Understanding the molecular basis for improved egg quality in maiden and repeat spawning Atlantic salmon (*Salmo salar*) maintained at elevated temperature

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Abstract

Tasmanian Atlantic salmon (*Salmo salar*) broodstock can encounter temperatures above 20 °C, which has a marked negative effect on reproductive development. Broodstock management strategies are needed in order to maintain egg quality in the face of thermal challenge either due to seasonal fluctuation or climate change, where temperatures are expected to rise even further. In Tasmania, the Atlantic salmon industry uses maiden fish for approximately 75 % of egg production due to their smaller size and the lower cost associated with their husbandry relative to repeats. However, maidens appear to be more susceptible than repeat fish to the effects of elevated temperature. The salmon industry's ability to cope with rising temperature is hindered by the lack of understanding of the effects of thermal challenge on the endocrine system, which ultimately determines egg quality. Therefore the aims of the present research were to: (1) determine how temperature influences endocrine function and reproductive development in maiden and repeat spawning female Atlantic salmon; (2) understand the molecular mechanisms that determine egg quality in broodstock maintained at elevated temperature; and (3) develop management strategies to maintain endocrine function and egg quality under thermally challenging conditions

Using available immunological assays, we have confirmed thermal inhibition of plasma 17β-estradiol (E_2), *vitellogenin* (Vtg) and testosterone (T) in Atlantic salmon maintained at 22 °C relative to 14 °C during the vitellogenic growth phase. Then, using quantitative real-time polymerase chain reaction (qPCR) assays developed and validated in the present study, we found evidence of thermal impairment of *vtg*, *zona pellucida b* (*zpb*), *zona pellucida c* (*zpc*), *p450 aromatase a* (*cyp19a1a*) and *cholesterol side chain cleavage protein* (*cyp11a1*) gene expression in fish maintained at 22 °C relative to 14 °C. On the other hand, basal levels of plasma *luteinizing hormone* (Lh) and hepatic gene expression levels of *estrogen receptor alpha* (*erα*) were not suppressed as a result of thermal challenge. Plasma *follicle stimulating hormone* (Fsh) levels increased as a result of maintenance at 22 °C, a phenomenon which has not been described previously for salmonids.

Impairment of hepatic and gonadal gene expression at 22 °C resulted in a reduction in egg fertility and embryo survival in maidens and repeats relative to the corresponding

control group at 14 °C. Consistent with industry observations, maiden fish were more sensitive than repeats to thermal challenge, even though both groups had similar plasma gonadotropin and steroid profiles (Fsh, Lh, T and E_2) and expression patterns of all hepatic and ovarian genes throughout maintenance at 22 °C. In Atlantic salmon, February corresponds to a period of increased thermal sensitivity and even short thermal exposures at this time can have lasting implications on egg quality (King et al., 2007). The only difference in endocrine profiles among maidens and repeats occurred in February, whereby maidens displayed suppressed levels of plasma Vtg relative to repeats. This may have contributed to the differential response of maidens and repeats to high temperature in terms of egg viability.

The next step was to develop and test alternative management techniques that maintain endocrine function and egg quality in the face of thermal challenge. Our first strategy was to test whether prolonged use of a gonadotropin-releasing hormone analogue (Gnrha) during the vitellogenic growth phase could offset the inhibitory effects of thermal challenge in maiden and repeat spawners. Gnrha-treatment during vitellogenesis did not compensate for the negative effects of thermal challenge (22 °C versus 14 °C) on timing of ovulation, egg size, egg fertility or embryo survival in any fish. The lack of effectiveness was reflected by the endocrine data, where plasma Fsh levels were not different between Gnrha-treated and untreated groups at 22 °C. Furthermore, plasma E_2 levels were unchanged in Gnrha-treated fish at 22 °C, and subsequent transcription of *vtg*, *zpb* and *zpc* was not enhanced in Gnrha-treated fish relative to untreated fish at 22 °C.

As a second strategy, we conducted a pilot study in juvenile Atlantic salmon that aimed to determine whether direct E_2-administration would generate appropriate plasma E_2 levels and stimulate downstream vitellogenesis and zonagenesis at 22 °C. E_2-treatment induced *vtg* gene expression and protein synthesis in juveniles maintained at 14 and 22 °C. E_2-treatment induced *Zp* gene expression at 14 °C, and to a lesser extent at 22 °C. Since E_2-treatment stimulated vitellogenesis and zonagenesis (to some extent) in juveniles at 22 °C, we tested a similar protocol in maiden spawning broodstock due to the industry's preference for using maidens. In this experiment, *vtg* gene expression and plasma protein levels were higher in E_2-treated maiden broodstock at 22 °C than untreated broodstock at 14 and 22 °C. As a result, E_2-treated fish at 22 °C had a raster rate of follicle growth and larger eggs at spawning relative to both untreated groups. In contrast, E_2-treated and

untreated fish had similar *zpb* and *zpc* gene expression levels at 22 °C, and gene expression for these groups was suppressed relative to the control group at 14 °C during late vitellogenesis. Hence, E_2-treatment failed to upregulate *zpb* or *zpc* gene expression, and there is mounting evidence suggesting that Vtg and Zps respond differentially to E_2-treatment at elevated temperature.

Egg fertility was highest in the control group maintained at 14 °C, followed by the control group at 22 °C, then the E_2-treated group at 22 °C, despite the stimulatory effect that E_2-treatment had on Vtg synthesis and oocyte growth. The reduction in egg quality in both groups of fish maintained at 22 °C relative to 14 °C may have occurred due to thermal inhibition of *zpb* and *zpc* gene expression. We observed that plasma cortisol levels were elevated in E_2-treated fish after the second pellet implantation relative to all other groups. It is likely that the second implantation was perceived as somewhat stressful, and this may have also led to a further reduction in egg quality in E_2-treated fish.

In conclusion, we have provided evidence that female maiden Atlantic salmon are more susceptible to the effects of elevated temperature. We also provide direct evidence that thermal impairment of endocrine function occurs at multiple levels of the reproductive axis. Treatment with Gnrha was not able to offset the impacts of elevated temperature. In contrast, E_2-therapy was effective in maintaining vitellogenesis and oocyte growth at 22 °C. However, stimulation of Vtg alone was not sufficient to maintain egg quality. The lower egg quality at higher temperature is apparently due to the impairment of zonagenesis. Our results suggest that it is difficult to overcome the impacts of higher temperature on the reproductive axis using a single hormonal treatment. Hormonal therapies in the future should simultaneously stimulate vitellogenesis and zonagenesis in order to maintain egg quality, and it is worth investigating whether a higher dose of E_2 could stimulate zonagenesis. It is evident that the method of hormonal delivery is of considerable importance and implantation techniques also warrant further investigation. Understanding the regulatory mechanisms governing the expression of Zp genes is justified, which could aid in the development of new management strategies that are essential for maintaining egg quality at high temperature without the added cost of intensive temperature control.

