Assessment of key reproductive markers after hormonal induction of spawning, using gonadotrophin-releasing hormone in female yellow belly flounder (*Rhombosolea leporine)*:

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Abstract

Yellow belly flounder (YBF) (*Rhombosolea leporina)* are of interest to the New Zealand aquaculture industry as a novel culture species. This is due to their high commercial value and low trophic feeding level. However, when held in captive settings, YBF are observed to undergo reproductive failure. GnRHa has been used as a spawning inducing agent within many cultured fish species. Flounder gonadotrophin levels were traced after induction and oocyte development was histologically assessed. At pituitary level it was seen that the GnRHa induction resulted in increased follicle stimulating hormone (FSH) levels. Luteinising hormone (LH) was seen to be unaffected. However, neither of these changes were significant $P>0.05$. These weak results were most likely caused by seasonality.

Oocyte development was seen to follow a similar trend to other flounder and flatfish species, when oocyte size, development stages and features were tracked using histological analysis. Relationships between Gonadosomatic index (GSI) and gonadotrophins FSH and LH displayed weak correlations ($P>0.05$). This again could be linked to seasonal variability in temperature and photoperiod. The presence of large amounts of atretic oocytes observed in the gonads indicated that the ovary had already spawned for the season before initial capture, or that oocytes had been aborted. A likely cause of this would be captivity -induced stress, or fish having previously spawned before capture.

All fish that ovulated were part of the GnRHa treated group. Fish were strip-spawned, fertilised and embryonic development was tracked. Incubation temperatures were 17 and 19°C. It was established that of the two temperatures, 17°C was optimum. A larger percentage of these embryos survived from fertilisation to hatch, which took approximately 68 hours. Bacterial infection was seen to be a problem in eggs incubated at the higher 19°C temperature. Egg quality was assessed by looking at the 8 cell blastomere stage, and grading charts were made for reference. these assessed blastomere size, shape, symmetry and cohesion.

It is suggested that future research efforts focus on seasonal variations of gonadotrophin levels and gonadal development, in order to gain clearer understanding of these seasonal effects. Effects of cortisol on the reproductive axis would also be a beneficial research area. This would provide insight into how stress affects sex steroid production and the maturation of the gonad and oocytes in YBF.

Acknowledgments:

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Kent Jeffries.

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All live animal research was conducted at Toi Ohomai Institute of Technology aquaculture facility as part of a project approved by the Toi Ohomai Institute of Technology Animal Ethics Committee (Approval number-2016-01).

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Introduction/ Background

1.1 Aquaculture globally

Human pressure on marine resources has continued to intensify, with a declining trend globally seen in wild fisheries. As a result, aquaculture has become one of the most promising avenues for increasing marine fish production (Naylor & Burke, 2005). Similar to agriculture, aquaculture has become a potential solution for providing fish products to meet global needs. This is of huge significance as wild catch per-unit effort is decreasing and world-wide fish stocks are being exhausted (Wooton & Smith, 2014). In light of this, aquaculture has become the fastest growing primary industry in the world. Farmed fish production is now greater than conventional fisheries' catch rates, with the vast majority of culture occurring in Asia (Gjedrem, Robinson & Rye, 2012). If the aquaculture industry continues to expand at the current rate, production will exceed 132 million tonnes of fish and shellfish, and 43 million tonnes of seaweed in 2020 (Klinger & Naylor, 2012). However, for continued growth of this sector to be sustained, management and culture methods which maximise efficiency and ensure a constant supply of juveniles are needed (Naylor, Goldburg, Primavera & Kautsky, 2000). The domestication of various other novel species is also important to reduce the pressure on wild stocks and maintain their availability in the global market place.

1.2 New Zealand finfish aquaculture

New Zealand's marine finfish aquaculture industry is small compared to that of many other countries. It is based primarily on farming of king salmon in sea cages, around sheltered bays along the South Island coast (Forrest et al., 2007). However there has been recent interest in the upregulation of marine farming as a means for sustainable development in the marine space. This would provide potential employment and economic opportunities. It would also boost production of fish to meet an ever-increasing demand for marine protein. This is all part of a push to drive a blue revolution, taking farming to the ocean (Rennie, White & Brabyn, 2009).

The location and geography of New Zealand lends itself well to marine farming. It has 13,000km of coastline ranging from sub-tropical in the north to cooler temperate environments in the south (Hayden, 1989). Although New Zealand shows a huge potential for successful mariculture, until now there have been very few species of finfish trialled or farmed in New Zealand waters. The majority of New Zealand aquaculture revolves around king salmon, greenlip mussels and pacific oysters (Hayden, 1989). However, there has been recent increased interest and government schemes are emerging to grow New Zealand's aquaculture sector. This has increased motivation to expand on the number of species currently cultured in NZ. A large focus is being put on finfishes, including yellowtail kingfish and Groper (Hapuka) (Forrest et al., 2007).

1.2 Flounder

Flatfish, including flounder are a group of marine or brackish-water dwelling finfish that support a popular table-fish market worldwide. They are popular with recreational and commercial fishers alike (Daniels & Watanbe, 2011). Flounder and flatfish species are among the few finfish species that are currently subject to significant stock enhancement efforts in Europe, Asia & North America. This is an attempt to remedy wild population numbers which are steadily declining. Within the New Zealand setting there are 11 recognised flatfish species. Four of these species are flounder, including the sand flounder/ dab *(Rhombosolea plebeia),* greenback flounder (*Rhombosolea tapirine)*, black flounder *(Rhombosolea retiaria),* and the yellow belly flounder (*Rhombosolea leporine)* (Colman, 1974). The two of these flounder making up the largest of the commercial and recreational catch percentage are the sand and yellow belly (Colman, 1973). The YBF is the most preferred of the two, due to its larger size. It represents a lucrative set-net fishery, particularly in the Firth of Thames in the North Island which yields between 100,000 – 250,000 kilograms of fish annually (Colman, 1974). This fishery is driven by a large domestic and international demand. This, coupled with attributes including a high flesh value, low trophic feeding level, high fecundity, and fast growth rate (Colman, 1974), are what make YBF a potential candidate as an aquaculture species for the waters around New Zealand.

Yellow belly flounder inhabit shallow coastal water throughout the whole of New Zealand's North and South islands (Colman, 1973). Like most other flounder species, they are batch spawners, and ooctyes mature and ovulate in groups within the ovary. This means they are able to spawn multiple times throughout a season (Canario & Scott, 1990). During spawning season, YBF migrate out of the estuaries to deeper water where they congregate to spawn (Colman, 1973). Males at first maturity in most flounder species are seen to be significantly smaller than females. It has been observed that YBF males mature at approximately 23-25 centimetres, whereas females are generally mature once they reach 26 centimetres (Colman, 1972).

1.3 HPG axis

Species domestication for use in modern agriculture requires the management and understanding of all life stages, and control over reproductive processes (Cnaani & Levavi-Sivan, 2009; Rainis & Ballestrazzi, 2009; Pillay, 1990).

Reproduction within fish is controlled through the interconnection of multiple tissues and organs through the hypothalamic-pituitary-gonad (HPG) axis. Also involved are the hormones which allow the axis to function (figure 1) (Hachfi et al., 2012).

The HPG axis enables vertebrates to have neuroendocrine control over complex tasks such as growth, metabolism, osmoregulation and reproduction (Sower, Freamat & Kavanaugh, 2009; Zohar et al., 2010). Understanding the functioning of the HPG axis is therefore of critical importance for the development and improvement of cultured fish stocks. It allows identification of the key neuroendocrine events which take place (Cnaani & Levavi-Sivan, 2009).

Physiologically, before the hypothalamus there is the reception of external cues. An example is sunlight/photoperiod and temperature at the pineal gland, which then trigger the hypothalamus to start producing gonadotrophins (Cabrita, Robles & Herraez, 2008; Sower, Freamat & Kavanuagh, 2009). The release of gonadotrophins is the central key component to the HPG axis. Gonadotrophins are released from the pituitary gland and activate varying aspects of gonadal development stages and processes (Davies, Bromage & Swanson, 1999).

Key hormones such as gonadotrophin releasing hormone (GnRH), luteinizing hormone (LH) and follicle-stimulating hormone (FSH), control or regulate gametogenesis and steroidogenesis. Understanding the synthesis and release of such key hormones is of huge importance in developing methods to control the reproductive potential of domesticated stocks (Sower, Freamat & Kavanaugh, 2009). Without these hormones, maturation of the gonad cannot take place. Commonly within aquaculture ventures, failure at ovulation and spawning occurs due to failure of the pituitary to release gonadotrophin hormones (Mehdi & Ehsan, 2011).

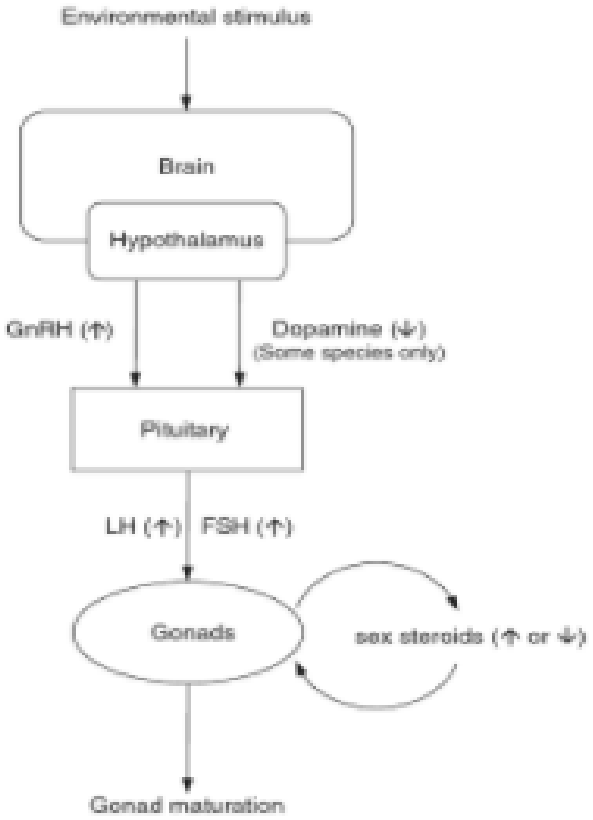

Figure 1- A simplified diagram showing the Hypothalamus-Pituitary-Gonad axis, which regulates reproduction and gonadal maturation within all teleost fishes. Arrows indicate positive feedback loops and indicates negative feedback loops (Wooton & Smith, 2014).

1.4 GnRH controlled reproduction

Within most aquaculture procedures, pharmaceutical methods of controlling sex and reproduction in finfish species are widely used in fish production (Patino, 1997). Chemicals in the form of hormones are used in every stage of the reproductive cycle. They are useful for altering sex before the gonads have formed, right through to the use of gonad maturation hormones, such as GnRH, to induce final gonad growth and spawning (Bromage & Roberts, 1995; Patino, 1997).

Gonadotrophin releasing hormone agonists have been widely used to stimulate the pituitary to release LH. LH is required to induce final oocyte maturation (FOM) of ovulation and spermation within cultured fish. This potentially fixes the issue of reproductive failure within aquaculture settings (Mylonas, Fostier & Zanuy, 2010; Mylonas & Zohar, 2000). This method of inducing gonadal maturation has been achieved in females of 40 different species (Mylonas & Zohar, 2000). It has been found to bring about (FOM), ovulation and spawning in multiple fish species with different reproductive methods.

This includes fish with synchronous, group synchronous and asynchronous ovarian development (Mugnier et al., 2000; Mylonas, Fostier & Zanuys, 2010). Within males, more than 20 species have been tested with results of increased sperm production for up to five months after the initial treatment (Mugnier et al., 2000; Mylonas, Fostier & Zanuys, 2010).

This gonadal maturation is a result of the feedback loop between production of GnRH in the hypothalamus in the brain and the reception of the GnRH on the anterior of the pituitary (Burger et al., 2004). This results in the production of gonadotrophins FSH and LH within the pituitary. These two gonadotrophins are essential for fertility. They contain the same genetic α- subunit (CGA), yet have a different β-subunit, which makes them unique for their distinctive roles in gonadal maturation (Thompson & Kaiser, 2014). After production in the pituitary, FSH and LH then carry out their effects in the ovaries if the fish is female and, in the testes, if the fish is male, leading to steroidogenesis and gametogenesis (Burger et al., 2004; Thompson & Kaiser, 2014).

1.5 Aims:

This thesis aims to trial GnRHa as a potential therapy to overcome stress-related reproductive failure in YBF, as well as to describe embryonic development in this species. Gonadotrophin releasing hormone analogue will be used in testing to try and bring about final maturation of the gonad through hormonal feedback loops. Using GnRH should produce follicle stimulating hormone and luteinising hormone, both crucial gonadotrophins in gonadal development. It is hypothesised that with the aid of GnRH there will be a larger percentage of fish which enter final stages of gonadal maturation. These fish that have entered ovulation can then be strip spawned.

Eggs can then be fertilised with the use of sperm from strip spawned males. The developmental stages leading to hatch can then be recorded, as reference points for normal embryonic development in the future.

To determine effectiveness of GnRHa as a spawn inducer, qPCR will be used. The use of qPCR allows comparison of gonadotrophin levels within the pituitary and the gonad of both GnRH -treated fish and control implanted fish.

Ovarian oocyte development will also be recorded to characterise key features and determine whether oogenesis can be associated with gonadotrophin levels and GSI.

1.6 Research Questions/hypothesise

Hypothesis 1: GnRH induction will induce spawning in yellow belly flounder through the production of reproductive markers.

Hypothesis 2: Reproductive markers (FSH and LH) will be influenced by GnRHa treatment.

Hypothesis 3: Embryonic development will be influenced by temperature with an increase in temperature resulting in an increased development rate.

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Chapter Two- Ovarian development in yellow belly flounder following gonadotrophin releasing hormone analogue treatment.

1.0 Introduction

1.1 Overview of the HPG axis

Within all vertebrate animals, gonadal maturation starts with the reception of external stimuli which in interpreted within the hypothalamus. Such external stimuli can include temperature and photoperiod. Environmental cues help to direct the timing of gonadal tissue growth as well as spawning (Crim, 1982; Sancho, Solow & Lobel, 2000; Hellqvist, Schmitz, & Borg, 2008; Miller et al., 2009; Muncaster et al., 2010; Taranger et al., 1998). Environmental stimuli create a cascading effect of endocrine signalling from the brain to the gonad along the brain-pituitary-gonad (BPG) axis (Bromage, Porter & Randall, 2001; Cardinaletti et al., 2010; Muncaster et al., 2010). The result of this signalling is the maturation of the gonads, producing viable gametes whether that be sperm or eggs.

From the hypothalamus, the production of gonadotrophin releasing hormone (GnRH) is stimulated. This secretion of GnRH is then received on the exterior of the pituitary, which results in the production of both gonadotrophins (GTHs): follicle stimulating hormone (FSH) and luteinising hormone (LH) (Patino & Sullivan, 2002). FSH and LH are key hormones controlling reproduction in all vertebrates, and are regulators of gametogenesis and steroidogenesis (Guzman et al., 2009). The functionality of these two GTHs is genetically linked to their differing β subunits. Follicle stimulating hormone is a glycoprotein involved in the initiation of gametogenesis, alongside the regulation of growth and early oocyte development within the gonad. FSH is predominantly produced during vitellogenesis and spermatogenesis, whereas LH regulates maturation phases, including spermiation and ovulation, through gonadal membrane receptors (Gómez et al., 1999; Guzman et al., 2009; Mateos et al., 2002; Swanson, Dickey, & Campbell., 2003). Once these gonadotrophins bind to their particular receptors, they stimulate the gonadal production of sex steroid hormones.

This is achieved through feedback loops, such as oestradiol 17β (E2) produced in the granulosa cells, formed in response to a bioconversion of 11 ketotestosterone (11-KT) via the aromatase enzyme (Yaron & Levavi, 2011). Testosterone is produced from the conversion of cholesterol in the thecal cell layer. Oestradiol within females promotes oogonia proliferation and vitellogenesis. The period leading up to final oocyte maturation (FOM) is largely driven by E2. However final maturation is driven by the production of progesterone hormones under stimulation of LH. While the steroid responsible for maturation may differ between species it is often generically termed maturation- inducing steroids (MIS) (Nagahama, 1994; Muncaster et al., 2010; Yaron & Levavi, 2011). Therefore, the gonadotrophins FSH and LH provide a critical role in co-ordinating gametogenesis by enabling communication between the upper and lower HPG tiers of the reproductive axis.

1.2 Oocyte development and maturation

Species-specific knowledge of ovarian maturation, follicle growth and ovulation within teleosts is important for both the aquaculture, fisheries and environmental sciences (Patiño & Sullivan, 2002). The process of oogenesis can be summarised as the process by which primordial germ cells (PGCs) progress through follicle stages to become mature oocytes ready to be ovulated and fertilised (Patiño & Sullivan, 2002). Ovarian follicles consist of an oocyte surrounded by an internal layer of granulosa cells and an external layer of thecal cells (Nagahama, Chan, & Hoar, 1976). Within oogenesis, follicle growth may be classified into five key stages; Oogenesis, primary follicle growth, cortical alveolus stage, vitellogenesis, and maturation/hydration (Wallace & Selman, 1981; Tyler & Sumpter, 1996; West 1990). These stages are clearly visible with light microscope analysis of ovarian histological samples.

Primary growth/ previtellogenic oocytes (PVO): these serve as a reserve of spawning material for future breeding sessions or seasons. They contain no yolk, and are the baseline stage for oocyte development. Previtellogenic oocytes can be found in the gonad year-round, regardless of seasonality (Murua & Saborido, 2003).

