FOM in yellowbelly flounder. Similar studies in meagre (*Argyrosomus regius*) and spotted rose snapper (*Lutjanus guttatus*), show that too low concentrations of GnRHa result in reduced or absent spawning, while excessive volumes lead to desensitisation, diminished spawning, and reduced egg and larval quality (Fernandez-Palacios et al., 2014; Ibarra-Castro & Duncan, 2007). Moreover, the type of GnRHa treatment influences hormonal expression dynamics. This study utilised a single injection to induce maturation over a 5-day period, during which all treatment groups responded to the GnRHa. Similar research in Senegalese sole (*Solea senegalensis*) showed that GnRHa was no longer detectable in the plasma at days 3, 7, and 14, depending on whether it was administered via single

injection, microspheres, or implant (Guzmán et al., 2009). Likewise, in female European sea bass (*Dicentrarchus labrax*), microspheres induced up to four separate spawning events during a 21-day experimental period, while a single injection induced only one (Forniés et al., 2001). These findings demonstrate that both delivery method and dosage have a substantial impact on study outcomes, highlighting the need for further optimisation to develop a robust protocol for yellowbelly flounder.

4.1.3 Experimental limitations and biological variability

Considerable variation in pituitary gonadotropin expression levels was evident between treatments. This was likely an artefact of the small sample sizes used per treatment group (4–6 fish). Here, individual variability can disproportionately magnify results. Consequently, no statistically significant differences were observed between day 1 and day 5 treatments. Increased sampling to include days 2, 3 and 4 may have helped strengthen observable trends however, this would require an additional 48 fish. While increased sample sizes could help reduce variability, ethical, logistical, and financial constraints limit the number of animals that can be used. Variation is to be expected when working with animals and especially when measuring dynamic processes such as gene expression across individual scales. The use of wild-caught females without adequate acclimation, unlike in similar studies (Guzmán et al., 2009; Pankhurst & Fitzgibbon, 2006; Lim, 2016), as well as handling stress, may have contributed to variable results and limited ovulation. When fish are stressed, cortisol is produced via the hypothalamic–hypophyseal–interrenal (HHI) axis, which can cross-talk with the reproductive axis, leading to issues with reproductive performance (Murugananthkumar & Sudhakumari, 2022). Ellis-Smith (2022) reported that wild-caught yellowbelly flounder were slow to recover from capture and disturbance-related stress, causing a likely failure of FOM, ovulation, and spawning despite GnRHa treatment. Similar results were observed in this study.

4.2 Future recommendations

This study served as an initial investigation to characterise pituitary gonadotropin expression. It should be recognised that measuring gonadal receptor expression to assess the overall functionality of the yellowbelly flounder gonadotrophic system was beyond the scope of this study and may be addressed in ongoing and future research. Nevertheless, it is strongly recommended that future

work include sex steroid measurements to gather a more comprehensive characterisation of the HPG axis. For example, Lim (2016) utilised plasma levels of E2, T, and 17α,20β-DP to determine whether GnRHa or hCG was more effective in inducing ovulation in starry flounder (*Platichthys stellatus*). That study concluded that GnRHa administration exhibited higher steroid levels and more effective sustained ovulations compared to those treated with hCG. Similarly, Poortenaar and Pankhurst (2000) investigated changes in E2, T, and 17α,20β-DP levels in relation to oocyte diameter and stage progression, comparing the effects of different administration methods (injection versus pellet), hormone volumes, and hormone types (LHRHa versus hCG) in greenback flounder (*Rhombosolea tapirina*). Their results showed that both the LHRHa (50 µg/kg) injection and the LHRHa pellet induced greater increases in oocyte diameter, accompanied by higher plasma and ovarian levels of E2, T, and 17α,20β-DP, compared to the higher-dose LHRHa (100 µg/kg) injection. These studies highlight the importance of integrating plasma hormone analyses to enable a broader understanding of the endocrine function across the entire HPG axis.

The results of pituitary gonadotropin expression in yellowbelly flounder differ to that of classical salmonid models, but reflect that of other multiple-batch group synchronous spawning fish. In the current study, pituitary *fshβ* expression was at peak levels greater than that of *lhβ* during the maturational stages of oogenesis. Understanding the association between pituitary gonadotropin expression and actual plasma gonadotropin levels in relation to gonadal development would be highly beneficial. While this would require the development of a suitable assay to measure plasma gonadotropins, it would contribute to the current understanding of the gonadotropic system function in fish with multiple-batch group synchronous ovarian development.

It would also be recommended to use greater fish numbers per treatment in future studies to reduce variability in gene expression data and improve statistical power. Given that the flounder in this study showed a trend of greater gonadotropin expression at a lower GnRHa dosages (25 µg/kg and 50 µg/kg), then a future study with limited fish should focus on these two GnRHa concentrations using greater numbers of fish per treatment and incorporating an additional time point, to include day 0, day 3 and day 5. However, future work would ideally use hatchery-raised sibling fish that have a common environmental history to help reduce individual influences and elucidate the true effects of GnRHa treatments.