Statement of originality

The submitted work does not contain material which has been previously published or written by any person other than the candidate except where due and proper reference has been given in the text. Additionally, work in this thesis has not been submitted to meet the requirements of any other agree or award at this, or any other institution.

For a detailed description of the candidate's contribution to each co-authored manuscript, please see the sections titled 'list of publications, presentations and awards'. Co-authors provided supervision, funds, expertise, laboratory and field assistance and logistics that made the research and publications possible.

Kelli Anderson

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List of publications, presentations and awards

Accepted and anticipated publications

***Chapter 3**- Hepatic reference gene selection in adult and juvenile female Atlantic salmon reared under thermal challenge*

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Author contributions: Anderson, K.C (90 %) and Elizur, A (10 %). **Kelli Anderson conducted all of the laboratory work and data analysis associated with this research and drafted the manuscript for publication.** Abigail Elizur provided principal supervision and constructive feedback on manuscript drafts.

***Chapter 4-** Thermal impairment is differentially expressed in maiden and repeat spawning Atlantic salmon*

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drafted the manuscript. Harry King took part in experimental design, was responsible for salmon health and maintenance, provided supervision and gave feedback on the manuscript. Patricia Pankhurst did the laboratory work associated with the detection of plasma proteins. Nicole Ruff was responsible for fish health and maintenance. Abigail Elizur took part in experimental design, provided principal supervision and manuscript feedback.

Chapter 5- *Effect of thermal challenge on plasma gonadotropin levels and ovarian steroidogenesis in female Atlantic salmon*

Anderson, K.C., Swanson, P., Pankhurst, N.W., King, H., Elizur, A. (2012) Effect of thermal challenge on plasma gonadotropin levels and ovarian steroidogenesis in female maiden and repeat spawning Tasmanian Atlantic salmon (*Salmo salar*). *Aquaculture* **in press.**

Author contributions: Anderson, K.C (60 %), Swanson, P (10 %), Pankhurst, N.W (10 %), King, H (5 %), Abigail (15 %). All authors took part in the field work component of this research (except for Penny Swanson). **Kelli Anderson took part in experimental design, drafted the manuscript and conducted all of the laboratory work and data analysis (gene isolation and cloning, qPCR development and validation, RNA extractions, cDNA synthesis and qPCR analysis). Kelli carried out the Fsh and Lh radioimmunoassays in conjunction with Penny Swanson (and did the associated data analysis).** Ned Pankhurst and Abigail Elizur took part in experimental design, provided supervision and manuscript feedback. Harry King provided supervision and was responsible for animal maintenance and health.

Chapter 6- *The effect of Gnrh treatment during vitellogenesis on the reproductive physiology of thermally challenged female Atlantic salmon*

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Chapter 7- *Effect of elevated temperature on estrogenic induction of vitellogenesis and zonagenesis in juvenile Atlantic salmon*

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were involved in experimental design, provided supervision and manuscript feedback. Patricia Pankhurst carried out all ELISA and RIA laboratory work. Nicole Ruff was involved in experimental set-up and monitored animal health.

Chapter 8- *Effects of E_2-treatment on the reproductive physiology of thermally challenged maiden Atlantic salmon*

Pankhurst, N.W., Anderson, K.C., King, H., Pankhurst, P., Swanson, P., Ruff, N., Elizur, A. (2011) Estrogen therapy offsets thermal impairment of vitellogenesis but not zonagenesis in maiden spawning female Atlantic salmon. This paper will be submitted for publication in due course.

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Presentations

Anderson, K., Elizur, A., King, H., Pankhurst, P., Ruff, N., Pankhurst, N. Effect of elevated water temperature on the reproductive physiology of female Atlantic salmon (S*almo salar*): understanding the molecular basis for improved egg quality. Talk presented at the **Australasian Aquaculture International Conference and Trade Show**, Hobart, Australia; 23-26 May 2010.

Anderson, K., King, H., Elizur, A., Ruff, N., Pankhurst, T., Pankhurst, N. The effect of stock age and elevated water temperature on reproductive physiology and egg quality of female Atlantic salmon (*Salmo salar*). Talk presented at the **Australia Marine Science Association, South East Queensland Postgraduate Student Workshop,** Moreton Bay Research Station, North Stradbroke Island, Australia; 22-24 May 2009.

Anderson, K., King, H., Ruff, N., Pankhurst, P., Elizur, A., Pankhurst, N. Effect of elevated water temperature on the B-P-G axis and reproductive physiology of female Atlantic salmon (*Salmo salar*). Talk presented at the **University of the Sunshine Coast Research Conference: Research to benefit society**, Sippy Downs, Australia; 9th November 2009.

Anderson, K., King, H., Ruff, N., Pankhurst, P., Elizur, A., Pankhurst, N. Effect of elevated water temperature on the B-P-G axis and reproductive physiology of female Atlantic salmon. Talk presented at the **Skretting Australasian Aquaculture International Conference and Trade Show**, Brisbane, Australia; 3-6 August 2008.

Awards

Competitive travel grant from the Australian Seafood Centre for Research Cooperation (CRC) to travel to the United States of America an train at the Northwest Fisheries Science Centre (2010, AU $4500)

1st prize at the University of the Sunshine Coast's Research Conference (2009) for best student presentation

4th prize at the Skretting Australasian Aquaculture, International Conference and Trade show (2008) in the category 'best student abstract'

Competitive Australian post graduate award (APA scholarship, AU $20,007 per annum) in 2008

PhD 'top-up' scholarship from the Australian Seafood CRC in 2008 (AU $5,000 top-up, $5,000 operating and $1,000 travel per annum)

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The use of gene names and abbreviations has been standardised according to the Zebrafish Model Organism Database (ZFIN, http://zfin.org). Briefly, full gene names are lowercase italic, and gene symbols are three or more italicized lowercase letters. The protein symbol is the same as the gene symbol, but non-italic and the first letter is uppercase.