Cortical alveoli (CA): these are characterised by the presence of yolk proteins/oil globules (cortical alveoli vesicles) contained in the cytoplasm of the oocyte. These vesicles are associated with oocyte growth, where they increase in size and number to form peripheral rows. Cortical alveoli vesicles release their contents inside the egg membrane during fertilisation (Murua & Saborido, 2003). If oocytes reach CA development stage it is a good indicator that oocytes will continue development through the remaining maturation phases (Murua & Saborido, 2003; Watanabe et al., 1998).

Vitellogenesis (Vt): this stage can be characterised by the visibility of the "true" yolk vesicle which forms inside the cytoplasm of the oocyte. Vitellogenesis results in the oocyte increasing dramatically in size as the yolk accumulates. This stage ends when the oocytes have reached their mature, fully developed size and undergo maturation. This is initiated by maturation inducing steroids (MIS) (Masui & Clarke, 1979; Murua & Saborido, 2003).

Maturation & hydration: Maturation is indicated by the migration of the nucleus to the oocyte's animal pole. Once this migration has occurred the first meiotic division takes place. Hydration is the final step before ovulation, where the oocyte rapidly takes in fluid through the follicle and the yolk/oil droplets coalesce (Fulton, 1898; Murua & Saborido, 2003).

These oocyte stages can then be linked to GnRHa or sham treatment to look at the effects of GnRHa on oocyte development. Size of oocytes and development of key processes such as vitellogenesis can be used as indicators of GnRHa induction success, with size increases being associated with oocyte development (Scott, Witthames, & Vereirssen, 1999). The effects of varying dosages of GnRHa can be tested by looking at concentration of GnRHa ($\mu g/kg^{-1}BW$) in relation to oocyte maturation.

2.0 Methods

2.1 General/sample collection

Ovarian samples were provided from adult captive yellow belly flounder that had been treated with either a gonadotrophin releasing hormone analogue (GnRHa) or sham implant in July 2018. These fish had been captured from the Tauranga harbour using dragnets, and then transported to the Toi Ohomai Institute of Technology aquaculture facility. The fish were then sedated in an aerated, anaesthetic seawater bath containing 2 phenoxy-ethanol ($0.5ml^{-1}L$) until loss of equilibrium. Once anaesthetised, they were administered an individual numbered plastic floy tag, and an intramuscular implant consisting of a pellet containing GnRHa mixed in a cholesterol and cellulose matrix. The dosages ranged from 150 to $610\mu g/kg^{-1}$ of body weight or sham (matrix only) treatment. Fish of both treatments were then divided evenly across 3 1600 litre recirculating seawater tanks.

Fish were removed from the tanks after 5 days of treatment. They were given an anaesthetic overdose in 2 phenoxy-ethanol (cessation of gill ventilation for at least 30 seconds followed by swift decapitation with a sharp knife.) Organs involved in the HPG axis (Brain, pituitary, & ovary) were then dissected out and stored. Ovarian tissue was fixed in 10% neutral buffered formalin for 24 hours, and then transferred to 70% ethanol before being sent away to the University of Otago, Department of Anatomy histology lab for histological preparation. The samples were embedded in paraffin and sectioned at 5µm, using a microtone. These samples were then mounted on glass slides and stained with haemotoxylin and eosin for light microscopy.

2.2 Histology

The histological slides were analysed for the current study using a light microscope (Leica DFC290) with a camera attachment. This microscope used different magnifications to look at ovarian maturation stages. Literature (Muncaster et al., 2010; Murua Saborido, 2003), was used as a guide to assess ovarian staging. All images used for this study were at 100X magnification. A stage micrometer was also photographed to serve as a size reference for the photos when being analysed.

2.3 Data analysis

Data analysis was conducted using windows Microsoft Excel. Gonadosomatic index was calculated for fish using the equation below.

$$Ig = \frac{\text{Gonad weight}}{\text{(Body weight-gonad weight}} \times 100$$

Regression analysis tests were run, and t-tests (assuming equal variances) were used to test for statistical difference between trial groups.

Image J software (ij152-win-java) was used to analyse photographic results. Scale bars were created using a stage micrometer as a reference for measure. This was also used to calibrate length, so that oocyte diameter could be measured for each of the ovarian stages. Ten oocytes at each of the development stages PVO, CA, EV, and LV were taken, and averages were calculated to give relative sizes. Photoshop (adobe version CS6 extended (13.0)) was then used to collate all images for comparison and form the results- based photographic figures used in this chapter.

3.0 Results

Regression analysis of length vs weight were run, resulting in a positive correlation between the length-weight relationship (figure 2.1). As length increased, there was a subsequent increase in the weight of individual fish (R^2=0.93). Lengths between both treatment groups were then analysed with a t-test to assess size variation between trial groups. Statistically no difference was found between the 2 treatment groups ($P<0.05$, T-stat=1.42). Fish ranged in length from 28cm to 46cm. Weight ranged from 205.6g for the smallest fish to 883.6g for the largest fish.

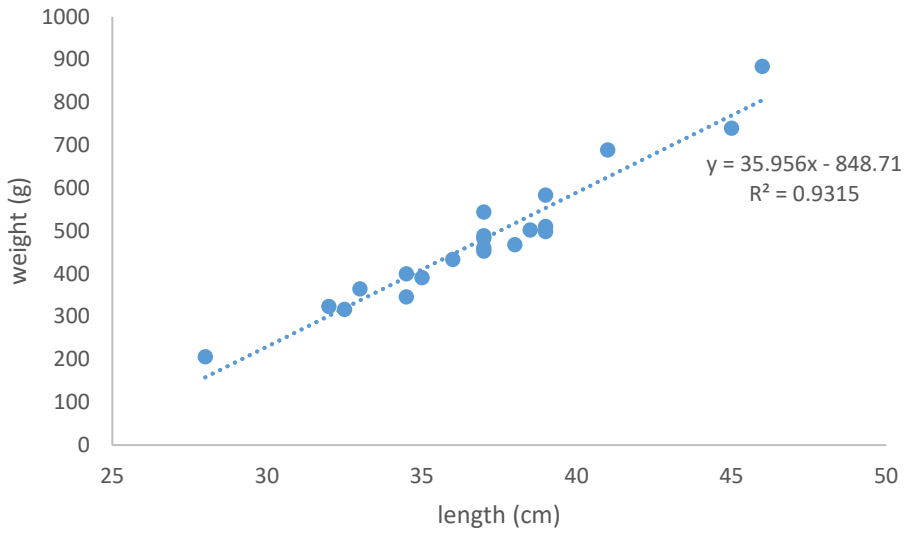

Figure 2.1: Regression analysis of individual fish length against weight, with a positive correlation supported by an R2 value of 0.93.

Oocyte maturation stages within each of the samples were analysed. More of the GnRHa fish were seen to be entering vitellogenesis compared to that of the sham implanted fish. Three GnRHa treated samples were found to contain early vitellogenic (EV) oocytes.

This compared to an absence of EV oocytes found in the sham samples. The sham samples predominantly contained pre-vitellogenic oocytes (figure 2.2). Late vitellogenic oocytes were also seen to be more common in the GnRHa treated fish ovaries. Two GnRHa treated ovaries contained LV oocytes compared to one sham fish ovary which contained LV oocytes. The presence of cortical alveoli was seen in two samples, one from each of the trial groups. However only the latest dominant stages of development have been documented in figure 4.

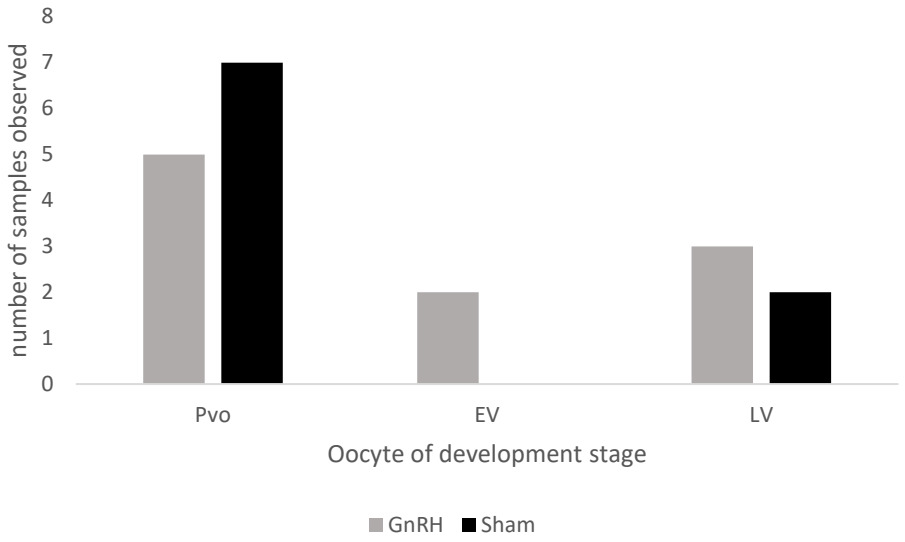

Figure 2.2: Dominant stages of oocyte development found within samples of both GnRHa and sham treated fish.

Dosage of implanted GnRHa was calculated for all fish within the GnRHa treatment group. Dosage was seen to have an effect on the developmental progression seen within the oocytes. Larger dosages (µg/kg^{-1}BW) were associated with fish exhibiting poorer oocyte development (figure 2.3). It was observed that dosages associated with vitellogenesis were in the mid range 200 µg/kg^{-1}BW. The average dosage found in EV oocytes was 263 µg/kg^{-1}BW, and in LV oocytes was 266 µg/kg^{-1}BW. This was a lot lower than average dosages associated with PVO, where the average dosage was 386 µg/kg^{-1}BW (figure 2.3). Samples containing PVO's were seen to have a larger range of dosages (169-610 µg/kg^{-1}BW). However, fewer intermediate dosages were seen in the vitellogenic oocyte stages of mid 200 µg/kg^{-1}BW. However this is unsupported statistically ($P>0.05$).

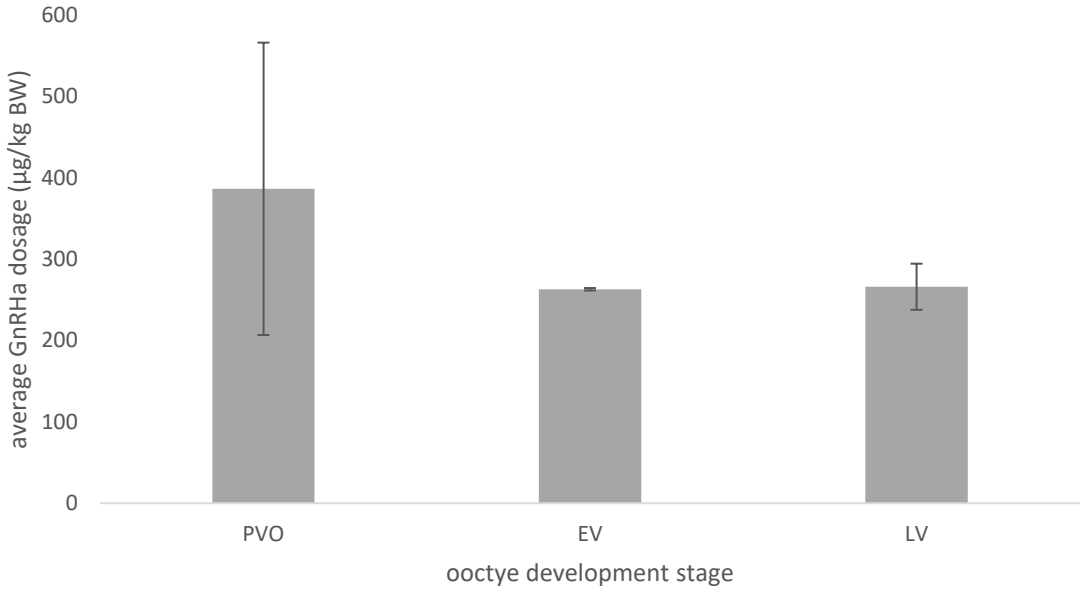

Figure 2.3: Relationship between GnRHa dosage and stages of oocyte development in the gonad at harvest error bars±SD.

Ooctyes were observed going through the various stages of maturation from primary growth to late vitellogenic/ early maturity (figure 2.4). Oocytes greatly increased in size as they progressed through maturation stages (figure 2.6 & table 1). Previtellogenic ooctyes averaged 60±10µm and grew rapidly, to over 5 times the size at LV stages, where oocytes averaged 320±40µm. The largest variation in size between stages was seen between early vitellogenic and late vitellogenic, where oocytes more than doubled in size (table 1). Maturation stages could also be classified by internal oocyte features. Cortical alveoli could be seen to have lipid vesicles within the cytoplasm. In EV oocytes there is recrudescence as the yolk begins to form, and LV can be identified by the presence of a “true” yolk.

Table 2.1: Ovarian stages and corresponding average ooctye size associated. PVO: previtellogenic ooctye, CA: Cortical Alveolus oocyte, EV: early vitellogenic, LV: Late vitellogenic

Ovarian stage	size(µm)±SD
PVO	60±10
CA	100±10
EV	150±20
LV	320±40

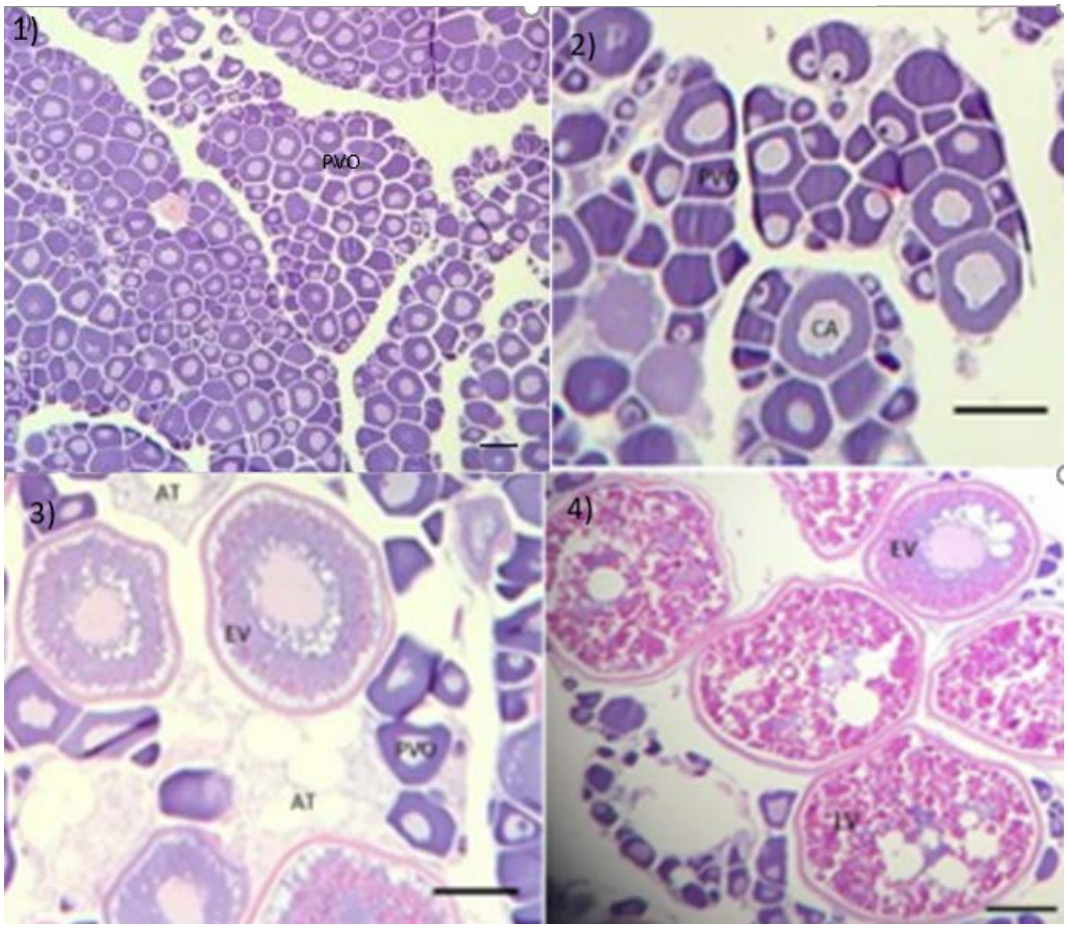

Figure 2.4: Stages of ovarian development within yellow belly flounder 1) primary growth displayed. 2) cortical alveoli 3) early vitellogenic 4) late yolk(vitellogenic) with some indication of maturation due to germinal vesicle migration beginning. AT, atretic oocyte; PVO, previtellogenic oocyte; CA, cortical alveolus oocyte; EV, early vitellogenic; LV, late vitellogenic. Scale bars=0.1mm.

Oocyte features were seen to also change with maturation (figure 2.5) Within PVO the features were consistent; a small nucleus surrounded by cytoplasm. In further developed oocytes including vitellogenic oocytes, the presence of oil globules, vitellogenin vesicles and follicle cell layers can be made out. This shows maturing development within the ovary. The appearance of multiple stages of oocyte development within this gonad is characteristic of group synchronous spawners.