Further investigation into the mechanisms underlying an apparent downregulation of gonadotropin expression following treatment with 100 µg/kg of GnRHa could yield interesting information on the pituitary effects of GnRHa dosage. This would likely involve *in vitro* studies of possible GnRH receptor desensitisation and downstream limitation of gonadotropin expression. This phenomenon remains understudied and under-reported in GnRHa studies, yet likely has important implications for the optimisation of induced reproduction in fish.

4.3 Conclusion

This study had two main aims: 1.) to characterise *fshβ* and *lhβ* expression in wild yellowbelly flounder at different stages of reproductive development; and 2.) to quantify pituitary *fshβ* and *lhβ* expression over a five-day period following injection with different concentrations of gonadotropin-releasing hormone analogue (GnRHa). Results showed that both pituitary *fshβ* and *lhβ* expression peaked in fish with post-vitellogenic ovaries. Expression of *fshβ* was greater than *lhβ*, collectively, the data reflects the multiple-batch group synchronous ovarian development and spawning strategy of this species.

Doses of 25 µg/kg or 50 µg/kg GnRHa elicited the greatest pituitary gonadotropin expression, while doses of 100 µg/kg may have had the opposite effect. These findings support ovulation and oocyte data from previous induced reproduction studies in this species. Collectively, the information encapsulated in this study provides a valuable stepping-stone toward a deeper understanding of the broader HPG axis within this species.

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Appendices

Appendix 1 - Chapter 2 analysis

Table 1. One-way ANOVA of normalised fold expression of follicle-stimulating hormone (*fshβ*) across each oocyte stage (Stage I; Previtellogenic, Stage II; Cortical Alveolar, Stage III; Vitellogenic, Stage IV; Germinal Vesicle Migration).

Source	Sum of squares (SS)	Degrees of freedom (v)	Mean squares (MS)	F statistic	p-value
Treatment	56.4591	3	18.8197	3.5738	0.03
Error	121.1196	23	5.2661		
Total	177.5787	26			

Table 2. One-way ANOVA of normalised fold expression of luteinising hormone (*lhβ*) across each oocyte stage (Stage I; Previtellogenic, Stage II; Cortical Alveolar, Stage III; Vitellogenic, Stage IV; Germinal Vesicle Migration).

Source	Sum of squares (SS)	Degrees of freedom (v)	Mean squares (MS)	F statistic	p-value
Treatment	13.3325	3	4.4442	3.1145	0.046
Error	32.8194	23	1.4269		
Total	46.1519	26			

Appendix 2 - Gel electrophoresis

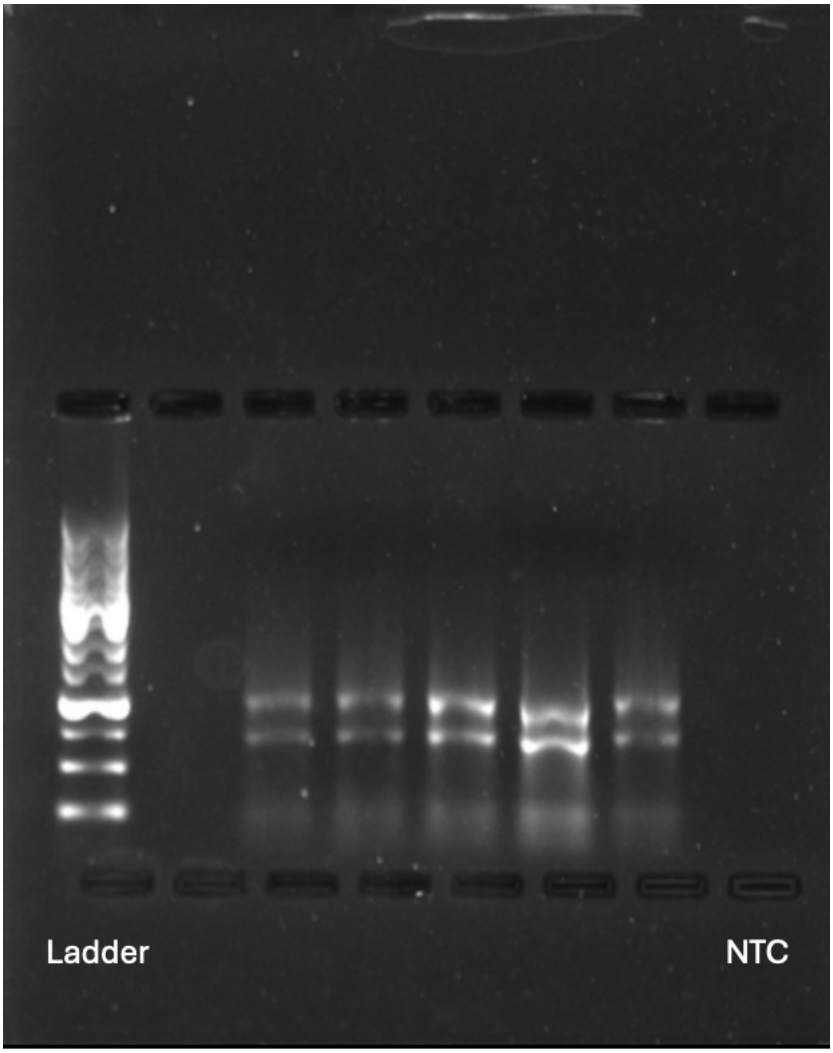

Figure 1. An example of gel electrophoresis showing the quality of RNA samples used in this study, extracted with Directzol™ RNA Miniprep kit.