20β-hsd	*20β-hydroxysteroid dehydrogenase*
BPG-L	Brain-pituitary-gonad-liver axis
CFCS	Consensus furin cleavage site
CO_2	Carbon dioxide
CPC	Pore canals of the zona pellucida
Cyp19a1a	*p450 aromatase a*
Cyp11a1	*Cholesterol side-chain cleavage protein*
E_2	Estradiol-17β
EE_2	17α-ethinylestradiol
Ef1α	*Elongation factor 1 alpha*
EHP	External hydrophobic patch
Er	*Estrogen receptor*
Erα	*Estrogen receptor alpha*
ERE	Estrogen responsive element
F	Cortisol
Foxl2	*Forkhead transcription factor*
FRDC	Fisheries Research & Development Corporation
Fsh	*Follicle stimulating hormone*
Fshr	*Follicle stimulating hormone receptor*
GL	Granular outer surface of the zona pellucida
Gnrh	*Gonadotropin-releasing hormone*
Gnrh1	sbGnrh, seabream Gnrh
Gnrh2	cGnrh, chicken Gnrh
Gnrhr	*Gonadotropin-releasing hormone receptor*

GSPs	Gene specific primers
Gth	Gonadotropin
GVBD	Germinal vesicle breakdown
GVM	Germinal vesicle migration
Hprt1	*Hypoxanthine phosphoribosyltransferase 1*
Hsps	Heat stress proteins
IHP	Internal hydrophobic patch
Kiss1	*Kisspeptin*
Kiss1r	*Kisspeptin receptor*
Lh	*Luteinizing hormone*
Lhrha	*Luteinizing hormone releasing hormone* analogue
MIH	Maturation inducing hormone
MPF	Maturation promoting factor
PS	Perivitelline space
Saltas	Salmon Enterprises of Tasmania
SEM	Scanning electron microscopy
SSD	Soft shell disease
Star	*Steroidogenic acute regulatory protein*
T	Testosterone
Tbp	*Tata binding protein*
TMD	Transmembrane domain
Vtg	*Vitellogenin*
Vtga	*Vitellogenin a*
Vtgb	*Vitellogenin b*
Zp	Zona pellucida (matrix or protein depending on context)
Zpa	*Zona pellucida a*
Zpb1	*Zona pellucida 1*
Zpb2	*Zona pellucida 2*
Zpba	*Zona pellucida ba*
Zpbb	*Zona pellucida bb*
Zpc	*Zona pellucida b*
Zpx	*Zona pellucida x*

Chapter 1- General introduction

1.1 Atlantic salmon aquaculture in Tasmania

1.1.1 A brief history and current status of Tasmanian Atlantic salmon farming

Atlantic salmon were first brought to New South Wales, Australia from the River Philip in Nova Scotia, Canada in the mid-1960's (Reilly et al., 1999). Four importations of approximately 100,000 ova were used to establish the first ever breeding population in Australia. Then, in the mid-1980's around 570,000 ova were taken from Gaden Hatchery in New South Wales to Tasmania to found the now very valuable Tasmanian Atlantic salmon aquaculture sector (Jungalwalla, 1991, Reilly et al., 1999). The first commercial harvest of 53 tonnes took place in the summer of 1986/87 (Tassal, 2008). In 2006/07 Atlantic salmon overtook the rock lobster fishery in Western Australia as Australia's most valuable single-species fishery, and is now worth approximately $323 million per annum, harvesting 29700 tonnes of fish in 2009 (ABARE and FRDC, 2010) and employing in excess of 1200 people (Battaglene et al., 2008). For continued strong industry performance, reliable and predictable production of high quality smolts is required.

1.1.2 Atlantic salmon farming in Tasmania

Since the Atlantic salmon industry was established in Tasmania, farming techniques have changed to optimise breeding conditions and subsequent production of smolts. In the past it was common practice to transport broodstock from growout to fresh-water two to three months prior to spawning (Jungalwalla, 1991). However, now broodstock are maintained in fresh water from hatching to allow continuous control of water quality parameters (Battaglene et al., 2008). Typically, female broodstock spawn in onshore hatcheries between the start of May and the first week of June (Jungalwalla,

1991), after the water temperature is gradually ramped down to 8 °C which encourages final oocyte maturation and ovulation (King and Pankhurst, 2000). Eggs are then incubated until 'eye-up' in June or July, then first-feed occurs sometime in August or September (Jungalwalla, 1991). Fry are progressively moved into larger tanks starting in October or November and by December they weigh between 2-3 g, and are held in large production tanks (Jungalwalla, 1991). Approximately one year later, from mid-September to the end of October, juvenile fish undergo the smoltification process and are then transferred to marine farms for grow-out (Jungalwalla, 1991). Tasmanian seawater temperatures are generally favourable for salmon growth, and because of this, salmon usually reach market size (~3.5 kg) by the middle of the following summer (~15 months) (Jungalwalla, 1991). Fish are then harvested before the development of secondary sexual characteristics and a corresponding reduction in flesh quality which makes them undesirable to consumers (King, 2001). The entire production cycle takes approximately 30 months (Battaglene et al., 2008).

1.1.3 Tasmanian Atlantic salmon broodstock

Atlantic salmon are cold-water fish endemic to the northern Atlantic Ocean. They spawn naturally on the North American and European continents (Pennell and Barton, 1996). In the northern hemisphere Atlantic salmon spawn sometime between October and January depending on regional variations in environmental conditions (Pennell and Barton, 1996). The Tasmanian stock of Atlantic salmon is known to spawn in May and June which is the austral autumn-winter period (Jungalwalla, 1991). Spawning patterns and reproductive development of Atlantic salmon is similar between northern hemispheres and southern hemisphere, albeit 6 months out-of-phase (King and Pankhurst, 2003). As a result of translocation to the southern hemisphere, Tasmanian Atlantic salmon frequently experience seawater temperatures between 8-18 °C (Jungalwalla, 1991); although temperatures may exceed 20 °C (King and Pankhurst,

2000) which is at the upper limit of thermal tolerance (22-23.5 °C) for the species (Pennell and Barton, 1996).

While elevated temperature helps Tasmanian Atlantic salmon to achieve high growth rates (Jungalwalla, 1991), it can also have a marked effect on sexual maturation and reproduction (King and Pankhurst, 2003, King and Pankhurst, 2004). A major consequence of exposing Atlantic salmon to elevated temperature in the southern hemisphere is that reproductive milestones are reached at a younger age compared to their northern hemisphere counterparts (Jungalwalla, 1991, King and Pankhurst, 2003). Nova Scotian stock are considered to be late maturing stock with most fish maturing after two sea winters; whereas 95 % of Tasmanian Atlantic salmon mature sexually after just one winter at sea as grilse and spawn at three years of age (Jungalwalla, 1991, Tarangera et al., 1999). In Tasmania, approximately 75 % of smolt production comes from 'maiden' or first-time spawning fish (King, H 2007, pers. comm., 6 February) for various reasons. Firstly, post spawning mortality is an unpredictable feature that can occur once maiden fish have completed their first reproductive cycle. In ideal conditions, the level of post spawning mortality should be lower than 5%. However, if post spawning mortality is higher, there may not be the required number of repeat spawning fish for the following production year. Secondly, even if post spawning mortality in maiden fish is low, there is a large cost and risk associated with holding maiden fish at an intensive onshore hatchery facility for an extra production year until they become repeat spawning fish. Specifically, repeat spawning fish are approximately double the weight of maiden spawning fish, and therefore require twice as much feed at a hypothetical feed conversion ratio of 1:1 (this equates to an additional cost of $3 per kg). Feed constitutes approximately 1/3 of production costs (with another 1/3 going to operating and another 1/3 to labour), so the total cost per kilogram for rearing repeat spawning fish is approximately $9 AU. Therefore, if a 5 kg maiden fish becomes a 10 kg repeat spawning fish, there is an additional cost of $45 AU per fish. If maiden spawning fish are able to produce enough high quality eggs to meet industry demand, then this this figure represents an additional cost with negligible return. However, if egg

production in maiden fish is unreliable, then investment in the maintenance of repeat-spawning fish may be worthwhile. Furthermore, due to the size difference between maiden and repeat spawning fish, the husbandry of repeat spawning fish is more difficult which is also a consideration.