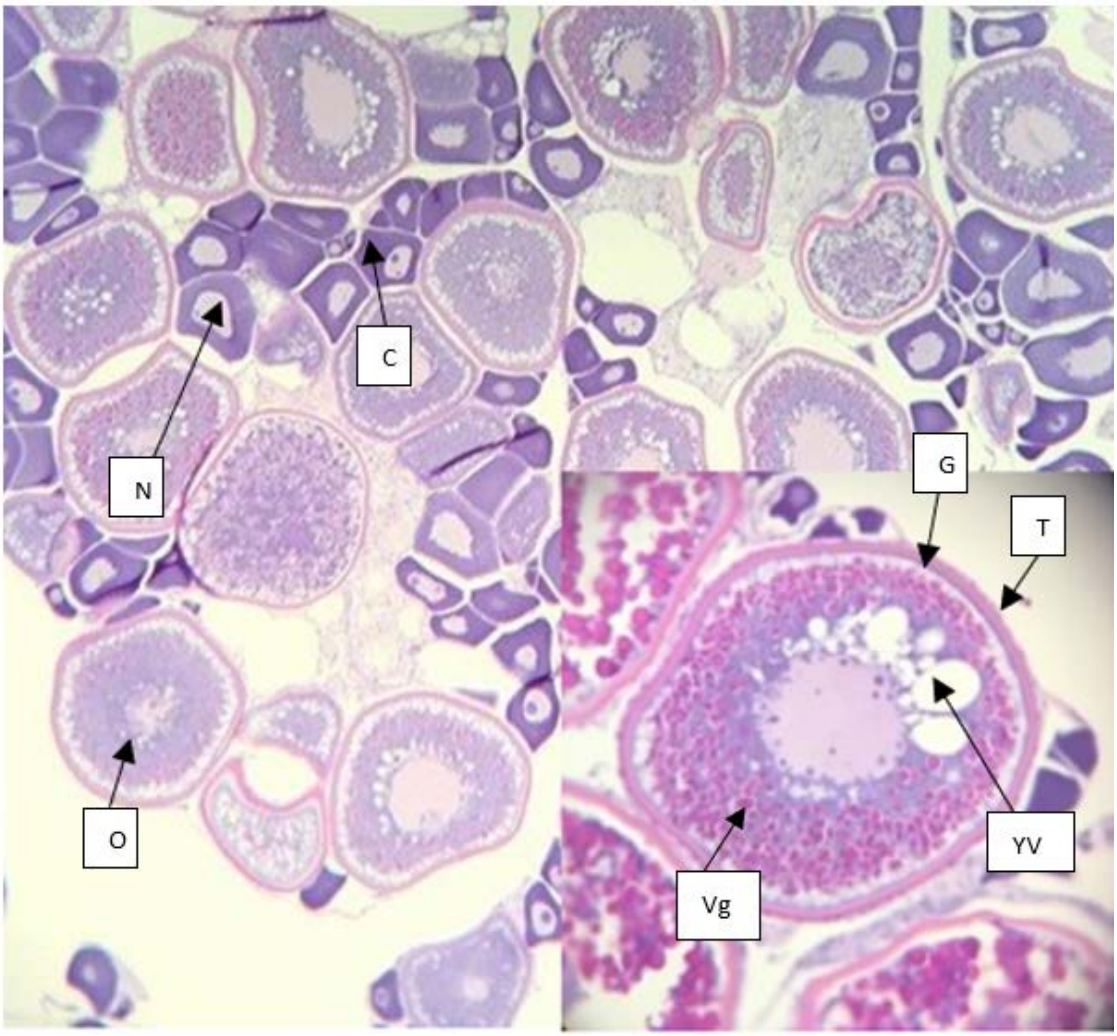

Figure 2.5: varying stages in oocyte maturation and labelled key features. O; oil droplet, N; nucleus, C; cytoplasm, G; granulosa layer, T; thecal layer, YV; yolk vesicle, Vg; vitellogenin globule.

4.0 Discussion

4.1 Size weight relationship

Size and weight were seen to have a strong positive correlation with each other R^2= 0.93 (figure 2.1). This showed that with increased length (cm) there is a corresponding gain in weight (g). Trial groups were compared, and found that p>0.05, showing that bias could be removed from the treatment groups. From other studies there have been similar findings in flatfish length-weight relationships. In a trial conducted by Bayhan et al (2008), where 7 species of flatfish were trialled, a positive correlation between length and weight was found for each species, with R^2 values ranging from 0.74 to 0.98.

4.3 Histological stages

Histologically, sample gonads were seen to range from primary follicle growth, where the ovary is largely or primarily made up of PVO, right through to vitellogenesis/early maturation with LV oocytes (figure 2.2). Size was seen to increase dramatically throughout oocyte development. The average previtellogenic oocyte was 60±10µm, with the average size of late vitellogenic oocytes being 320±40µm (table 1). These sizes were similar to those observed in other flatfish species. In summer flounder studies, oocytes within the vitellogenic phase of oocyte maturation had an average oocyte diameter of 301µm before hydration, and rapid uptake of fluids leading into final maturation (Merson et al., 2000). Greenback flounder also showed a similar range of oocyte sizes, ranging from 100µm to 450µm (Sun & Pankhurst, 2004). Korean flounder species *Liopsetta obscura* also showed very similar oocyte sizes throughout the developmental stages, where PVO had diameters measuring from 18 to 55µm. This diameter was seen to grow significantly at cortical alveoli, where it was between 170 and 210µm with the formation of yolk vesicles. Late vitellogenic oocytes had diameters between 250 and 300µm (Yamamoto, 1956).

With many flatfish species having group-synchronous ovarian development, there are cohorts of varying oocyte stages present within the ovary (figure 2.4). This is the most common form of ovarian development within teleost fishes (Wallace & Selman, 1981). Between PVO and CA stages the recrudescence of yolk granules can be seen within the cytoplasm (figure 2.1 & 2.2). At PVO stage the main features

of the oocyte are the presence of a nucleus and cytoplasm (figure 2.3) (Murua & Saborido, 2003). However, when oocytes reach vitellogenesis, the oocyte can be broken into many regions and features become clearly visible. These features include the presence of both thecal and granulosa layers, as well as oil and vitellogenin vesicles (Figure 2.4) (Murua & Saborido, 2003).

4.4 Effects of GnRHa on oocyte development

A greater number of trialled fishes were seen to enter vitellogenesis when implanted with GnRHa (Figure 2.2). This was seen in three GnRHa samples which contained gonads primarily made up of oocytes in the early vitellogenic phase. Fewer of the GnRHa treatment fish gonads consisted predominantly of PVO oocytes, compared to that of the sham implanted fish. Atretic oocytes were seen to be more common in the sham implanted fish when compared to the GnRHa treated fish.

GnRHa induction recruits' batches of oocytes into vitellogenesis, as has been observed in this trial. This occurs through the production of FSH and stimulation of final oocyte maturation (FOM) from vitellogenic batches of oocytes within asynchronous ovaries. This has been observed in the ovaries of greenback flounder implanted with luteinising hormone releasing hormone (LHRH) (Barnett, & Pankhurst, 1998a). Winter flounder implanted with GnRHa were seen to have increased levels of E2 and testosterone. This leads to a larger percentage of oocytes reaching maturity (Harmin et al., 1995). Oocyte size/maturation levels have been found to increase significantly after GnRHa implantation. This was observed in plaice when hydration of the oocyte was achieved after treatment with GnRHa (Scott, Witthames, & Vermeirssen, 1999). These oocyte sizes were consistently larger than those produced by the control group fish during the trial. This indicated more advanced oocyte maturation being associated with GnRHa implantation (Scott, Witthames, & Vermeirssen, 1999). Similar findings have also been expressed within sea bass where, with induction of LHRH, there is an increase in oocyte diameter, which can be associated with development stage and maturation (Alvarino et al., 1992).

Treatment dosage of GnRHa was seen to affect oocyte development (figure 2.3) however unsupported statistically($P>0.05$). Dosages most related to vitellogenic

oocytes were seen to be approximately 260 µg/kg^{-1}BW, whereas larger dosages were seen to have the opposite effect. Dosages of over 300 µg/kg^{-1}BW were associated predominantly with previtellogenic oocytes (figure 2.3). Similar findings have been seen in spotted rose grouper, (Ibarra-Castro, 2007), where the optimum dosage range was 240-280 µg/kg^{-1}BW. Anything significantly above or below this range was obserevd to have a lesser effect on gonadal maturation. Over-dosage of GnRHa has been associated with the desensitisation of the pituitary to gonadotrophes, as seen in goldfish that were under continuous administration of GnRHa (Habibi, 1991). This may be the case when higher dosages are administered to the YBF, resulting in the lack of gonadal development, despite the stronger concentration per kilo of body weight. However optimum dosage is seen to be highly variable between species (Barnett & Pankhurst, 1999; Mylonas, Hinshaw & Sullivan, 1992). Effective dosages of GnRHa in implantation form for plaice were 50 µg/kg^{-1}BW (Scott, Witthames, & Vermeirssen, 1999), whereas dosages of above 300 µg/kg^{-1}BW were seen to still elicit oocyte development in dusky grouper (Marino et al., 2003).

4.5 Conclusion

Oocyte development within YBF is largely controlled by a cascade of hormonal cues originating from the hypothalamus in the brain. This produces GnRH. Pituitary FSH and LH are then responsible for the varying stages of gonadal maturation. Oocyte development results from this study are similar to those found in the literature surrounding oocyte development in other flatfish species. However, these findings are highly affected by seasonality. Vitellogenesis was seen to be more prevalent in the ovaries of GnRHa inducted fish, showing that GnRHa is effective as an oocyte development and maturation inducer. Dosage was seen to significantly effect oocyte development where optimal dosage within this trial was seen to be approximately 260 µg/kg^{-1}BW with anything over 300 µg/kg^{-1}BW seen to elicit little change within the gonad. This is possibly due to desensitisation of the pituitary. Future research may need to look into seasonal variability in both hormone and gonadal development. Pilot studies from this investigation showed improved responses to hormonal manipulation throughout the months of June/July.

This could potentially be an interesting area for further future investigation into the relationship between gonadotropins and gonad development. Further research should be undertaken to study dosage and its effects. This would then potentially allow optimum GnRHa dosages to be confirmed for administration to YBF, to elicit the best oocyte development and maturation.

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Chapter three- Effects of GnRH on Gonadotrophin Gene regulation and the use of PCR to evaluate reproduction targets at hormone level

1.0-Introduction

1.1 qPCR

Real-time polymerase chain reaction (PCR) within the biological science fields has become a widely used method for biological investigations (Franzellitti et al., 2015; Valasek & Repa, 2005). This is due to its ability to identify and quantify extremely small changes within nucleic acid sequences (Valasek & Repa, 2005). These changes within gene expression can then be used to describe and extrapolate higher level changes of biological organisation within varying developmental, and physiological sequences (Franzellitti et al., 2015; Valasek & Repa, 2005).

The method of PCR requires four main components; template DNA, primers, nucleotides, and DNA polymerase. All four components are functionally important with DNA polymerase being the major functional enzyme which links together individual nucleotides to form the PCR product. These nucleotides consist of four genetic bases which are the building blocks for all genetic material. These are adenine, thymine, cytosine and guanine (A, T, C, G) (Garilbryan & Avashia, 2013). Primers are short DNA sequences. They specify that the exact product to be amplified should be complementary to the target DNA's base sequence, allowing for the product to be formed (Garibryan & Avashia, 2013).

These products are then made quantifiable through the use of fluorescent chemistries which relate concentration of PCR product with fluorescent intensity. The greater the fluorescent intensity the larger the quantity of PCR product produced (Wong & Medrano, 2005).

The use of primers within PCR methods is extremely important and multiple primers are commonly used to ensure robust results. The comparison of amplification efficiency between primers is also important, as variation in primer efficiencies may result in strong bias within results.

Having a primer set that encompasses the portions of desired genetic code is important for the production of reliable results (De Beeck et al, 2014). The use of gene sequences which are already known (housekeeping genes) is therefore useful as a normalisation method. This can be used to compare against other PCR reactions to validate results and show consistency between results generated within PCR trials run (Kozera & Rapacz, 2013).

1.2 GnRH, FSH & LH Regulation

Gonadotrophin releasing hormone (GnRH) is a key regulator of sexual development and reproduction within all vertebrates (Hildahl et al., 2011; Mateos et al., 2002). It is a decapeptide produced in the hypothalamus, through the reception of external cues such as photoperiod and water temperature (Thompson & Kaiser, 2014). GnRH regulates the pituitary secretion of gonadotrophin hormones' follicle stimulating hormone (FSH) and luteinising hormone (LH). It achieves this through anterior binding to its high affinity transmembrane receptor GnRH-R (Burger et al., 2004). Both of these gonadotrophins have the same α-subunit, but have unique β-subunits (Thompson & Kaiser, 2014). This stimulation of these gonadotrophin hormones is then controlled through steroidal feedback loops (Mateos et al., 2002). Follicle stimulating hormone and LH then exert their effects within the testis or ovary of the fish bring about steroidogenesis and gametogenesis (Burger et al., 2004; Thompson & Kaiser, 2014). In female fish FSH is highly expressed during the vitellogenesis phase of gonadal maturation. Luteinising hormone is seen to be most elevated during final maturation of oocytes (Hirai, Oba & Nagahama, 2002). The stimulation of GnRH release from the hypothalamus can be triggered by environmental cues such as water temperature and photoperiod (Bromage, Porter & Randall, 2001; Damasceno-Oliveira et al., 2014). Seasonal fluctuations can have a major influence on the timing of gametogenesis, vitellogenesis and the production of the gonadotrophins FSH and LH. These seasonal variations (including photoperiod and temperature) can affect the production of FSH and LH differently. Therefore, understanding of reproductive cues is an essential starting point for the evaluation of FSH and LH within the reproductive cycle of teleost fish species (Damasceno-Oliveira et al., 2014).

Within the gonad, FSH and LH stimulate the proliferation and differentiation of germ cells (Dierich et al., 1998). FSH Receptors (FSH-Rs) in females, receive FSH in the ovary through the granulosa cells. The activation of FSH gonadotrophin receptors by FSH activates folliculogenesis (Dierich et al., 1998).

In males, the signalling of FSH is crucial for the initiation of spermatogenesis and the normal maintenance of sperm production within sexually mature adults (Oktay, Briggs, & Gosden, 1997). With FSH and testosterone stimulation, the seritoli cell can then provide support for the differentiation of germ cells, starting the spermatogenesis process (Dierich et al., 1998).

Within females, FSH in the ovary is received in the granulosa cells via FSH receptors (FSH-R) and is used to regulate follicle maturation (Ranniki, Zhang, & Huhtaniemi, 1995). During the reproductive cycle, the stimulation of FSH within the ovary reduces apoptosis events within cells. It also enhances proliferation and induces follicle maturation in the time leading up to ovulation (Dierich et al., 1998; Richards, 1994).

1.3 Gonadal somatic index

Gonadal somatic index (GSI) is a widely used measure to quantify changes in reproductive activity. Gonadosomatic index works on the assumption that there is an isomeric relationship between the total weight of an organism and gonad weight (Ebert, Hernandez, & Russell, 2011). As oocytes initiate development and progress through vitellogenesis, the overall mass of the ovary also increases towards the breeding season. Therefore, GSI can be used as a quick and convenient macroscopic indication of gonadal maturity and the relative proximity of spawning (Ebert, Hernandez, & Russell, 2011; Siikauvuopio & James, 2010).

The objective of the current study was to assess how the administration of GnRHa influences pituitary gonadotrophin expression in YBF, and whether there is a correlation between GtH expression and a macroscopic, morphometric analysis of gonadal development such as I_G.

2.0 Methods

2.1 Database Searching and Primer Design

Geneious v11.1.4 (Biomatters Ltd.) software was used to identify follicle stimulating hormone (fsh), luteinizing hormone (lh), beta-2microglobulin (B2M), succinate dehydrogenase complex subunit A (SDHA) and beta-actin (β-actin) genes from a previously constructed RNA-Seq transcriptome library. This was prepared by Dr. Steve Bird from gonad and pituitary tissues of wild-caught adult yellow belly flounder (*Rhombosolea leporine*). A protein sequence for the genes of interest was obtained from a closely related species (in the order Pleuronectiformes) This was then used to search the RNA-Seq library using the basic local alignment search tool (BLAST) programme, tBLASTn. The resulting hits were downloaded and aligned to the reference protein sequence. The nucleotide sequence for each of these was obtained and used to design primers using Primer3 0.4.0 (Rozen & Skaletsky, 2000) for use in quantitative PCR (qPCR). Primers were designed using the recommendations for typical primer design (Yang et al., 2006) as a guideline. Primers were designed with: a melting temperature (T_m) of 60-62°C and only 1°C between the forward and reverse, a length of ~20 nucleotides, a GC content of 40-60%, and a 3' end GC clamp to promote superior binding. The following tables detail the final primers used for qPCR (**Table 1**).

Table 1. Real-time PCR primer sequences used throughout this thesis. All primers were designed to specifically target *Rhombosolea leporina*. Abbreviations: (F) = Forward. (R) = Reverse complement. Tm = Melting temperature.