Appendix 3 - DeNovix® DS-11 spectrophotometer results

Table 1. DeNovix® DS-11 spectrophotometer results show the concentrations and purity of each RNA sample.

Sample name	A260	A230/A280	A260/A280	RNA concentration (ng/µL)
P.1	2.287	2.093	1.956	91.478
P.2	1.8724	2.011	1.923	74.898
P.3	3.1511	2.105	1.905	126.044
P.4	4.5447	2.084	1.953	181.786
P.5	2.2361	1.967	1.926	89.445
P.6	3.7947	2.208	1.922	151.79
P.7	1.355	1.964	1.984	54
P.8	3.086	2.196	1.938	123.438
P.9	2.6195	1.845	1.941	104.78
P.10	0.3701	1.592	1.965	14.805
P.11	0.7656	2.123	1.934	30.625
P.12	2.5795	2.102	1.931	103.18
P.13	2.3639	2.17	1.951	94.558
P.14	2.0023	2.069	1.941	80.094
P.15	3.1567	1.774	1.975	126
P.16	2.4714	2.035	1.924	98.855
P.17	3.5383	2.138	1.939	141.532
P.18	2.9998	2.111	1.907	119.991
P.19	2.4052	2.052	1.915	96.207
P.20	3.1903	2.179	1.939	127.613
P.21	3.6907	2.209	1.929	147.629
P.22	3.0468	2.199	1.943	121.873
P.23	2.653	2.207	1.955	106.121
P.24	0.0878	0.658	1.713	3.513
P.25	3.6766	2.21	1.957	147.064
P.26	1.2016	2.027	2.002	48.063
P.27	2.4019	2.126	1.923	96.076
P.28	2.7958	2.224	1.942	111.831
P.29	1.5918	2.18	2.014	63.67
P.30	2.9381	2.037	1.96	117.524
P.31	2.0034	2.154	1.906	80.137
P.32	2.0957	2.276	1.933	83.826
P.33	3.2402	2.196	1.951	129.607
P.34	2.869	2.185	1.943	114.762
P.35	1.8427	2.132	1.967	73.707
P.36	3.5864	2.209	1.917	143.457
P.37	4.4584	1.971	1.941	178.338
P.38	2.9869	2.276	1.971	119.474

P.39	1.7403	2.171	1.954	69.612
P.40	2.4385	2.114	1.847	97.539
P.41	4.1606	2.225	1.95	166.424

Appendix 4 - cDNA synthesis

Table 1. Reagent volumes used for qScript™ cDNA synthesis for each sample. Due to low concentrations of RNA, the maximum (16.00 µL) was used for cDNA synthesis with no water being used.

Sample name	RNA (µL)	Supermix (µL)	Water (µL)	Total (µL)
P.1	16.00	4	0.00	20
P.2	16.00	4	0.00	20
P.3	15.87	4	0.13	20
P.4	11.00	4	5.00	20
P.5	16.00	4	2.82	20
P.6	16.00	4	2.82	20
P.7	16.00	4	0.00	20
P.8	16.00	4	0.00	20
P.9	16.00	4	0.00	20
P.10	16.00	4	0.00	NA
P.11	16.00	4	0.00	20
P.12	16.00	4	0.00	20
P.13	16.00	4	0.00	20
P.14	16.00	4	0.00	20
P.15	16.00	4	0.00	20
P.16	16.00	4	0.00	20
P.17	16.00	4	0.00	20
P.18	16.00	4	0.00	20
P.19	16.00	4	0.00	20
P.20	15.67	4	0.33	20
P.21	13.55	4	2.45	20
P.22	16.00	4	0.00	20
P.23	16.00	4	0.00	20
P.24	16.00	4	0.00	NA
P.25	13.60	4	2.40	20
P.26	16.00	4	0.00	20
P.27	16.00	4	0.00	20
P.28	16.00	4	0.00	20
P.29	16.00	4	0.00	20
P.30	16.00	4	0.00	20
P.31	16.00	4	0.00	20
P.32	16.00	4	0.00	20
P.33	15.43	4	0.57	20
P.34	16.00	4	0.00	20
P.35	16.00	4	0.00	20
P.36	13.94	4	2.06	20
P.37	11.21	4	4.79	20
P.38	16.00	4	0.00	20

P.39	16.00	4	0.00	20
P.40	16.00	4	0.00	20
P.41	12.02	4	3.98	20