1.1.4 A current industry bottleneck: reliable smolt production

Salmon Enterprises of Tasmania (Saltas) is the leading producer of Atlantic salmon smolts in Australia. Their production data shows that current methods of smolt production have one significant drawback. Survival of eggs to the eyed embryo stage from maiden spawning fish can be as low as 30-50 %, compared to 60-80 % for eggs from repeat spawning fish. Yet as mentioned above, the majority of egg production comes from maiden spawners. Modelling of this uncertainty has shown that a shortfall in smolt production worth $15-20 million/annum may occur if maiden fish do not perform (King, H 2007, pers. comm., 6 February). It may appear that the lower reproductive performance observed for maiden fish occurs purely as a function of broodstock age. However, the combined effect of rearing temperature and age on reproductive performance is not well understood, although it has been established that elevated temperature can reduce reproductive performance in maiden spawning fish (King and Pankhurst, 2003, King et al., 2007). Therefore, it is unclear as to whether the differences in reproductive performance are due to age class, thermal challenge or a combination of the two.

The variable spawning success of Atlantic salmon broodstock has left the industry with the ongoing problem that egg production is not predictable, and this has resulted in a significant management challenge. To produce the required number of smolts, the number of maiden spawning fish which produce eggs of lower quality could be increased. However, this will result in the additional cost of expanding the scale of holding and rearing facilities. Another option is to increase the hold-over of maiden fish to become repeat spawners, and then use these fish for the majority of smolt

production. Thus far, the significant costs and risks associated with holding fish for an extra production year have made this an undesirable option (King, H 2007, pers. comm., 6 February). Still, industry (Saltas) production data and experimental results show that egg survival can be improved through thermal management (Taranger and Hansen, 1993) and breeding with repeat spawners. In fact, it is expected that in the future all broodstock holding facilities in Tasmanian Atlantic salmon hatcheries will be thermally regulated with the introduction of recirculation systems and temperature control (Battaglene et al., 2008). Unfortunately, the initial capital investment required to set-up a thermally controlled broodstock facility, and the ongoing cost of cooling large volumes of water on a yearly basis is significant (King, H 2007, pers. comm., 6 February). This cost may be out of reach for some smaller hatcheries; especially when faced with the prospect of global warming where even (seemingly) small increases in water temperature can have a biological impact and result in a significant increase in cooling cost. Therefore, the most favourable solution which has not been achieved thus far, is to rear maiden fish under conditions that do not increase production costs significantly, but will increase reproductive performance to a commercially acceptable level even when faced with high summer temperatures and climate change.

1.1.5 Climate change predictions for Tasmania

South east Australia is considered to be especially vulnerable to climate change (Cai et al., 2005); in fact, climate driven changes in zooplankton community structure and the distribution of various nearshore fish species have already been observed in waters off eastern Tasmania (Johnson et al., 2011). Changes in ocean ecology have been linked to a southern extension of the east Australian current resulting from wind stress caused by climate change (Battaglene et al., 2008, Johnson et al., 2011). Climate change is expected to continue in Tasmania with various temperature increases possible depending on annual carbon dioxide (CO_2) emissions and the prediction model used (Battaglene et al., 2008) (Fig 1.0). Middle-of-the-range climate prediction models (50th

percentile) show that under moderate CO_2 emissions, a 1.5 °C increase in air temperature can be expected by 2030 for most of Tasmania (Fig. 1.0). The rate of sea surface temperature increase in eastern Australia was 2.28 °C per century between 1944 and 2002 compared to a global average of ~0.6 °C (Battaglene et al., 2008). This shows that eastern Australia is warming at over three times the global rate, and for this reason global climate predictions are generally considered to be conservative in the context of Australian change (Battaglene et al., 2008). None the less, it seems that the rest of the world's salmon producers will look on to see how Tasmanian Atlantic salmon cope, especially since they are already farmed towards their upper limit of thermal tolerance (Battaglene et al., 2008).

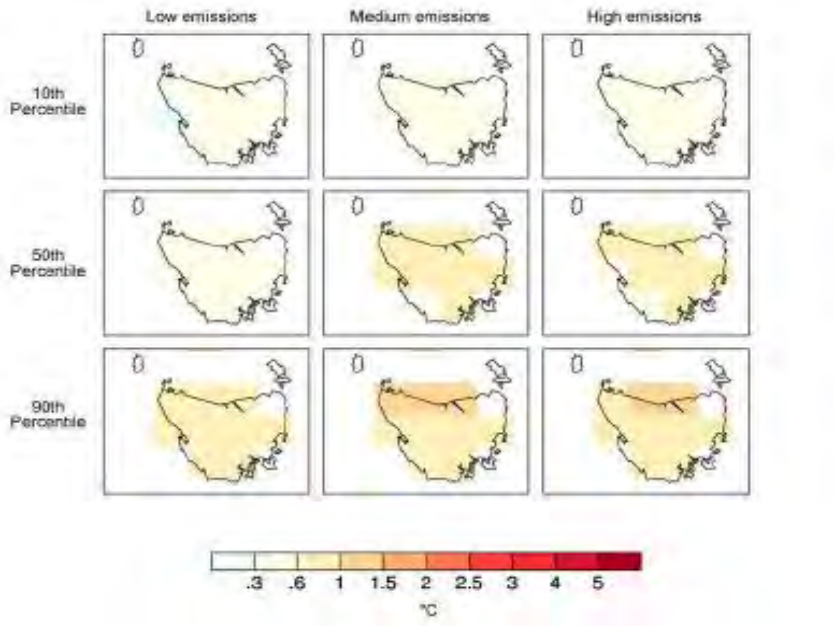

Figure 1.0 Predictions of change in summer air temperature by 2030

Projections are given relative to the period 1980-1999. The projections give an estimate of the average climate around 2030, taking into account consistency among climate models. The 50th percentile (mid-point of the spread of model results) provides the best estimate. The 10th and 90th percentiles (the lowest and highest 10 % of results) provide a range of uncertainty (figure and figure legend reproduced form Battaglene et al., 2008).