Primer name	Nucleotide sequence	Size	T_m (°C)	Gene target
rlBACTIN-RTF1	5′-TACAACGAGCTGAGAGTTGC-3′	114	54.8	*Actb*
rlBACTIN-RTR1	5′-GTTGAAGGTCTCGAACATGATC-3′		53.5	*Actb*
rlB2M-RTF1	5′-AACAGACAGACCTGGCTTTC-3′	97	63.8	*B2m*
rlB2M-RTR1	5′-ACTCTGCAAATGAACTTGTCATC-3′		63.0	*B2m*
rlSDHA-RTF1	5′-TCAGACTCAACATGCAGAAG-3′	93	61.4	*Sdha*
rlSDHA-RTR1	5′-TAGCATCCATCTTGTCGCATCC-3′		63.9	*Sdha*
rlFSH-RTF1	5′-TGTGTGCAGGACAGTGC-3′	117	62.0	*Fsh*
rlFSH-RTR1	5′-GACATCCATTAATGTGCTTCGC-3′		62.0	*Fsh*
rlLH-RTF1	5′-CAAATGGTGTCTCTGGAGAAGG-3′	124	62.0	*Lh*
rlLH-RTR1	5′-GCTGGTACATGTTGAGGAAGG-3′		63.0	*Lh*

2.2 RNA Isolation

Gonad and pituitaries were collected from fish that had been implanted with GnRH or a sham. They were used for isolation of total RNA using the Quick-RNATM MiniPrep Kit (Zymo Research) following the manufacturer's instructions. This RNA extraction protocol consists of three steps; sample lysis, sample cleaning (removal of genomic DNA) and RNA washing/purification. Approximately 50 mg of tissue was added to 2.0ml RNase/DNase free conical base screw cap tubes (Neptune). These contained 600µL of lysis buffer along with a mixture of 0.1mm and 0.5mm glass beads (BioSpec Productions). Samples were then homogenised using a bead beater (Alphatech Systems Limited) at a rate of 4800 oscillations per min for 20sec. Next, the lysate was centrifuged at 10,000g for 1 minute to clear the lysate. This was then transferred into a Spin-Away filter in a collection tube. The lysate then underwent centrifugation at 10,000g for 1 minute, which removes the majority of the gDNA. The flow through was used for the rest of the RNA purification. 95% ethanol was added at a ratio of 1:1 to the flow through. This was mixed well and then transferred to an RNA Zymo-Spin IIICG column in a collection tube. It was centrifuged at 10,000g for 30 seconds, where the total RNA became bound to the column matrix, and the flow-through discarded. The total RNA then underwent a DNase I treatment to remove remaining gDNA. To achieve this, 400µL of Wash Buffer was added to the column and centrifuged at 10,000g for 30 seconds. Secondly, a DNase I reaction mix was then prepared in a 2.0mL RNase and DNase free tube, containing 5µL of DNase I and 75µL of DNA Digestion Buffer. This was then added to the column matrix and left to incubate at RT for 15 minutes to degrade the gDNA. The column was then centrifuged at 10,000g for 30 seconds and the flow-through discarded. This ensured complete removal of gDNA and the DNase I reaction mixture. To clean up the RNA, 700µL of RNA Wash Buffer was added to the column, centrifuged at 10,000g for 30 seconds, discarded and repeated again with a volume of 400µL. The column was then centrifuged for an additional 2 minutes at 10,000g to ensure complete removal of the Wash Buffer. Lastly, the RNA-Spin column was transferred into a sterile collection tube. 50µL of DEPC water was added directly to the matrix of the column.

This was incubated for 1 minute at RT and then centrifuged at 10,000g for 30 seconds, eluting the RNA to be collected at the bottom of the tube. The column was discarded, and a NanoDropTM reading carried out to determine the quality and quantity of the RNA.

2.3 Spectrophotometric Analysis

A Thermo Scientific NanoDrop™ 2000 spectrophotometer was used to determine the concentration and purity of RNA, cDNA and plasmid DNA. In contrast to traditional spectrophotometers which use cuvettes and require large sample volumes, the NanoDrop utilises surface tension to hold samples in place between two optical fibres. It only requires 1 µl of sample. Initially Kimtech Science™ KimWipes™ were used to clean the optical fibre, after which the machine was blanked with 1 µl of DEPC-treated water. Next, 1 µl of each sample was loaded and read. Kimtech Science™ KimWipes™ were used to clean the optical fibre ends between each sample. The software calculated sample concentrations by comparing absorbance readings at wavelengths of 230, 260, and 280nm. A 260/280 ratio of 1.8-2.2 and a 260/230 ratio of 2.0-2.2 is generally accepted as a pure RNA sample, whereas ratios outside these indicate the presence of contamination (ThermoFisher Scientific, 2010). The RNA was immediately used for cDNA synthesis, with any remnants being stored at -80°C.

2.4 cDNA Synthesis

Synthesis of cDNA used the Quanta Biosciences qScript XLT cDNA SuperMix following the manufacturer's guidelines. To each PCR tube, 10µL of RNA (final concentration 1µg), 4µL of 5x qScript SuperMix (containing Oligo dT's and random primers) and 6µL of DEPC H_2O were added, giving a total volume of 20µL. The mixture was vortexed and centrifuged briefly to ensure components are at the bottom of each tube. The tubes were then incubated for 5 minutes at 25°C to allow primer annealing, 60 minutes at 42°C for reverse transcription to take place and finally for 5 minutes at 85°C to deactivate the enzyme before being stored at -20°C until used. Triplicate cDNA of all samples was synthesized, and then pooled for final volume of 60µl of cDNA. A 140µl volume of DEPC H2O was then added, bringing the total volume to 200µL total per sample. For each qPCR reaction, 5µl of cDNA was used as template.

2.5 Quantitative PCR (qPCR)

qPCR was used to monitor amplification of a targeted gene during a PCR reaction. This method can be used to quantitatively or semi quantitatively show amplification of genes relative to each other. It was performed using the Rotor-Gene 6000 (Corbett®) and a SYTO 82 MasterMix was prepared beforehand (**Table 2**). A 0.2mL RNase/DNase free PCR tube (Axygen®), 13.7µL of 50x MasterMix, 0.1µL of Hot Fire Pol® Taq Polymerase (Solis Biodyne) and 1.2µL of combined 10µM forward and reverse primer were used.

These were all added to 5µL of the desired cDNA template for a 20µL reaction. The amplification protocol used an initial amplification of 95°C for 15 minutes to activate the Taq and denature all the DNA. This was followed by 35-45 cycles of denaturation at 95°C for 15 seconds, annealing at 58°C for 15 seconds, and extension at 72°C for 30 seconds. After this a fluorescent reading was done. Fluorescent outputs were also measured for a melt analysis. This involved slowly increasing the temperature from 60 to 99°C to confirm that the amplified products are a single discrete species. All samples were run in duplicate. A negative control was also run containing DEPC water instead of cDNA template.

Table 2: Pre-made 50x qPCR MasterMix. This is combined with primers, Taq, and template to make up a final qPCR reaction of 20µL.

Reagent	**Per Reaction**	**Total (50X)**
SYTO 82	0.008µL	0.4µl
Buffer (10x)	2µL (1x)	100µl
$MgCl_2$ (25mM)	2µL (2.5mM)	100µl
dNTP (10mM)	0.4 µL (200µm)	20µl
H_2O	9.3µL	465µl

2.5.1 Primer Testing and Efficiencies

Prior to using any of the primers for the study of gene expression, primer efficiencies were investigated through amplification plots and melt curve analysis. Primer testing was run in duplicate alongside a negative control to ensure accuracy of the samples. cDNA used for this was pooled from control and treated samples. The negative controls contained DNase/RNase free water in lieu of cDNA templates to test for DNA contamination. Upon completion, an excel spreadsheet was generated in LinReg format. This is a file type readable by the LinReg software in order to determine the Ct values and the efficiencies of the primers.

The amplification plots and melt curves were examined and qPCR products were run on a 1.5% agarose gel to confirm amplification of a single product.

2.5.2 Gel Electrophoresis

A 1.5% agarose gel was prepared by dissolving 0.75g of AppliChem® agarose powder into 50mL of 1x SuperBuffer, containing 4 mM NaOH, 14.6 mM Boric Acid (Zhang et al., 2011) or 1x TAE, containing 10 mM Tris-HCL, 1 mM EDTA in a conical flask. This was mixed and microwaved at 700W for 2 minutes or until no crystals or powder remained visible. The solution was then cooled under running water for 2-3 minutes. After this 2µL of 10mg/ml Ethidium Bromide (EtBr) was added and mixed by swirling the flask. After the solution had cooled it was then poured into a gel mould, taking care not to introduce air bubbles. A 12-well comb was then placed into the gel to create wells for products to be loaded into. It was left to solidify for 20-30 minutes at RT, after which the gel was placed in an electrophoresis mini gel system (OWL® Separation System), and the combs removed. Into the gel tanks, either 1x SuperBuffer or 1x TAE was poured to cover the gel and fill the tank to the indicated level. A 10µL volume of the qPCR product was then mixed with 3µL of 6x loading buffer (30% glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol) and loaded into a well on the gel. Into the first lane, 5µL of iNtRON® 100bp ladder was loaded to act as a reference for product sizes. Once the gel was loaded, the electrodes and cover were attached to the tank. The voltage was set to 200V for 15 minutes for SuperBuffer, or 100V for 30 minutes for TAE. After electrophoresis, the electrodes were disconnected, and the gel

removed from the tank. An image was then taken using the Invitrogen® iBrightFL1000 gel imaging system and saved for later analysis.

2.5.3 Gel Electrophoresis

The geometric mean from three Housekeeping genes was used to normalize the expression of FSH and LH (Vandesompele et al., 2002). Fold change was determined using the delta-delta Ct method (Livak & Schmittgen, 2001) as the efficiency of each primer set was found to be 2 using the LinReg software. For statistical analysis a student's T-test was carried out using Microsoft Excel to determine the significance of the results, where values of $P<0.05$ were considered statistically significant. For the T-test a two-tailed distribution was used, and it was performed using 2 samples with unequal variants.

Results

3.1 qPCR

The efficiency of each pair of primers (Table 1) designed for qPCR first had to be determined. At 100% efficiency, primers double the amount of template DNA after each PCR cycle. However, each primer pair developed needs to be tested, as they can deviate from the ideal amplification. Therefore, it is important to calculate exact efficiencies for all sets of primers used within this study. The housekeeping genes b-actin, B2M and SDHA were the first to be validated and have their primer efficiencies determined, with LinReg reporting a primer efficiency of 2.0 for each one. In addition, melt curves were analysed and the amplified products were run on an agarose gel for b-actin (Figure 3.1 & 3.2), B2M (Figure 3.3 & 3.4) and SDHA (Figure 3.5 & 3.6), showing that each primer pair was only amplifying a single product. The efficiency testing of the FSH and LH primers was also carried out using the same approach, however the data is not shown.

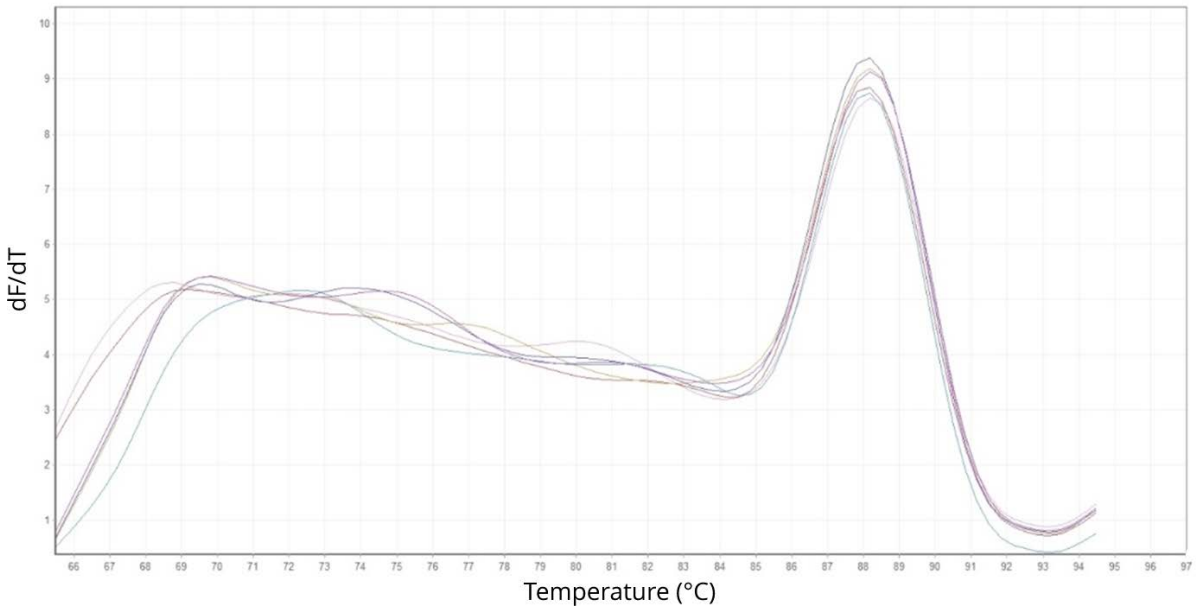

Figure 3.1: Melt curve obtained using β-actin primers.

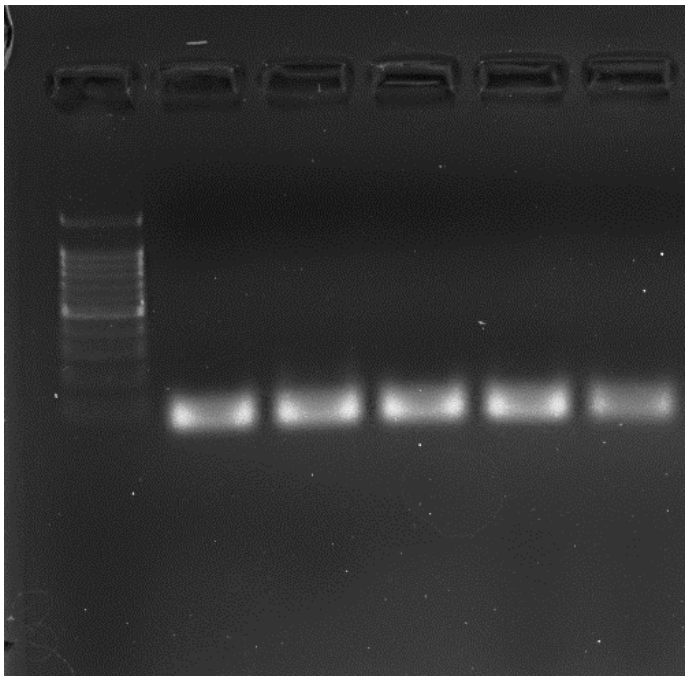

Figure 3.2: Image showing β-actin qPCR products that were amplified and run on a 1.5% agarose gel.

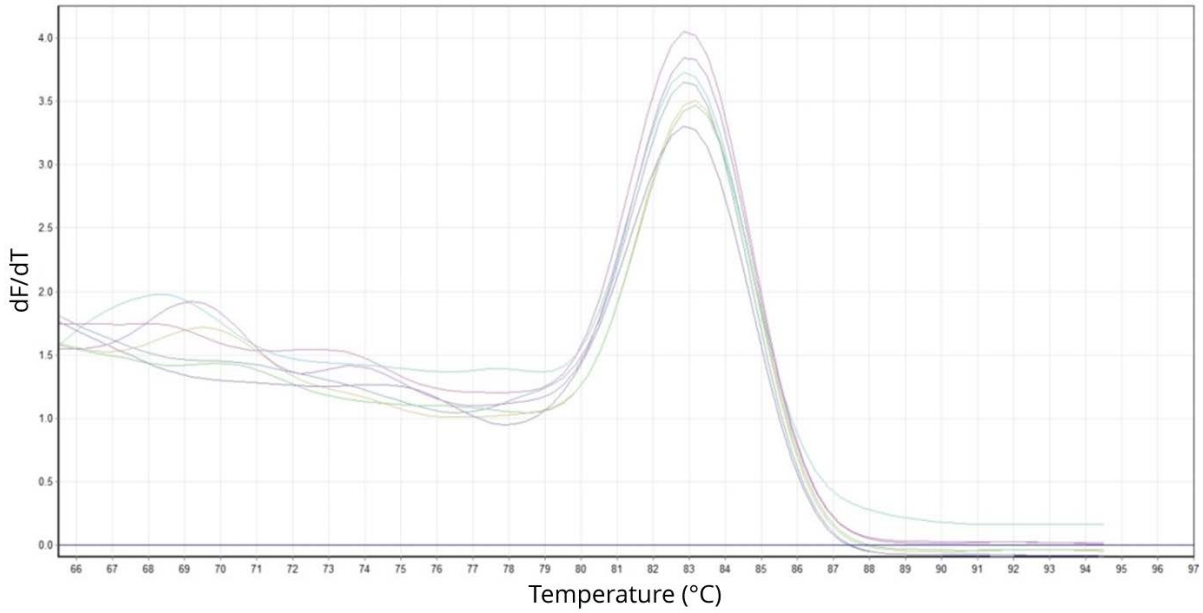

Figure 3.3: Melt curve obtained using B2M primers.

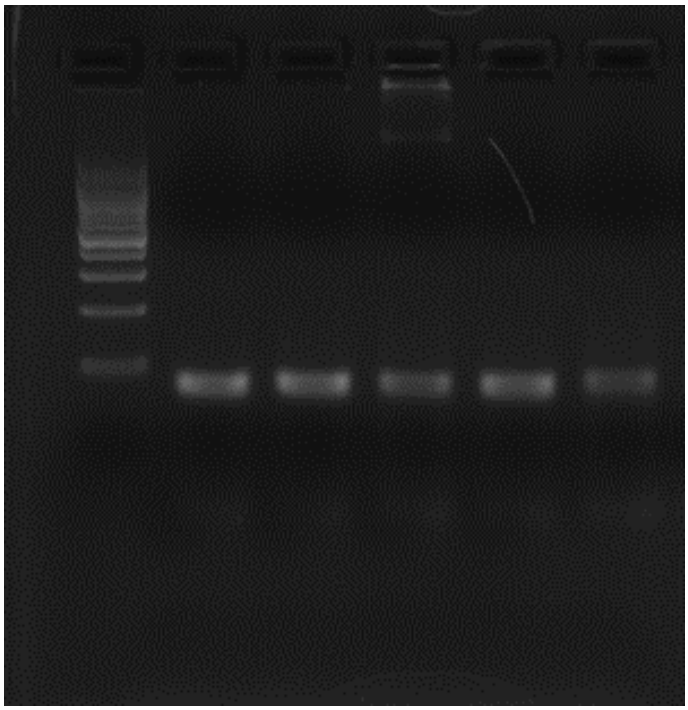

Figure 3.4: Image showing B2M qPCR products that were amplified and run on a 1.5% agarose gel.