1.1.6 Need for the research

In recent years it has become apparent that the Tasmanian Atlantic salmon industry must adapt their current method of production in order to withstand the impacts of thermal challenge on egg quality. Unfortunately, the industry's ability to adjust is hindered by a lack of understanding concerning the interactions between stock age, temperature, endocrine function and ultimately egg quality. Only when we have a better understanding of the molecular basis for reduced egg quality will we be able to develop alternate management strategies that compensate for endocrine suppression and maintain egg quality at high temperatures. The following literature review covers current knowledge concerning normal and thermally impaired reproductive development in teleosts, with specific reference to salmonids where information is available.

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Chapter 2- Literature review

2.1 Reproduction in teleost species

2.1.1 Oocyte development

Oocyte development in teleosts is a dynamic process and occurs continuously, but for the sake of classification can be split into several phases: oogonial proliferation, primary oocyte growth, cortical alveolus, vitellogenesis, maturation and ovulation (reviewed in Tyler and Sumpter, 1996). Primary oocyte growth refers to a period of development which begins at the chromatin nucleolar stage, progresses through the perinucleolar stage and ends during the early cortical alveolus stage (reviewed in Wallace and Selman, 1981). The transition from oogonial proliferation to primary oocyte growth is hallmarked by the commencement of meiosis (reviewed in Lubzens et al., 2010). This phase of development coincides with a period of significant heterogeneous and ribosomal RNA synthesis, and formation of the Balbiani body which is comprised of various organelles and fibrogranular material (Tyler and Sumpter, 1996, Patiño and Sullivan, 2002). An oocyte in the early primary growth phase is characterised by a spherical centrally located nucleus and a large basophilic nucleolus; the oocyte itself is enclosed by a thin layer of squamous follicle cells (Wallace and Selman, 1981, Koya et al., 2003). As the oocyte grows in size, multiple nucleoli will appear (perinucleolar stage Fig. 2.0) (Merson et al., 2000, Koya et al., 2003), an acellular zona pellucida will begin to develop (Tyler and Sumpter, 1996), and the components of the Balbiani body will disperse (Wallace and Selman, 1981).

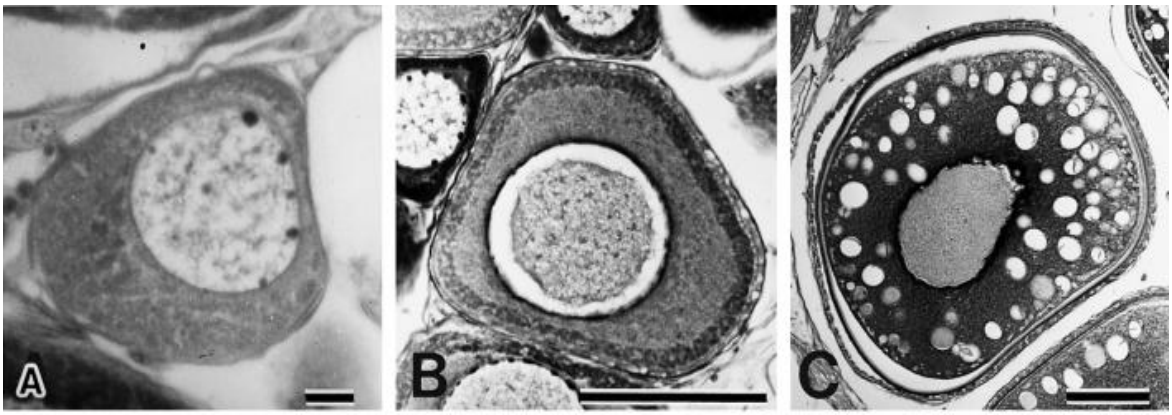

Figure 2.0 Pacific herring (*Clupea pallasii*) ovarian tissue stained with hematoxylin-eosin (A) Early perinucleolar stage (bar = 10 µm), (B) late perinucleolar stage (bar = 100 µm) and (C) cortical alveolus stage (bar = 100 µm) (Koya et al., 2003).

The shift from primary to secondary oocyte growth is associated with further enlargement of the oocyte and the appearance of cortical alveoli and small lipid droplets within the cytoplasm (Fig. 2.0) (Merson et al., 2000, Campbell et al., 2006, Lubzens et al., 2010). During the cortical alveoli stage (also known as primary vitellogenesis), the number of membrane-bound cortical alveoli will increase in size and number until they almost completely fill the cytoplasm (Koya et al., 2003, Campbell et al., 2006). Cortical alveoli are found in close proximity to Golgi apparatuses that are thought to play a role in the production of their contents (Lubzens et al., 2010) including a polysialoglycoprotein of high molecular weight (Tyler and Sumpter, 1996). Vitellogenic development occurs while the oocyte is arrested at the dipoltene stage of the first meiotic prophase (Lubzens et al., 2010). During this phase, cortical alveoli are progressively displaced to the periphery of the oocyte due to the central accumulation of yolk protein (Fig. 2.1) (Tyler and Sumpter, 1996), and the thickness of the zona pellucida increases (Koya et al., 2003). The uptake of *vitellogenin* (Vtg, 'egg yolk' precursor) results in rapid growth of the oocyte and is associated with a subsequent increase in gonadosomatic index (King and Pankhurst, 2003). Oocyte maturation is characterised by a decrease in the rate of, or complete discontinuation of endocytosis, the resumption of meiosis, migration of the nucleus to the periphery, subsequent germinal vesicle breakdown (GVBD) and in some species oocyte hydration (Lubzens et

al., 2010). During maturation, the first meiotic division is completed which gives rise to the first polar body that degrades after being expelled from the oocyte (Patiño and Sullivan, 2002). The now secondary oocyte progresses to metaphase II of meiosis (reviewed in Nagahama, 1994), the follicle tissue ruptures (Fig. 2.2) and the ovulated ovum will reside in either the abdominal cavity or ovarian lumen depending on the species (Lubzens et al., 2010). At this stage, the haploid ovum has completed meiosis II and the second polar body has degraded which signals the end of oocyte maturation and ovulation (Lubzens et al., 2010).

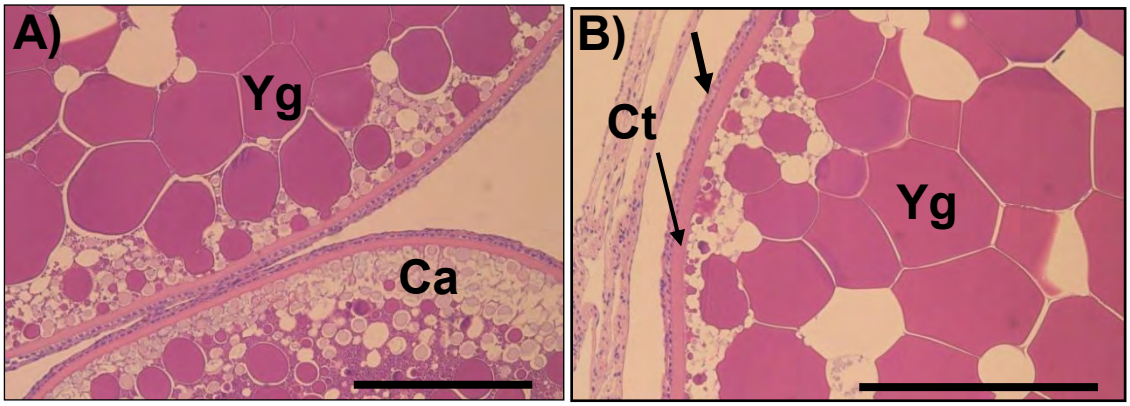

Figure 2.1 Vitellogenic Atlantic salmon (*Salmo salar*) oocytes

Yg = yolk globule, Ca = cortical alveoli and Ct = connective tissue. A) scale bar = 20 µm, B) scale bar = 50 µm. Sections stained with hematoxylin-eosin. Thin and thick arrows point to the zona pellucida and follicular layer respectively. Micrographs were kindly supplied by Prof. Ned Pankhurst and Dr. Harry King.

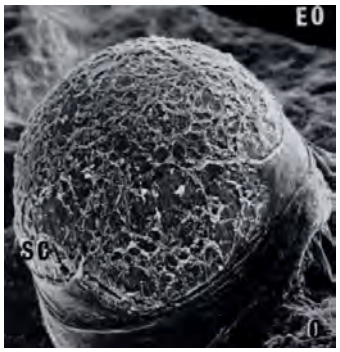

Figure 2.2 Scanning electron micrograph of a secondary oocyte during ovulation

A secondary oocyte from the lamprey (*Petromyzon marinus*, non-teleost) emerges from the follicular layer during ovulation. SC = adhesive coat attached to the zona pellucida, EO = extraovarian space and O = ovarian wall (Yorke and McMillan, 1980).

2.1.2 A brief overview of the brain-pituitary-gonad-liver axis

In mammals and non-mammalian vertebrates such as fish, reproductive processes including puberty are controlled by the brain-pituitary-gonad (BPG) axis which is a complex endocrine cascade involving many genes, receptors, enzymes and hormones (Martinez-Chavez et al., 2008, van Aerle et al., 2008). Puberty refers to the process by which a sexually immature individual acquires the ability to reproduce for the first time (reviewed in Okuzawa, 2002). The onset of puberty in females is hallmarked by the beginning of vitellogenesis, which results from activation of the BPG-liver (BPG-L) axis (reviewed in Okuzawa, 2002). In recent years it has become apparent that no single cue is responsible for 'switching on' the BPG-L axis; cues may come in the form of genetic, metabolic or environmental signals (reviewed in Roa et al., 2008). Regardless of what combination of signals trigger activation of the BPG-L axis, there is mounting evidence to suggest that the endocrine response is mediated by *kisspeptins* (Han et al., 2005, Messager et al., 2005, Kanda et al., 2008) (Fig. 2.3). *Kisspeptins* are a family of recently discovered ligands which, with its receptor Kiss1r (formerly referred to as Gpr54), are thought to be the 'gatekeepers' for both the initiation of puberty and regulation of reproductive development in mature animals (Roa et al., 2008). *Kisspeptins* are without a doubt the most potent known stimulators of the BPG-L axis discovered so far (reviewed in Tena-Sempere, 2006). In mammals *kisspeptins* stimulate gonadotropin-releasing hormone (Gnrh) neurons and subsequently trigger the release of Gnrh (Han et al., 2005, Messager et al., 2005), which before the discovery of *kisspeptins* was thought to be the 'major player' of the reproductive axis (Roa et al., 2008). The central role of Gnrh is to regulate the secretion of the gonadotropins (Gth), namely *follicle stimulating hormone* (Fsh) and *luteinizing hormone* (Lh) from the pituitary (Dickey and Swanson, 2000) (Fig. 2.3). Fsh initiates the gonadal biosynthesis of testosterone (T) and 17β-estradiol (E_2), the latter then plays a role in regulating gametogenesis through positive or negative feedback on the brain, depending of the stage of sexual maturation (Saligaut et al., 1998, Levavi-Sivan et al., 2006) (Fig. 2.3). In addition, E_2 is responsible for triggering the hepatic synthesis of the egg yolk precursor Vtg and *zona pellucida*

proteins (Zp) (Fig. 2.3) which will ultimately form the 'egg shell' surrounding the developing embryo (Celius and Walther, 1998). At the end of vitellogenesis, Lh stimulates the production of hormones involved in oocyte maturation (Planas et al., 2000) and is therefore important for successful ovulation.

2.1.3 Gonadotropin-releasing hormone

Gnrh is a key regulator of the BPG-L axis and directly stimulates the pituitary to produce Fsh and Lh (depending on reproductive stage) through hypothalamic nerve fibres, that in fish are directly connected to the pituitary (reviewed in Millar et al., 2004, Levavi-Sivan et al., 2010). In the pituitary, Gnrh binds to its respective G protein-coupled receptor (or receptors) and thereafter sexual development followed by maturation can take place (Levavi-Sivan et al., 2010).

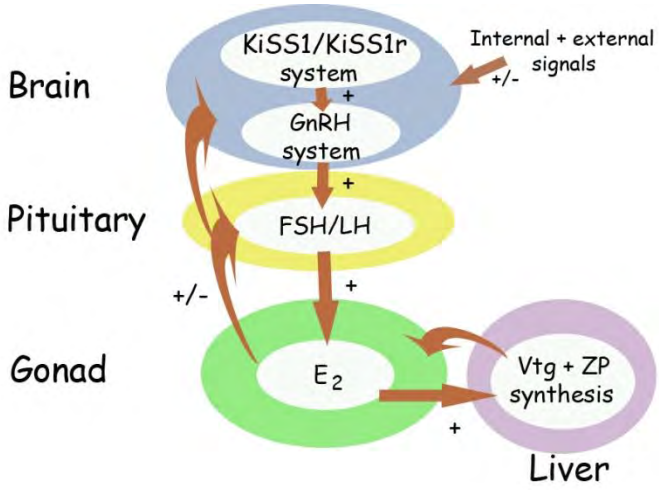

Figure 2.3 A simplified view of the BPG-L axis in fish displaying hepatic Zp synthesis
Internal and external signals have either a stimulatory (+) or inhibitory (-) effect on the initiation of reproductive development and the collective result is mediated by the Kiss1/Kiss1R system which modulates the synthesis of Gnrh. Gnrh in turn stimulates the pituitary to synthesise Fsh and Lh which trigger gonadal E_2 production (displayed), or final oocyte maturation (not displayed) respectively. E_2 regulates the hepatic synthesis of Vtg and Zp proteins which are then transported to the ovary via the blood. E_2 also has a role in controlling reproductive development through positive and negative feedback mechanisms (figure by Kelli Anderson).