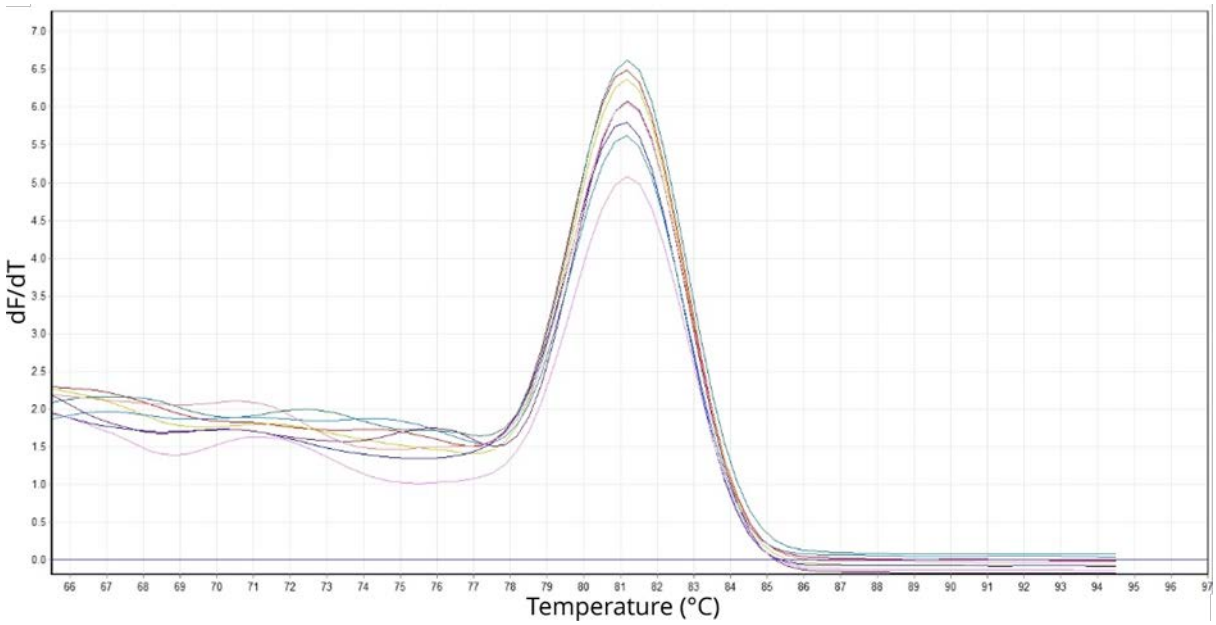

Figure 3.5: Melt curve obtained using SDHA primers.

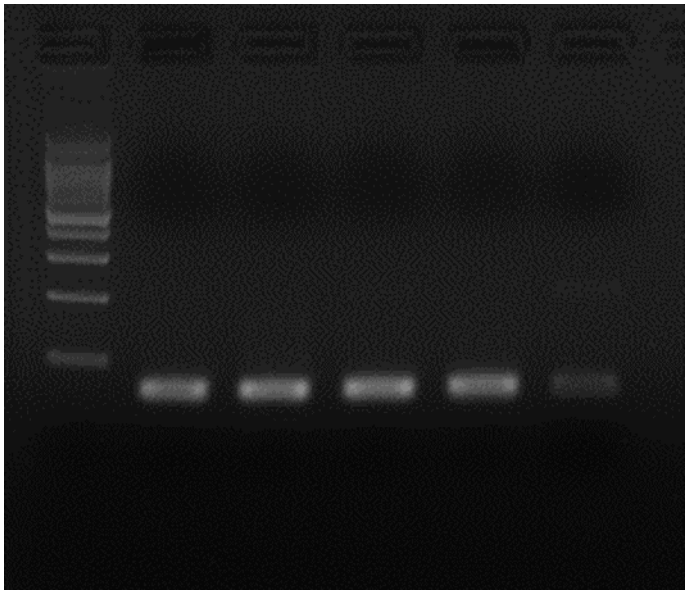

Figure 3.6: Image showing SDHA qPCR products that were amplified and run on a 1.5% agarose gel.

The geometric mean of the β-actin, B2M and SDHA genes were used to normalize the expression of the FSH and LH genes. As all the primers being used had an efficiency of 2.0, it was possible to use the delta-delta Ct (2–ΔΔCt) method to determine the fold change between the fish implanted with a sham or GnRH. To analyse the data statistically, a T test was applied to the data to derive a *P*-Value, to identify if the data was significantly different.

In the pituitary, FSH expression increased around 2-fold in the GnRHa implanted fish with a fold change value of 2.30 (Figure 3.7). Control fish were seen to have a smaller amount of quantified FSH in the pituitary with a fold change value of 1.24. However, this was not supported statistically (p= 0.07).

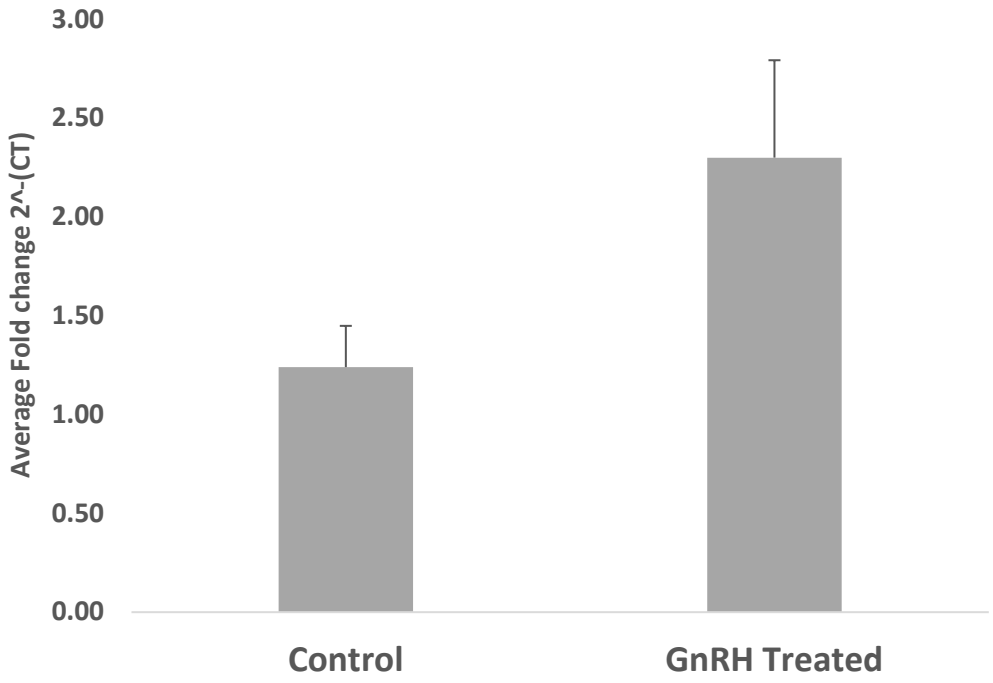

Figure 3.7: Mean relative pituitary FSH expression (fold change) in GnRH and sham treated yellow belly flounder (n=10), error bars are S.E.

Pituitary LH expression was slightly higher in the sham implanted fish (Figure 3.8). It was observed that there was a mean relative fold change of 1.32 in the sham treated fish. This compared to a fold change of 1.25 in the GnRHa treatment group. However, this was not supported statistically (p= 0.85).

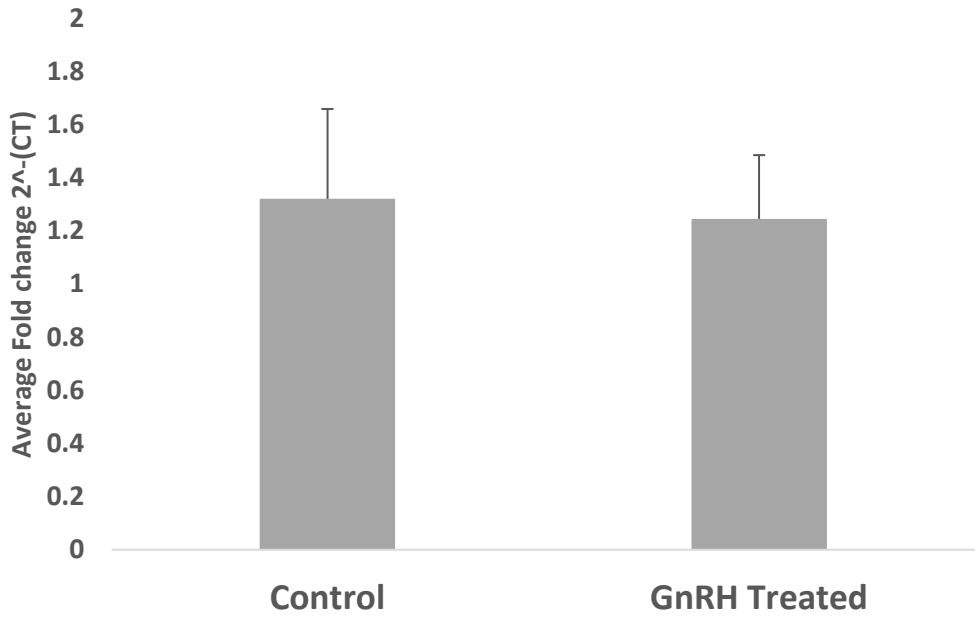

Figure 3.8: Mean relative pituitary LH expression (fold change) in GnRH and sham treated yellow belly flounder (n=10), error bars are S.E.

Pituitary LH levels were seen to have a positive correlation with GSI levels (figure 3.9). Gonadosomatic index increased with an increase in LH production at the pituitary level. It was seen that LH levels between individual samples ranged from 0.05 to 2.68±0.88SD. Gonadosomatic index ranged throughout the samples from 0.50 to 13.29±2.86SD. However, this was not supported statistically P>0.05.

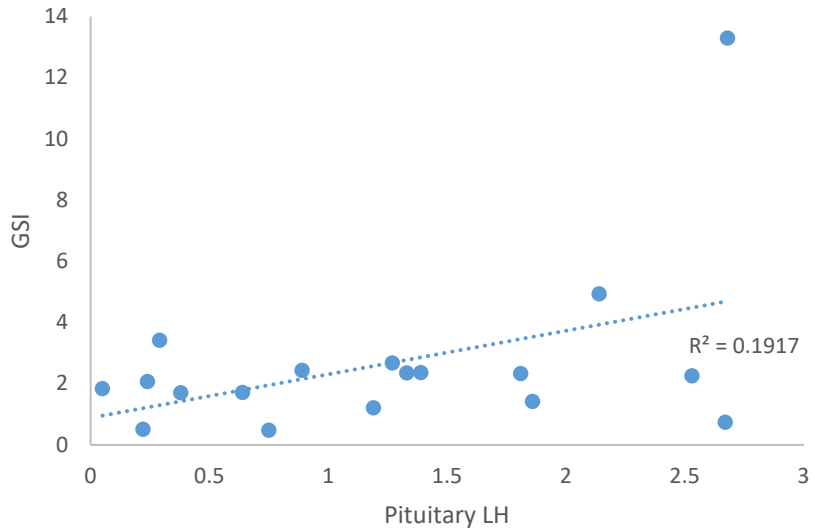

Figure 3.9: correlation between pituitary LH and GSI for both trial groups of fish.

No evidence for a correlation between Pituitary FSH and GSI was observed (R^2=0.0072). Pituitary FSH levels ranged within individual samples from 0.23 to 5.40±1.24SD (figure 3.11). Average FSH levels between the control groups showed that FSH was higher in the GnRHa treated fish with an average FSH level of 2.19± 1.69SD. Sham implanted fish were seen to have an average FSH level of 1.24±0.66SD. This result was statistically insignificant (P>0.05).

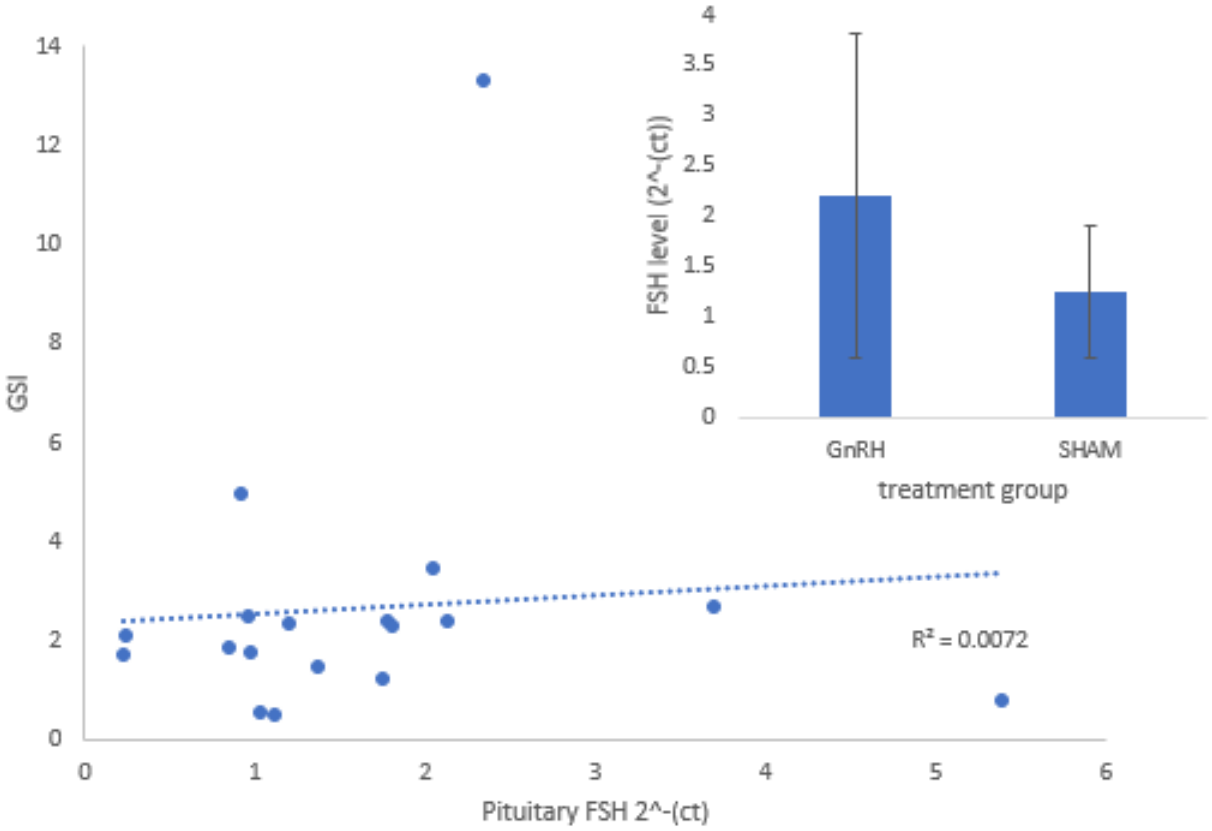

Figure 3.11: A) regression relationship between individual fishes pituitary FSH and GSI. B) Mean relative FSH (fold change) for GnRH and sham treated yellow belly flounder. error±SD.

4.0 Discussion

4.1 Primer efficiencies

To ensure bias within genetic samples is removed, samples need to be run against control genes (housekeeping genes), to normalise mRNA levels between individual samples run (Silver et al., 2006). These housekeeping genes need to be adequately expressed in the sample tissues, and show minimal variability in the expression of mRNA within samples. Within this study, the housekeeping genes used for normalisation consisted of 3 genes; B2M, SDHA, and β-actin. These genes, along with FSH and LH, were analysed using the PCR method, to ensure normal mRNA transcription was taking place. All housekeeping genes (B2M, SDHA, and β-actin), run on qPCR showed the presence of only 1 product being formed (figures 3.1-3.6). The presence of only one peak symbolises the presence of only one PCR product (Valasek & Repa, 2005; Vandesompele et al., 2002). This single peak was seen in the genes B2M, SDHA, and β-actin (figures 3.1 to 3.6), showing normalised PCR mRNA expression.

4.2 Gene regulation of FSH and LH in the Pituitary

Pituitary FSH was seen to have a greater fold change over the duration of the trial in the GnRHa treated fish when compared to the sham implanted fish. Gonadotrophin releasing hormone analogue was seen to have less effect upon LH levels in the pituitary, with a higher fold change seen in the control group compared to the GnRHa treatment group. However, neither were supported statistically ($P>0.05$). FSH was seen to have the greatest difference in fold change between the treatment groups (GnRHa and control). This shows insight into the role of GnRHa as a stimulatory cue on the pituitary. The GnRH stimulating the pituitary to produce FSH as a response. This is shown by the fold change response, which at 2.3 was approximately double that of the control implanted fish (Figure 3.7 & 3.8). This variation in the levels of both FSH and LH within the pituitary could be a result of the method in which the GnRHa was administered into the fish. With GnRH being a hormone released in a pulsatile manner from the hypothalamus, the regulation of the gonadotrophins FSH and LH can be impacted by the frequency of the GnRH release (Thompson & Kaiser, 2014).