To date, 14 Gnrh variants have been characterised in vertebrates while additional unique forms have been found in prochordates and invertebrates (reviewed in Lethimonier et al., 2004). Eight of these variants can be found in teleost fish which is the highest number of variants of all vertebrate classes (Lethimonier et al., 2004). Gnrh variants are traditionally named after the species from which they are characterised with the exception of mammalian Gnrh (reviewed in Somoza et al., 2002). Variants found in teleosts include: mGnrh (mammalian), cGnrh-II (chicken), cfGnrh (catfish), sGnrh (salmon), sbGnrh (seabream), hgGnrh (herring), pjGnrh (pejerrey, also called medaka, Gnrh) and wfGnrh (whitefish) (Somoza et al., 2002, Lethimonier et al., 2004, Jodo et al., 2005, Nocillado et al., 2007).

For the subsequent chapters Gnrh nomenclature has been adapted as in Lethimonier et al., (2004); sbGnrh will be referred to as Gnrh1 and cGnrh as Gnrh2. The names of other Gnrh variants will remain unchanged unless otherwise stated. Teleost fish express at least two Gnrh variants (Jodo et al., 2005) with a growing number of species expressing three forms (Lethimonier et al., 2004). It was once thought that the presence of more than two Gnrh variants in a single species was a characteristic reserved for more evolved teleosts (Lethimonier et al., 2004). However, three variants were found in Pacific herring which is considered to be a primitive fish (Carolsfeld et al., 2000). In most vertebrates, Gnrh1 is thought to be the major regulator of the BPG-L axis while Gnrh2 has been associated with sexual behaviour (Millar, 2003). The third variant (if present) may be any of the other Gnrh variants and is designated Gnrh3, although these forms appear to be less conserved across species (Lethimonier et al., 2004, Levavi-Sivan et al., 2004).

2.1.4 Gonadotropins

Fsh and Lh are glycoproteins synthesised and secreted by the pituitary in salmon (Dickey and Swanson, 1998). They are heterodimeric sharing a common α subunit and differing in their β subunit which determines their biological specificity and activity (Swanson et al., 1991, Dickey and Swanson, 1998, Dickey and Swanson, 2000). Unlike tetrapods

which only have one common α subunit, multiple α subunits have been characterised in several species of fish including salmonids (Dickey and Swanson, 2000). In salmonids, the Lh or Fsh heterodimer may be composed of either a α1 or α2 subunit together with its own unique β subunit (Dickey and Swanson, 2000). Fsh is responsible for stimulating oocyte growth by triggering the production of gonadal steroids while Lh causes a shift in steroidogenesis that promotes oocyte maturation (Nagahama et al., 1993). As such, gonadotropins have a critical function in endocrine stimulation and regulation.

2.1.5 Gonadal biosynthesis of testosterone and estrogen

The steroid hormone E_2 is produced via the stepwise metabolism of the base molecule cholesterol (reviewed in Payne and Hales, 2004). In the 2-cell type model which has been proposed for salmonids, *steroidogenic acute regulatory protein* (Star) transports cholesterol to the inner membrane of the mitochondria in the theca (outer somatic layer) which is the first site of enzymatic conversion, and the rate-limiting step of steroidogenesis (reviewed in Stocco, 2000, Nagahama et al., 1993). From here, *p450 cholesterol side-chain cleavage protein* (Cyp11a1) catalyses three sequential oxidation reactions to yield pregnenolone which is a precursor for all steroid hormones (Payne and Hales, 2004) (Fig. 2.4). After the production of pregnenolone, several enzymes are involved in the conversion of intermediate molecules and eventually T (Fig. 2.4). The thecal layer then secretes T which diffuses into the granulosa cells (inner somatic layer) through the basement membrane (Nagahama et al., 1993). In the granulosa cells, conversion of T to E_2 is catalysed by *p450 aromatase a* (Cyp19a1a, Fig. 2.4), a member of the cytochrome p450 superfamily (reviewed in Simpson and Davis, 2001).

Once synthesised, estrogen exerts its action through ligand specific receptors (Er) present in estrogen responsive tissues (Hawkins and Thomas, 2004). In teleosts, three estrogen receptors are present namely Erα, Erβb and Erβa (formerly Erγ) (Hawkins and Thomas, 2004). All receptors belong to a superfamily of ligand-activated-transcription factors (Hawkins and Thomas, 2004). As such, binding between E_2 and its receptor results in a conformational change and an E_2-Er complex is formed (reviewed in

Arukwe and Goksøyr, 2003). The complex is then transported to the chromatin where it modulates the expression of genes containing estrogen responsive elements such as Vtg (Arukwe and Goksøyr, 2003).