The transcription and secretion of FSH has been seen to respond to a decreased frequency of GnRHa release (Thompson & Kaiser, 2014). In contrast, LH responds better to a higher frequency of pulsating GnRH release, resulting in a greater amount of LH gene transcription (Dalkin et al., 1989; Thompson & Kaiser, 2014). FSH was found to be expressed in greater levels within the pituitary after treatment with GnRHa. This could be a result of the implants releasing a dose of GnRHa over a short period, resulting in a greater level being produced, as it is required in the earlier stages of oocyte maturation when compared to LH. For reliable LH production, it may require exposure to higher pulse frequencies of GnRH (Thompson & Kaiser, 2014). It plays out its role in the final stages of oocyte maturation. This is also supported by the results seen within the control implanted fish. In these fish there was little difference between the control and GnRHa treatments of LH, and a larger difference was in the FSH control and GnRHa treated groups. This is due to the control not receiving the hormonal cue of GnRH to stimulate the production of either of the gonadotrophins.

When fold changes were compared with other trials, gonadotrophin expression was seen to be low in comparison with other fish species. Coho salmon, when injected in the spring before breeding season, were observed to react with an upregulation of FSH showed by an average fold change of approximately 4 (Dickey & Swanson, 2000). This was almost double that observed in this trial on YBF, which had a fold change of 2.3. In a study conducted by Mateos et al (2000), varying results were found in sea bass when injections of GnRHa was administered at a time of sexual resting. It was observed that GnRHa induction resulted in a 2-fold increase of pituitary LH levels with no change in pituitary FSH. This was due to the high levels of sex steroids circulating resulting in FSH's downregulation (Mateos et al., 2000). These two examples show the importance of when to implant with GnRHa, depending on the response wanted, and could be reason for the results found within this trial.

During fish capture from this YBF study, an absence of fish in the shallow intertidal flats over the June and early July period was observed. This is presumably due to their spawning habits. They migrate to deeper water to spawn over the period from June to November (Colman, 1973). Fish caught within the month of May were observed to have good body condition compared to the fish captured in July. This variance in fish condition with month of capture could be because in May the fish were pre -spawn, and in July they were post-spawn fish. Loss of body condition (fat content) has been observed in Norwegian caplin. Caplin were seen to have an average muscular lipid loss of 38% from lipids being bio-converted from muscular lipids to ovarian lipids, and used in follicle maturation (Henderson, Sargent, & Hopkins, 1984).

4.3 Pituitary abnormalities

High pituitary levels of both FSH and LH were observed in an individual fish which, appeared to have no gonad during dissection. Follicle stimulating hormone within this fish was seen to have a fold change value of 3.99, and LH was observed to have a fold change of 2.69. The higher values observed in this abnormal fish could be a result of there being no gonad to produce steroidal feedback loops for the down-regulation of these gonadotrophins. Similar findings have been seen in castrated fish species such as catfish and salmonids, where the lack of gonadal tissue results in higher plasma and pituitary levels of LH (Rebers et al., 1997). This trend was also seen in castrated red seabream (*Pagrus major*) where there were elevated levels of FSH within castrated fish after GnRHa treatment. It was suggested that this was due to the lack of production of 11 ketotestosterone in the Testis, which down regulates FSH production (Yamaguchi et al., 2005). Rainbow trout have seen to have a 4-fold increase in plasma GtH levels after bilateral castration due to loss of feedback from the gonad regulating GtH production at pituitary level (Billard, Richard & Breton, 1977).

4.5 GSI in relation to gonadotrophin levels

Gonadosomatic index and gonadotrophins FSH and LH were observed to have similar correlative relationships. There was no correlation between FSH and GSI (figure 3.12) where R^2=0.007 and P>0.05. Luteinising hormone was also seen to have no significant correlation with GSI R^2=0.19. This showed that an increase in levels of both FSH and LH had little to no effect upon the overall GSI in individuals. Gonadotrophin LH is responsible for the maturation of the gonad, in which oocytes increase in size at a rapid rate due to hydration. These hormones are hypothesised to have a significant influence on gonad size (Ibarra-Castro, 2007). This is because of their flow-on effects causing oocytes to increase in size five-fold, from previtellogenic oocytes to late vitellogenic oocytes and hydration.

The correlation between gonad growth and overall organism weight, and gonad growth versus gonadotrophin (FSH and LH) levels has been identified within other fish species. Seasonal fluctuations such as temperature and photoperiod, throughout spawning months have led to strong relationships between body weight (g) and degree of gonadal development (Bromage et al., 1992; Thorpe, Miles & Keay, 1984). Fish exhibiting greater growth were found to be more advanced in gonadal maturation stages (Campbell et al., 2006). Upon dissection, this growth was also seen to be associated with the accumulation of cortical alveoli. This accumulation was in turn associated with an increase in pituitary FSH levels (Campbell et al., 2006). Although this strong correlation was not found for the YBF trialled within this experiment, seasonality and stress may have played a large part in the results gathered. In a previous pilot study in the months of May/June, it was observed that implanted fish had a much greater response to GnRHa implantation. In these implanted fish, a greater percentage (17.6%) were seen to reach full oocyte maturity and ovulate. This compared to only 5% of the fish reaching ovulation when implanted in August. The findings from Campbell et al (2006), also show seasonal trends with gonadal growth and expression of FSH in coho salmon. Follicle stimulating hormone and gonad growth both spike for only short periods of time before decreasing again, symbolising a distinct spawning season.

Along with low levels of FSH, there was a high presence of atretic oocytes in many of the ovaries of the sampled fish. These atretic oocytes are commonly associated with the end of a spawning season (Merson et al., 2000). This represents a gonad which has already spawned, or has aborted oocyte development. This aborted oocyte development within other fish species may be stress induced, as has been seen in North Atlantic cod. When stressed by poor nutrition and changing water temperatures aborted oocyte development was witnessed. As a result, these fish never spawned in the trialled season (Rideout et al., 2000). Stress related oocyte abortion could easily be related to flounder species. Flounder species suffer from high stress levels in captivity, and undergo reproductive failure as well as failure to feed, within captive settings (Garcis-Lopez et al., 2006; Guzmán et al., 2009). Atretic oocytes are also a strong indicator that the gonad is from a fish that has already spawned. This seasonal gonadal development has been observed in other flatfishes and teleost's alike (Merson et al., 2000).

Luteinising hormone is seen to have a minimally stronger relationship to GSI than FSH in this trial. However, any correlation was still very weak R^2=0.19. This weak relationship may be due to the fact that LH only plays a role in the final stages of oocyte maturation, once the majority of weight has already been gained within the gonad (Campbell et al., 2006). A positive correlation has been made linking LH, GSI and oocyte diameter within striped bass (Holland, Hassin & Zohar, 2001). It was observed that with higher levels of LH there was an increase in both GSI value and an associated increase in oocyte diameter these relationships were supported by P-values where $p<0.05$. This was observed to a lesser extent in this trial. However, the increase can potentially be associated with weight gained within the final stages of maturation. This weight gain is a result of hydration of oocytes where there is a large uptake of fluid into the oocyte (Murua & Saborido, 2003). Histologically within this trial, hydration was not observed, and therefore could be the reason behind the weaker correlation seen between LH and GSI, in comparison to other studies (figure 2.2).

Other related studies have also seen positive relationships between oocyte growth and concentrations of various important reproductive hormones such as vitellogenin, E2 and 11-KT (Sun & Pankhurst, 2004).

Conclusion

Gonadotrophins FSH and LH are crucial for the reproductive maturation process of the gonad. The release of these gonadotrophins is regulated by hypothalamic secretion of GnRH. qPCR is a method used in which levels of these hormones can be detected in minute quantities, through translation of the target DNA. This gives the ability to quantify the amount of target gene within a sample, and can be used for comparative analysis. Follicle stimulating hormone and LH were both seen to be present in the pituitaries and gonads of all trialled fish. Both gonadotrophins within the pituitary were seen to be in higher quantities within the GnRHa treated fish.

In conducting future research, larger trial groups should be used to ensure more robust conclusions can be drawn from results. qPCR could also be used to detect quantities of GtH-Rs within the gonad. This would allow researchers to obtain a more accurate representation of the link between the gonadotrophins being secreted at the pituitary, and their corresponding influence on the gonad. Seasonality could also be examined to see if there are difference in the effectiveness of GnRHa treatment across varying stages throughout the annual reproductive cycle. In this trial there was an absence of fish during the month of June within the capture site. This was presumably due to them leaving the flats and going to deeper water to breed. In the late July period they returned but in poor condition. Therefore, knowledge of seasonal times for optimal injection results would be useful in order to successfully inject YBF with GnRHa in the future. Ascertaining times when they will leave areas which are accessible to capture efforts would allow for them to be caught before this point when naturally they are readying for the spawning season.

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Chapter four- Embryonic development in yellow belly flounder and the use of embryonic scoring as a potential grading system

Introduction

Aquaculture importance & embryogenesis

The aquaculture industry and wild fish populations alike are heavily reliant on the production of high-quality gametes, in particular, eggs (Bromage et al., 1992; Brookes, Tyler & Sumpter, 1997). Egg quality can therefore be defined as the ability of an egg to develop into a normal embryo without abnormalities. Sperm quality can be defined as its ability to fertilise a normal egg producing a viable embryo and subsequently zygote (Bobe & Labbe, 2010). Poor egg quality is one of the major constraints in the expansion of aquaculture for many finfish species. In fish farming high quality eggs characteristically exhibit lower mortalities and deformation post fertilisation (Brookes, Tyler & Sumpter, 1997). This results in regular supplies of larvae and juveniles which are essential for aquaculture production (Bromage et al., 1992). To be useful and practical, assessments need to be made before the incubation process. This relies on a comprehensive understanding of egg development and early morphological patterns (Kjorsvik, Mangor-Jensen & Holmefjord, 1990).

It is therefore essential to assess egg quality as a measure of egg batch survival and brood quality. A common way of doing this is through the assessment of gametes. Assessments look at sperm analysis, egg morphology, fertilisation and critical stages throughout embryonic development. These critical stages include cleavage, blastula, gastrula and segmentation.

The first stage of embryonic development involves cell division called cleavage. This occurs in the blastodisc, a yolk free region at the animal pole of the egg. During this stage, several mitoses occur within the zygote to form the new embryo (west, 1990). Cells produced at this stage are blastomeres (Murua & Saborido, 2003). The embryo at this stage is called a morula- a compact mass of cells.

At the end of cleavage, these cells divide, each becoming smaller in size. A large number of oil granules pass out from the cell mass into the yolk where they combine into larger globules. Cleavage in teleost fish is limited to blastodisc. Multiple cells are formed, and only the blastodisc will become the embryo (Kimmel et al., 1995). At the end of segmentation, the blastodisc becomes symmetrical and thickens. This thickened sector is highly important as it is embryonic material from which the future embryo will develop. Its median plane becomes the median plane of the embryo (Ninhaus-Silveria, Foresti, & Azevedo, 2006).

Cleavages result in two kinds of cells; periblast or blastoderm. Blastoderm produces the embryo. Periblast cells lie between the yolk and the blastoderm cells, and they cover the entire yolk (Kimmel et al., 1995). After passing through the morula stage, the compactness of cells is lost and an internal fluid-filled cavity appears inside it, called the blastoceol (Arezo, Peveiro, & Berios, 2005).

The blastula turns into the gastrula via the process of gastrulation. Through this process a portion of the blastula wall invaginates inside the blastoceol, forming a tube called the archenteron; a primitive intestine (Ninhaus-Silveria, Foresti & De Azevedo, 2006). The cells of the inner side of the tube form a germ layer called the endoderm (Ninhaus-Silveria, Foresti & De Azevedo, 2006; Stahl & Kruse, 2008). The cells of the outer side form the ectoderm. This is the start of tissue differentiation in embryonic development. The blastopore formed during gastrulation is archenteron's external opening which forms one of the extremities of the digestive tract. This is either the mouth in protosome organisms, or the anus in deuterostome organisms.

Gastrulation ends with closure of the blastopore. At the same time as blastopore closure, the whole germ ring fuses with the developing embryo which is now well demarcated from the yolk (Stahl & Kruse, 2008). After this, optical vesicles develop, the head of the embryo becomes differentiated, brain develops, and heart and tail development occur.

After development of various organs, the embryo body becomes cylindrical and symmetrical. Connection between body and yolk sac gradually narrows to form a stalk. The yolk sac decreases in size as the embryo grows.

Finally, the embryo hatches into a free-swimming larva.

Larvae, if deformed, show signs of abnormalities by late segmentation, including spinal and body plan deformations. These deformities are often due to gaps in the knowledge of critical developmental stages. This is common in aquaculture, especially where the rearing process is new (Divanach et al., 1996). However, these malformations/deformities may have large consequences on economics, due to downgrading of final products and biological performances of reared fish. Ultimately this influences market production cost as well as market value (Divanach et al., 1996).

Gamete quality & Fertilisation

The rate of fertilisation can be used as another determinate for spawn quality. Rates of fertilisation can be used to detect egg or sperm quality (Kjorsvik, Mangor-Jensen & Holmefjord, 1990). Poor gamete quality may result in a poor hatch rate and fewer larvae being produced. It is therefore critical to determine fertilisation rates as a means of batch quality. This then determines whether individual batches of eggs are worth carrying through the incubation process.

However due to lack of research into YBF as an aquaculture species, fertilisation cannot be used as a single determinate for spawn quality. Further research is required to establish whether fertilisation rates, hatch rates, percentage deformities and embryo symmetry can be confirmed as strong determining factors of gamete/spawn quality.

Activation of the cortical reaction, which occurs after fertilisation, sometimes fails in various species of teleost fish (Kjorsvik, Mangor-Jensen & Holmefjord, 1990). This incomplete activation can result in poor growth within the egg, and sometimes a complete failure for the egg to develop, resulting in termination. Incomplete activation is therefore a strong sign of poor egg quality. This can be seen in various flatfish species such as turbot (Kjorsvik, Stene & Lonning, 1984; Kjorsvik, Mangor-Jensen & Holmefjord, 1990).

Embryonic scoring

In most fish species blastomeres, cells formed by cleavages of fertilised eggs, size and shape are uniform during the cleavage stage of embryonic development (Shields, Brown & Bromage, 1997). However, deformities within blastomere morphology can be seen as a result of physical and chemical stressors that come in contact with the egg. This provides a unique opportunity to grade egg quality, as deformities within embryonic development are genuinely associated with low egg quality (Shields, Brown & Bromage, 1997). Without a way to score spawn quality, viability of larvae cannot be guaranteed. This hugely impacts aquaculture production. It becomes a time and resource drain as futile effort is spent on egg batches with low survival potential (Bardon-Albaret & Saillant, 2017).

Abnormalities within the 8 cell blastomere stage are a useful indicator of low egg quality (Shields, Brown & Bromage, 1997). The assessment of these fertilised eggs is generally based on 5 blastomere characteristics.

These include shape, size, cohesion of cells, symmetry and cell boundary definition (Shields, Brown & Bromage, 1997). Within this study embryos were scored at the 8-cell stage, and cleavages from the first to the 64th were documented. This is similar to the study done by Kjorsvik, Hoehne-Reitan, & Reitan (2003).

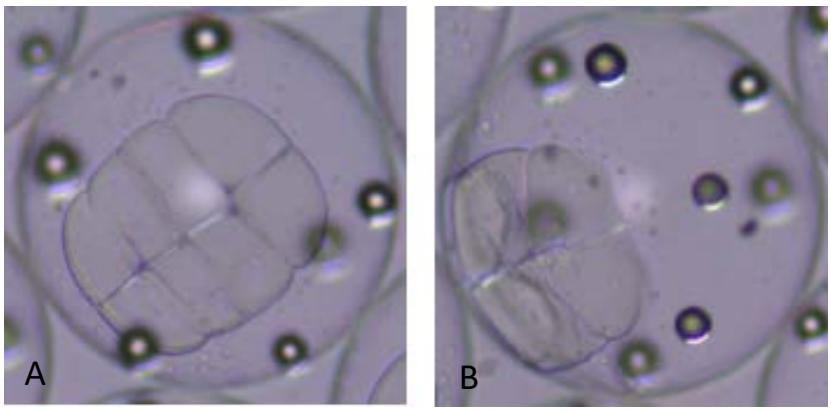

Figure 4.1: Early development of yellow belly flounder eggs at the 8 cell blastomere stage. High quality embryos (A) carry out symmetrical cleavages, when compared to poor quality eggs (B) which are observed to have irregular cleavages resulting in large amounts of malformation.

Methods

Collection

During the months of May/June, YBF were collected from within the Tauranga harbour by dragnet method, in conjunction with another study being conducted out of Toi Ohomai Institute of Technology. Fish were transported a short distance and held within 1600 litre tanks at the Toi Ohomai campus. These tanks were run on a recirculating system and the bottoms were sand covered. Fish were given an acclimatisation period of a week before being handled. This allowed for recovery from capture, transport and handling stress.

Implantation

Fish were anaesthetised in a water bath using 2-phenoxy-ethanol (2-PE) in a ratio of 0.5ml of 2 PE per litre of water. They were then given a plastic floy tag using a floy tagging gun halfway down the body on the dorsal side of the fish, 1 centimetre in from the dorsal fin. Fish were then assigned to trial groups depending on the floy tag colour. These were either the treatment group (GnRHa implanted) or sham group (sham implant containing no hormone). These treatments were delivered in an implant gun. Treatments were injected under the skin an inch down from the head and 2 centimetres to the left of the spine. They were administered in the form of slow release pellets that had 500µg of GnRH per gram of weight.

Observation, strip spawning & fertilisation

Fish were checked daily for signs of gonadal growth and ovulation. When fish were displaying an extended gonadal bulge, starting in line with the gonadal pore and working its way back towards the tail, this was an indication that they were ready to ovulate. These fish were then tested for ovulation by gently pressing on the gonadal region. If eggs were passed out the gonadal pore this was an indicator that ovulation had started and strip spawning could commence. The fish were then sedated in a saltwater bath containing 2-phenoxy-ethanol in a ratio of 0.5:1000. This was the recommended treatment ratio seen in Weber et al 2010, with his study involving anaesthetics in Senagol sole. Once fish's inhalation rates and movements had slowed, they were removed from the aesthetic bath.

They were dried carefully using paper towels to remove all water off the skin. Having done this, the fishes eyes were covered and they were placed on the edge of the working bench. Carefully using the index finger and thumb, the gonad region was rubbed from the tail up to the duct where the eggs would be released into a clean collection container. This same procedure was also used on the males to collect milt.

Once this was done, egg volumes were taken. The eggs were then placed into a jug containing 500ml of water, where 50µL of sperm was then added. The sperm and eggs were mixed for two minutes. Following this the eggs were washed to prevent polyspermy. This was done by sieving the eggs through a fine 200-micron mesh sieve. Fertilised eggs were then disinfected in a glutaraldehyde saltwater bath containing 400mg of glutaraldehyde per litre of seawater. Eggs were mixed in this solution for ten minutes then rinsed thoroughly using filtered seawater. They were then added to incubators and allocated to various temperature treatments.

A sample of the eggs was taken to look at fertilisation rate. This was determined by looking at 100 eggs and noting the percentage of fertilised to unfertilised. This count was done 4 times and an average of the four counts was taken as the percentage of fertilised eggs for each batch.

Eggs were divided into differing temperature treatments of 17 and 19 degrees Celsius. Each batch was monitored for development progress using a light microscope (Leica DFC290) with a camera attachment. Photos were taken each time changes were observed in the developing embryos. The time was documented to give reference to the length of time between fertilisation, different embryonic development stages and hatch. Deformities were photographed to be used in making up a blastomeric scoring chart. Development was considered deformed if the cell cleavages were observed to deviate from normal uniform cleavages, or other morphological differentiation was observed, such as deformation of the body axis. Each time the embryos were seen to have any morphological changes photos were also taken and times documented.

Results

Embryonic development

Embryonic development was recorded photographically. Development reference photo grids were made up to document major embryonic stages and descriptions were given (figure 4.2 & table 1). It was observed that the timing of stages varied with temperature. Cleavage stages were photographed from the first division to the 3rd division (8-cell) (figure 2). Further documentation of gastrula, blastula and segmentation were recorded and times taken to calculate times between fertilisation, stages and hatch. At blastula the formation of the blastocyst is clear, sitting separated from the blastoceol by the syncytial layer (figure 2(4)). Gastrula development was classified once cell layers could be seen, with epidermal and endodermal layers being observed. The presents of a rudimentary primary gut can be made out and both animal and vegetal poles are clear (figure 2(5)). At segmentation, general body plan can be made out with the presence of both eye buds and tail bud being formed (figure 2(6,7)). Towards late segmentation the outline of the yolk sack can be clearly made out. Oil droplet averages were also counted within each of the stages to see if they were to differ throughout development.

It was seen that in the 8-cell blastula stage (Figure 4.2, image 3) there was the largest average number of 9.14 oil droplets per egg. Oil droplet number then slowly decreased towards hatch with the fewest droplets being seen in the late segmentation stage where there was an average of 4.93 oil droplets per egg (Figure 4.2, image 7).

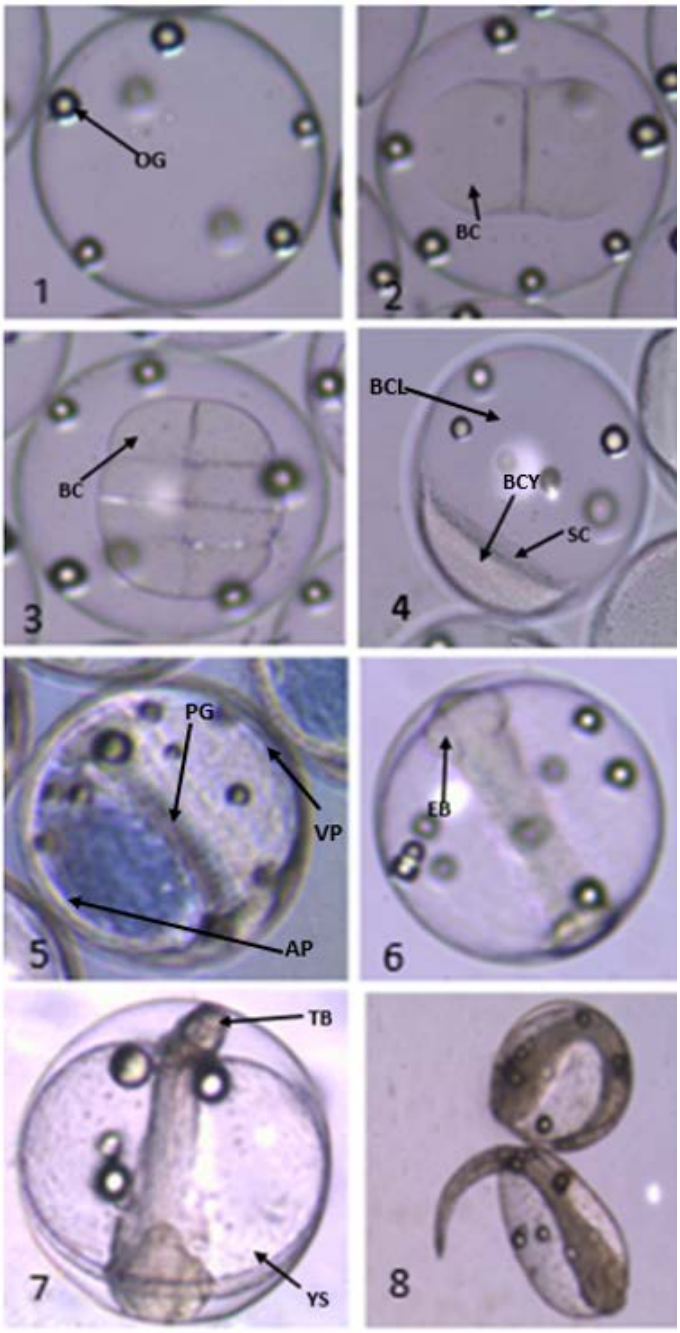

Figure 4.2: Normal embryonic development of yellow belly flounder at crucial embryonic stages, incubated at 17 degrees Celsius. 1) fertilised egg (OG, oil globule) 2) first cleavage of cells bring it to the 2 cell blastomere stage (BC, Blastomere cell), 3)3rd cleavage/ 8 cell blastomere (BC, Blastomere cell), 4) blastula (BCL, Blastoceol; SC, Scncitial layer; BCY, Blastocyst), 5) Grastrula (VP, Vegetal pole; AP, Animal pole; PG, primary gut), 6) early segmentation as body plan forms (EB, eye bud), 7) Late segmentation (YS, yolk sack; TB, tail bud), 8) hatch.

Table 1: Brief descriptions of how each Developmental stage has been classified within this study from cleavage to hatch.

Development Stage	Description of stage
Cleavage	• Occurs from the first meiotic division to the formation of the blastodisc.
Blastula	• Formation of blastocyst • Sencitial layer present • Blastocoel • Animal and vegetal poles established
Gastrula	• Visible tissues layers present consisting of the epidermis, mesoderm and endoderm. • Formation of primary gut • Clear cell polarity
Segmentation	• Formation of tail and eye buds • Embryo largely encompasses yolk sack
Hatch	• Embryo breaks through Chorion becoming larva

Time taken to reach each crucial embryonic stage was recorded for both temperature treatments trialled (17 and 19ºC). It was observed that the time taken from fertilisation was approximately 68 hours for the 17ºC treatment (figure 1). However, this time was significantly reduced with the raised temperature of 19ºC.

Time taken to reach the 8 cell blastomere was also seen to be significantly shorter with raised temperature. The batch incubated at 19ºC took Approximately 2 Hours and the 17ºC took approximately 2.5 hours to reach this stage.

Table 2: Time taken from fertilisation for each of the critical embryonic development stages to be observed. (embryos incubated at 17°C).

Development stage	Time (hr:min)
Fertilisation	0.00
First cleavage	1:40
8-cell blastomere	2:20
Blastula	18:25
Gastrula	27:45
Early segmentation	29:00
Late segmentation	31.50
Complete embryo	48:50
Hatch	68:10

Blastomere scoring

Blastomere scoring was based upon various levels of malformation observed at the 8 cell blastomere stage within development embryos. Scoring was ranked from one to five. one indicated a perfect embryo with good cell parameters of size, shape, symmetry, cell cohesion and clarity of cell boundaries. A score of five indicated high malformation within all of these aspects (Figure 4). It was observed that with good quality eggs, each of the 8 cells were easily identifiable with little to no overlap. As quality decreased, uniformity, cell overlap and shape irregularity became greatly increased (Figure 4.3).

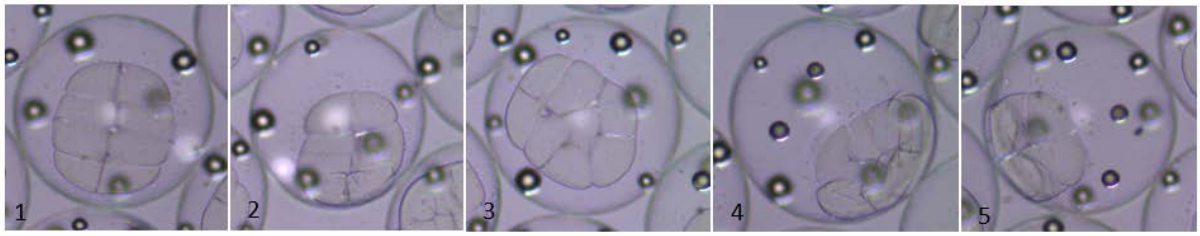

Figure 4.3: Blastomere scoring reference chart where 1) is a well-developed cleavage with good symmetry between cells, within the embryo to 5) which shows severe deformation in cleavages at the 8-cell stage with little symmetry. Symmetry between cells decreases with an increasing scoring number. Where grading is based upon uniformity of cells; Size, shape, symmetry, cohesion, and clarity of cell boundaries.

Temperature related development impacts

Rate of development was seen to vary between the two temperature treatments (figure 4.4). It was observed that at a higher temperature of 19ºC, embryos developed at a much quicker rate. Both the 17ºC and 19 ºC embryos developed over the same time to the first cleavage, but each cleavage after this was achieved at faster rates by the eggs fertilised at 19 ºC. The eggs incubated at a higher temperature were seen to reach the 64-cell stage approximately 70 minutes earlier than the eggs incubated at 17 degrees.

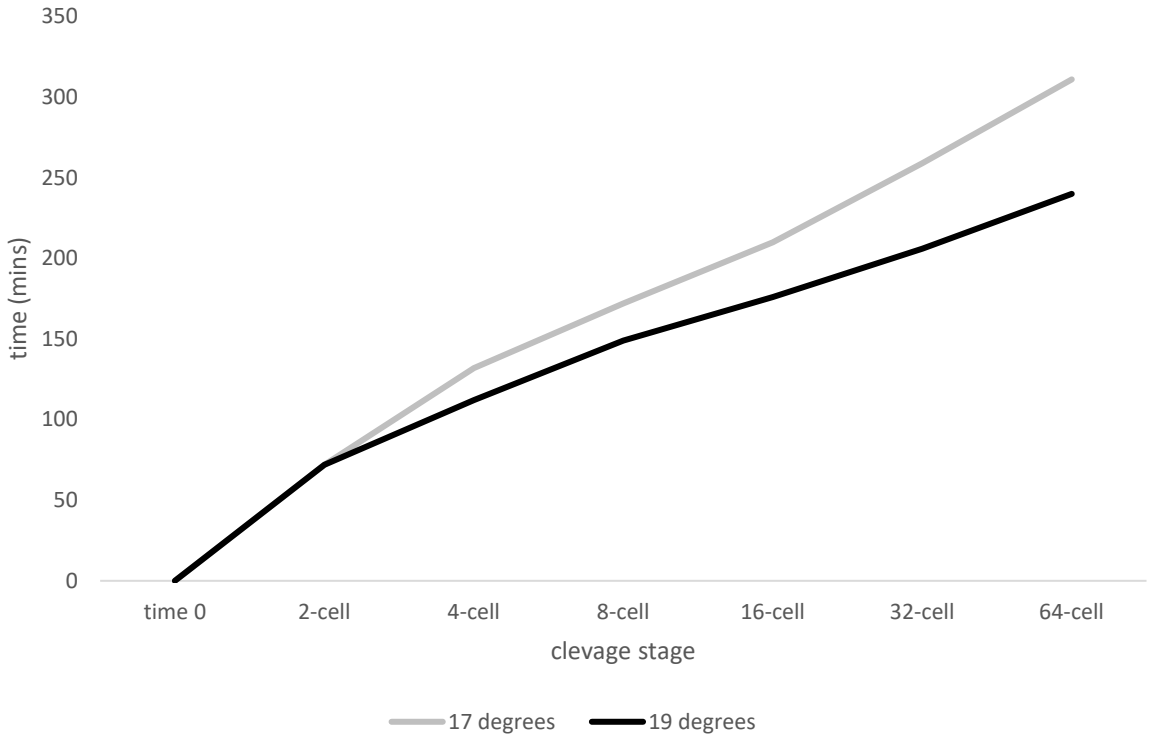

Figure 4.4: rate of embryonic cleavages at two trialled temperature 17ºc and 19ºc for YBF.

Discussion

Egg Quality and embryonic development

Egg quality can be determined by internal properties of the egg. These properties include symmetry, cohesion, shape and oil droplet size and number, as well as the environment within which the egg was fertilised and incubated (Brooks, Tyler, & Sumpter, 1997). It is hard to narrow down the determinants for the quality of individual batches of eggs as they are affected by multiple factors. It is therefore crucial for hatcheries to run at optimal external parameters for the development of normal embryos (Kjorsvik, Mangor-Jensen & Holmefjiord, 1990). The development of normal embryos can be affected by a multitude of factors, including the condition of broodstock at the time of spawn. Other studies have found that parental nutrition and stress levels can tamper with the integrity of embryos (Kjorsvik, Mangor-Jensen & Holmefjiord, 1990; Rosenthal & Alderdice, 1976). A study by Tyler & Dunn (1976), observed that within another species of flounder, the winter flounder, food restrictions and feeding regimes were related to the viability of embryos. They found large potential for improvement around captive reproduction. More studies need to be focussed around how feed can affect fecundity, maturation and oocyte quality (Kjorsvik, Mangor-Jensen & Holmefjiord, 1990). In another species of flatfish, the turbot, it has also been observed that captive reproduction can be extremely problematic when compared to round-fish species within similar conditions (Devauchelle et al., 1988).

A study conducted by Devauchelle et al (1988), concluded that problems with captive spawning and embryo development could be due to the vast difference between captive conditions and tank simulated conditions. It was suggested that more emphasis be placed around studying temperature during spawning periods. They observed that at above or below optimum temperature there were a greater percentage of deformities within embryos. With flounder frequenting subtidal areas of harbours and mud flats, this could make their spawning habits susceptible to tidal influences. As flounder within this trial were held in fixed volume tanks there was no tidal influence. Therefore, this lack of tidal influence may have interrupted natural feeding and spawning behaviour for fish in this study.

Flatfish within other studies have been extremely affected by tidal influence. This has been observed with the use of hydrolic pressure to simulate changes in water level. It was found that within flounder species there are fluctuations in flounder pituitary FSH when they are held within cyclic-hydrolic pressure conditions (Damasceno-Oliveira et al., 2014). With flounder being found in largely tidal areas, the lack of tidal influences in aquaculture could be an added factor to stress induced poor-quality gametes, resulting in lower fertilisation, hatching and survival rates.

Gonadotrophin releasing hormone analogue induction has also been seen to produce a lower quality of gametes than if fish are to spawn naturally. This has been hypothesised to be caused by disruption of the timing of FOM and ovulation, as these processes are sped up with the implantation of GnRHa (Mylonas, Hinshaw & Sullivan, 1992).

Embryonic development was characterised into 8 different key stages within this study from fertilisation to hatch. After fertilisation, cell cleavages were seen to occur for approximately 16.5 hours. Each cell formed represents a blastomere, and with each mitoses blastomeres grow smaller in size where the cluster off cells start to form the embryo (Ninhaus-Silveria, Foresti & De Azevedo, 2006). Moving into the blastula phase there is an observed division between the cluster of cells forming the embryo and a large fluid filled space which is known as the blastoceol which later becomes the yolk sac (figure 4.2, image 4). The blastoceol was separated from the blastocyst via the syncitial layer which is a membrane-enclosed layer that will later be involved in segmentation and the formation of the embryo (Arezo, Pereiro & Berois, 2005; Kimmel et al., 1995) Gastrulation can then be observed (figure 4.2, image 5) where there is the start of tissue differentiation. This can be seen through the layering of cells and formation of band like cell clusters. These layers consist of an epiderm, mesoderm (which becomes the embryo) and endoderm (Arezo, pereiro & Berois, 2005; Verissimo, Gordo & Figueiredo, 2003). It is here that the start of the digestive tract (figure 4.2) is formed shown by a tube which runs through the middle of the cell separated by a fluid filled sac (blastoceol) and the blastocap/blastoderm.

This early body plan can be classified as the somite and will continue to grow until becoming a fully formed embryo (Ninhaus-Silveria, Foresti & De Azevedo, 2006). Both the animal pole and vegetal pole are clearly defined, with the animal pole located at the top of the blastoderm and the vegetal pole located at the blastoceol end. At the end of gastrulation moving into segmentation there is a clear formation of the head and eyeballs (figure 2, image 6). The body plan of the larvae throughout these later stages becomes more and more apparent as the larvae grows closer to hatch, with the formation of the tail bud and clear definition of the embryo's yolk sack being seen in the late segmentation stage (figure 4,2 (7)). There is a reduction in the size of the yolk sac which can be seen when comparing figure 4.2, images 6 &7. In image 6 the yolk sac takes up the majority of the egg's internal space around the forming larvae. In contrast, in late segmentation (image 7, figure 4.2) the yolk sac can be seen to be pulling away from the external egg walls as the larvae uses it for growth. During late segmentation the tail also forms and can be seen to start wrapping around the diminishing yolk sac.

Oil globules were found to vary over the duration of the embryo's development. Within the majority of marine teleost fish species there is an observed decrease in the number of egg globules over the developmental stages between fertilisation and hatch (Holt et al., 1981). This trend was seen in the trialled fish embryos. There were the greatest number of oil globules present in the cleavage stages of development (figure 2, image 3) with an average of 9.14 oil globules per egg. This was seen to slowly decrease up until the late segmentation phase (figure 4.2, image 7) where an average of 4.93 oil globules per egg was found. These oil droplet numbers were high when compared to other studies done on teleost fish species. Ahlstrom & Moser (1980) found 25% of pelagic fish eggs lacked oil globules, 15% had 2 or more and 60% had a single oil droplet. Multiple oil globules however are common within select teleost fish species. Flatfishes as a whole are observed to have the largest number of oil globules recorded in teleost fish species (Ahlstrom & Moser, 1980). Within the early stages of development, the cohesion of multiple oil droplets can create a smaller number of larger droplets within the yolk. This may help with absorption when used as a fuel source for driving embryonic development (Ahlstrom & Moser, 1980).

Maternal nutrition, condition and stress

Parental nutrition and stress levels can tamper with the integrity of embryos (Kjorsvik, Mangor-Jensen & Holmefjiord, 1990; Rosenthal & Alderdice, 1976). A study by Tyler & Dunn (1976), observed that within another species of flounder, the winter flounder, food restrictions and feeding regimes were related to the viability of embryos. They found large potential for improvement around captive reproduction, with more studies needing to be focussed around how feed can affect fecundity, maturation and oocyte quality (Kjorsvik, Mangor-Jensen & Holmefjiord, 1990).

During this thesis trial conducted with YBF, treatment fish were observed to undergo stress- induced starvation. Over the course of the experiment there was no feeding observed from any of the fish, even with multiple sampled diet types. Diets trialled included squid, brine shrimp, mussel, crabs and blood worms. The lack of feeding may be stress- induced starvation and may have led to limited survival of embryos. Similar findings have been found in salmonids. When fish were injected with maternal cortisol, (cortisol taken from the blood plasma of females with mature ovaries) a reduction in offspring survival was noticed, and a reduction in the embryo's yolk sac volume. This led to the prevalence of morphological malformations within the egg and larvae (Eriksen et al., 2006). As seen in this thesis trial, fertilisation rates were relatively high with an average of 74% successfully fertilised. However, within a commercially operating facility an acceptable fertilisation rate would be considered anything greater than 80%. Trial fertilisation rates would need to be improved before commercial prospects were considered. Therefore, a way to control parental stress and related feeding behaviour is a must, before reliable yellow belly flounder can become a genuine aquaculture prospect.

Maternal age has also been documented to affect the quality of spawn. Age affects the lipid metabolism of ooctyes whilst in the ovaries of many fish species, including that of flounder (Shatunovskiy, 1988). Because fish were captured from the wild in this thesis trial, age was unidentified. Therefore, spawn quality may have become variable between different cohort groups of fish due to age and metabolic efficiency when producing gametes.

This theory was observed in a study conducted by Kamler and Zuromska (1979) where a definitive difference in egg quality was seen between differing age groups of tench. Middle aged spawners produced the highest spawn quality, while first time spawners and older spawners produced a lower spawn quality. It has also been noted that inter-batch variability between spawnings within a season could contribute to spawn variability in yellowtail flounder (Manning & Crim, 1998). This could be particularly relevant to this thesis study as fish were caught within spawning season and may have already completed their first spawning cycle before capture. Therefore, batches of eggs that were tested could have been received from fish that had already had multiple spawning sessions within the season.

Kamler and Zuromska (1979), compared the age differences among female spawners and described a clear difference in egg quality depending on the age of the female spawner. It was observed that the middle-aged spawners produced eggs of best quality.

Poorer egg quality is also found in first-time spawners of the female tench (Tinea tinea: Zuromska and Markowska, 1984). This may be due to a noticeable difference in cholesterol levels between middle-aged female and first-time spawning female fish. First time spawners were seen to have higher levels of cholesterol within multiple flounder species (Brooks, Tyler & Sumpter, 1997). Therefore, a knowledge of age within spawning fish can help when establishing a broodstock pool from which to choose gametes. Allowing fish of the correct age, in the best condition, to be picked for spawning increases the possibility of high-quality gametes being produced.

A study conducted by Benoit & Pepin (1990), also found that the interaction between maternal fish and the environment, particularly temperature, needs to be considered. They observed that development time from fertilisation to hatch was seriously affected by varying external parameters influencing broodstock fish. Variations in egg size were seen to be a factor attributed to differences in incubation period.

Embryonic development and scoring

The normal development of embryos and developmental changes that can lead to malformation are related to both natural internal and external factors (Cameron & Berg, 1992). Amongst the natural factors that cause mutation are low oxygen, temperature that deviates from optimum and salinity levels. These factors may all contribute to increases in the proportion of mutated embryos within each batch (Braum, 1973; Cameron & Berg, 19992). An example of such mutations within the cleavage stages can be seen in figure 3.

Deviation from normal embryonic development was seen to occur at the highest rate when temperatures deviated from the optimum of 17°C (figure 4.4). This sort of linkage has been observed in other flounder species such as the winter flounder. When trialled at differing incubation temperatures, there were observed differences in survival, hatching rates and yolk absorption, in addition to poor quality of genetic content (DNA and RNA) (Buckley, 1982). It was this difference in embryos' yolk absorption and variance in genetic quality which decreased the survival rate of those embryos and larvae that were incubated above and below optimum temperature. This elevated temperature is also closer to optimal for the growth of bacteria, causing bacterial infection. This bacterial infection was observed within both trial temperature groups, however was more prevalent and occurred faster within the 19-degree treatment group. This infection led to the loss of all embryos before hatch.

Time taken from fertilisation to hatch was seen to be approximately 68 hours which was observed to be similar to that of other flatfishes such as dab. Dab have an approximate incubation period of 3 days (72 hours) (Canario & Scott, 1990). After fertilisation, the egg enters the zygote period before going into the cell cleavages. Embryo size has also been seen to affect incubation periods, and egg size can be influenced by parameters to which the female fish is exposed whilst undergoing oocyte maturation in the ovary (Benoit & Pepin, 1990).

Use of a scoring system and mutation

The use of a scoring system to determine egg quality at early stages of embryogenesis of blastomere morphology creates a great baseline for grading fertilised eggs. This system looks at the number of normal cleavages at the 8-cell stage (Kjorsvik, Hoehne-Reitan & Reitan, 2003). Morphological aberrations can be seen generally within the early cleavages until the formation of the blastodisc. Aberrations are then seen with the formation of the head and growth around the yolk (Cameron & Berg, 1992). Therefore, these two stages of embryonic development represent useful tools for grading of embryos. The reduction of malformations in embryos and larvae is of crucial importance to aquaculture given the reduction in survival and market value of deformed fish (Divanach et al., 1996). Within YBF, it was observed that malformation could be directly linked to temperature. The percentage of malformed blastomeres was seen to increase as temperature deviated from the optimum of 17°C. In (Figure 4.4) deviation from the normal can be seen in embryos numbered one to five. The number one is considered normal, and five is on the extreme side of malformation. These malformations were characterised by deformities in the blastomeres and misshapen blastodiscs. These early stages of deformities commonly lead to body plan defects and retardation of the larvae. Common problems were spinal and notochord deformities, deformation to the head and eyes, and fin deformations, which subsequently gravely reduce hatching success and larvae survival (Cameron & Berg, 1992; Westernhagen et al., 1988). It was observed there were varying degrees of retardation within eggs in this trial with retardation being deviation from the mean in terms of shape, size, cohesion of blastomeres and symmetry (figure 4). The more of these components that deviated from normal the less likely the embryo was to hatch, and larvae to survive.

Conclusion

Studying embryonic development within YBF is crucial if wanting to maximise its farming potential. It has been observed that YBF embryonic development is affected by multiple parameters, including parental parameters during oogenesis. Maternal stress and broodstock cortisol levels, as well as feeding regimes may all contribute to varying embryo quality. These should therefore be carefully controlled for the best possible production of gametes. Under optimum temperature, deformities are reduced during development. 17 °C has been determined to be the best incubation temperature, resulting in the fewest embryos suffering deformities before hatch. The effects of maternal parameters combined with temperature are an avenue for further research, with the goal of reducing the percentage of deformities within embryos and larvae. Scoring systems are also a valuable tool when grading batches of embryos. This allows for easy assessment and decision making as to whether to carry out incubation on various batches in hatchery settings. Lower quality embryos can be identified at the 8-cell blastomere stage through loss of symmetry, variation in size and loss of cohesion of cells. This observation can lead to a more efficient hatchery system where low-quality batches can be disregarded, leaving room for better quality batches.

Due to lack of research around the aquaculture of yellow belly flounder, more research is needed into maternal stress and broodstock management to further reduce stress-induced embryonic conditions that lead to low quality larvae. Therefore, further trials should be run to work out how to maximise hatchery efficiency for yellow belly production.

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5.0 General Discussion

The primary aim of this study was to assess the effectiveness of GnRHa as a spawn inducer in YBF. This was done through the tracking of the reproductive marker hormones FSH and LH, in conjunction with gonadal growth and gonadal histology. This potentially aiding in future efforts to commercialise yellow belly as an aquaculture species. Results and literature reviews have shown that there is a need for new aquaculture species in New Zealand. Currently the industry relies heavily on three species: green lipped mussels, pacific oysters, and king salmon. Therefore, there is a need to diversify if imposed future aquaculture targets are to be met. Yellow belly flounder have been identified as a potential novel aquaculture species due to their high market prize, low trophic level feed regime, and desirable growth rates. However, under captive settings YBF tend to undergo reproductive failure, as a result of stress disruption to the BPG axis. This is common among many flounder species within aquaculture settings (Barnett & Pankhurst, 1999). Therefore, the use of spawn inducing agents such as GnRHa have potential to bridge this crucial link in completing the domesticated lifecycle of this species.

Gonadotrophin releasing hormone analogue was seen to have varying success when tested in this trial. The production of gonadotrophins FSH and LH when analysed through PCR methods showed no statistical difference between both GnRH and sham injected trial groups ($P>0.05$). However, there was an observed difference seen between both FSH trial groups. The GnRHa treated group had a fold change of 2.3 compared to the sham implants which observed a fold change of 1.2. When compared to literature these fold changes were particularly low, with GnRHa induction exhibiting fold changes exceeding 10-fold for FSH in some fish species (Mateos et al., 2002). Other forms of spawn inducing agents have been used on flounder, such as the green back which elicit large gonadal responses such as human chorionic gonadotrophin (hCG) (Barnett & Pankhurst, 1999). This showing that GnRHa induction can elicit a powerful hormone response resulting in the upregulation of gonadotrophins at a pituitary level. These low fold changes may be a reason why only a small percentage of fish injected were seen to ovulate (5%).

Under histological analysis it was also observed that many of the sample gonads had varying degrees of atresia. Atresia is commonly associated with the end of a spawning season; symbolising ovulation has taken place or oocyte development have been aborted. This highlights the importance of deciding when to inject GnRHa in trying to induce spawning. Within this experiment fish may have already spawned for the season resulting in reduced or unsuccessful responses to the GnRHa induction. In the pilot study conducted in the months of May/June it was observed that a higher percentage of fish (17%) ovulated, all of which were fish assigned GnRHa treatment. Seasonal variation in the production of gonadotrophins has been observed in other literature where there are seasonal spikes surrounding the breeding season before levels are seen to drop for the remaining months (Campbell et al., 2006). However, histologically there was an observed difference between the sham and GnRHa treatment groups. Gonadotrophin releasing hormone analogue treatment was seen to result in a greater number of ovaries entering vitellogenic phases of development. This has been a common result within other fish species trialled on implanted GnRHa in literature, including plaice, where GnRHa induction resulted in the hydration of oocytes (Scott, Witthames, & Vermierssen, 1999). Oocyte development also lead to oocyte maturation within winter flounder, and increased egg quantity at spawn (Berlinsky et al., 1997).

Both FSH and LH were found to have weak positive correlations with GSI. With elevated levels of either gonadotrophin being associated with a higher GSI. Gonadosomatic index can be correlated with gonadal maturity as it is an indicator of the gonad weight as a percentage of overall weight. Gonads were seen to range from immature, containing largely PVO oocytes, through to Late vitellogenic. Although there was little correlation found in this study, other studies have found strong correlative links between LH and GSI (Holland, Hassin, & Zohar, 2001). This is probably because LH is the key driving hormone in the final stages of maturation, including oocyte hydration, where the oocyte grows hugely in size and weight alike. Studies have also found positive correlations between other key hormones such as E2 and 11-KT and GSI (Sun & Pankhurst, 2004).

These can potentially be linked to both FSH and LH which trigger hormones for the production of both E2 an 11-KT.

Histologically, oocyte development was found to be similar to other flatfish species studied, with oocyte sizes and key developmental features confirming what was found in studies of other species. Cortical alveoli presence was characterised by the presences of yolk granules (Murua & Saborido, 2003; Wallace & Selman, 1981). Cortical alveoli have been linked to FSH secretion due to their role as a follicle stimulating hormone. However cortical alveoli were observed in only 10% of the samples showing again that FSH secretion was at a low level even after injections of GnRHa. Vitellogenic oocytes were seen to progress from containing yolk vesicles in early vitellogenic stages, through to the formation of true yolk in late vitellogenic oocytes. This is the point before hydration and ovulation, where, in other species, oocyte diameter is seen to increase again.

All fish that ovulated during this trial were from the GnRHa treatment group. However, the percentage of fish to reach ovulation and ready to be strip spawned was higher within the pilot study group sampled in May/June. This again proves the importance of seasonality and how it needs to be factored in when looking to induce spawning through GnRHa implantation.

Embryos used in the embryonic chapter were taken from fish stripped in the May/June period. When incubated it was the higher temperature of 19°C which caused a faster embryo development rate. However, these higher temperatures were also seen to have higher percentages of malformations, and no embryos incubated at 19°C were seen to reach hatch. Embryo incubated at lower temperatures were seen to have slower developmental rates. However, they suffered less malformation and bacterial infection took over at a slower rate. Similar findings have been seen in other studies looking into optimising temperature for embryonic development where there are a large number of deformed embryos when temperature deviates from optimum (Buckley, 1982; Canario & Scott, 1990).

Embryonic malformations can be predicted through scoring of the 8 cell blastomere stage, looking at uniformity of size, shape, cohesion, symmetry of the cells. This can help with the grading of embryos and the making of hatchery-level decisions as to whether to carry certain batches of embryos through to hatch.

Limitation and recommendation

The studies conducted in this thesis may have yielded better results with the addition of seasonal variability data on the induction of GnRHa through an annual cycle. This would allow for analysis of when flounder reach their peak periods for hormonal induction of GnRHa. It would enable the best results to be gained, with a maximal percentage of fish reaching final oocyte maturation. This may also help with correlative data between gonadotropin levels and developmental stages.

The large size range of the fish may have affected results, as this was the factor influencing dosage of GnRHa the most. In future, dosage should be set according to body weight to reduce such a range of dosage/kg BW. Effective dosages of GnRH should also be studied as it was observed that dosages of around 260µg/kg^{-1}BW led to higher rates of vitellogenesis than any dosages that were significantly lower or higher than this. Similar dosages have been seen to be effective on species such as the spotted rose (Ibarra-Castro, 2007). However effective dosages have been seen to be highly variable between different species, and therefore should be an area of focus in future research involving YBF and GnRHa treatment.

Statistically results would have also benefited from larger sample populations. This would have allowed for the disregarding of anomalous results, as well as greater certainty to be placed on conversions such as GSI recordings. Due to so few fish being recorded ovulating, it was hard to gather a realistic GSI picture giving relation to oocyte growth and gonadotrophin levels.

Future research may benefit from studying the effect of stress on flounder within captive settings, as this stress may also play a role in the aborting of oocyte development, leading to a better understanding of the relationship between cortisol and its effects on gonadal maturation.

The assessment of gonadotrophin receptors within the gonad, through the use of qPCR along-side that of pituitary levels or both FSH and LH would give a clearer picture into the effects of these gonadotrophins at the gonad level.

Trials into the disinfection protocols and treatment dosages of disinfecting agents would also shed helpful insight into bacterial control when incubating embryos. This was the limiting factor upon being able to bring embryos through to hatch in this trial.

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