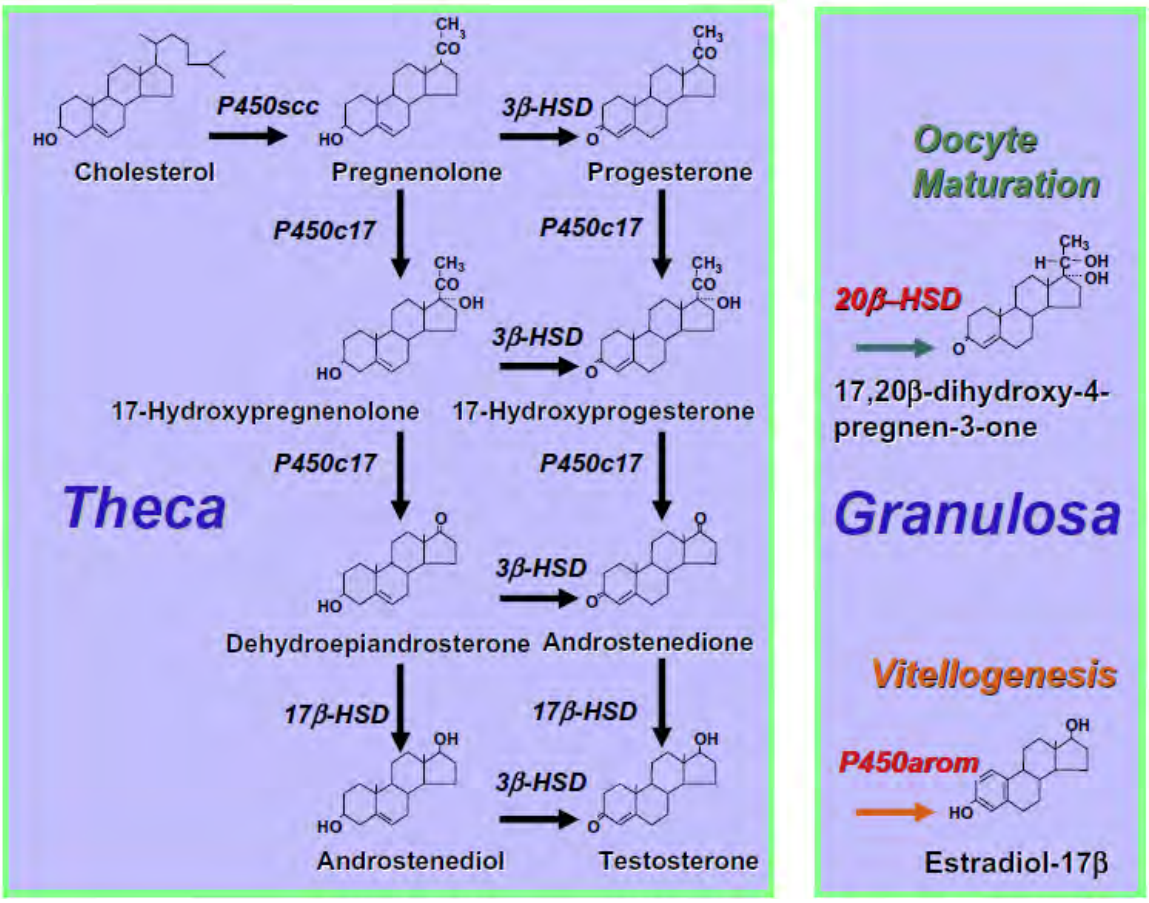

Figure 2.4 Ovarian steroidogenesis in teleosts

The stepwise synthesis of testosterone from the base molecule cholesterol in the thecal layer of the follicle, and its conversion to E_2 by the enzyme *p450 aromatase* during vitellogenesis (Lubzens et al., 2010). The enzymes shown are: *p450 side-chain cleavage* (p450scc = Cyp11a1 in text), *17-hydroxylase/C17-C20-lyase* (p450c17), *3β-hydroxysteroid dehydrogenase* (3β-hsd), *17β-hydroxysteroid dehydrogenase* (17β-hsd), *20β-hydroxysteroid dehydrogenase* (20β-hsd) and *p450 aromatase* (p450arom = Cyp19a1a in text).

2.1.6 Egg yolk production: vitellogenin genes and vitellogenesis

The egg yolk precursor protein Vtg plays a crucial part in the process of reproduction as it will ultimately become the sole source of nourishment for developing embryos (Hiramatsu et al., 2002). Vtg is a large, dimeric, lipophosphoglycoprotein hepatically produced by all oviparous animals including mammals, egg-laying vertebrates and invertebrates such as molluscs (reviewed in Babin et al., 2007). After synthesis, Vtg is released into the blood stream where it plays a role in transporting mineral salts, lipids, carbohydrates and phosphate groups (Mommsen and Walsh, 1988); Vtg is then rapidly and specifically taken up by the ovaries via receptor mediated endocytosis, and the entire process is called vitellogenesis (Tyler et al., 1988). In the developing/maturing ovary Vtg is proteolytically cleaved to yield a suite of egg yolk proteins (collectively called vitellins) including a large lipoprotein (lipovitellin) and phosvitin (Matsubara et al., 1999, Finn, 2007a). These proteins are stored in yolk granules or globules in the oocyte cytoplasm throughout reproductive development (Koya et al., 2003).

Early research suggested that only one form of the Vtg protein was responsible for embryo nutrition in teleost species. However, more recent research has provided evidence suggesting that Vtg belongs to the large lipid transfer protein superfamily which generally contains between 1-6 genes (reviewed in Buisine et al., 2002). Yet, there are exceptions to the rule; in a single cluster of the rainbow trout (*Oncorhynchus mykiss*) genome, 20 Vtg genes and ten pseudogenes have been found (Buisine et al., 2002). Despite this, only two distinct (and 'complete') genes (Vtga and Vtgb) have been characterised in teleosts thus far; these genes contain domains coding for lipovitellin, phosvitin and a β-component (Matsubara et al., 2003). Interestingly, distinct but 'incomplete' Vtg genes have also been discovered; in zebra fish (*Danio rerio)* a novel Vtg gene was characterised which lacks a phosvitin domain and is thought to be a primitive form of the gene (Wang et al., 2000). In barfin flounder (*Verasper moseri*) Vtga and Vtgb play separate roles in oocyte hydration and delivery of nutrients to the embryo (Matsubara et al., 1999). In this species Vtga has been shown to degrade within the post-vitellogenic oocyte and provide diffusible nutrients for embryo nourishment and

free amino acids which increase the eggs buoyancy by driving water influx (Matsubara et al., 1999). In contrast, it has been suggested that Vtgb degrades less readily and the remaining heavy chain of the protein is needed in the later stages of embryo development (Matsubara et al., 1999).

The regulation of Vtg genes is generally considered to be straight forward. E_2 regulates the production of Vtg in the liver by forming an E_2-Er complex that is translocated from the cytoplasm to within the nucleus where it directly binds to the *cis* region of cognate DNA sequences called estrogen responsive elements (Kumar and Chambon, 1988, Porte et al., 2006). In tilapia (*Oreochromis aureus*), the Vtg gene promoter contains a non-consensus TATA box that is able to drive basal transcription and two imperfect EREs that have similar binding affinities to consensus EREs (Teo et al., 1998). Similarly the promoter region of rainbow trout Vtg contains a non-palindromic ERE that differs from the consensus by three base pairs (Bouter et al., 2010). The binding of the E_2-Er complex to a responsive section of a gene's promoter is referred to as the 'classical' or 'genomic' pathway of estrogen action (Porte et al., 2006).

2.1.7 Egg protection: zona pellucida genes, zonagenesis and the zona pellucida

All ovulated vertebrate eggs are surrounded by an extracellular matrix of glycoproteins (reviewed in Spargo and Hope, 2003) which is loosely referred to as the 'egg shell' in many species. While the egg shell is believed to have the gross structure and function among vertebrates, its nomenclature varies greatly between classes of animals and even species (Spargo and Hope, 2003). In amphibians the egg shell is known as the vitelline envelope, in reptiles and birds the perivitelline envelope, in mammals the Zp and in fish the chorion (Spargo and Hope, 2003). In the interest of standardising the nomenclature across all species and research papers, the egg shell will be referred to as the Zp in this thesis as suggested in Spargo and Hope (2003).

The Zp in vertebrates serves many functions and its integrity is critical for the survival of offspring (Cousins and Jensen, 1994, Babin et al., 2007). The Zp prevents polyspermy after fertilisation (Renard et al., 1990), provides mechanical and microbial

protection for the embryo (Kudo, 1992), and mediates the diffusive exchange of gasses (Schmehl and Graham, 1987). In fish, the Zp contains a narrow channel called the micropyle which serves as a sole entry point for sperm (Schmehl and Graham, 1987, Amanze and Iyengar, 1990). At the point of egg activation, the micropyle closes (Schmehl and Graham, 1987) and the Zp becomes turgid and very insoluble due to water influx and structural changes of Zp matrix proteins (Masuda et al., 1991). Hardened teleost eggs can then support up to 100 times more weight than unhardened eggs (Schmehl and Graham, 1987), have the ability to trap contaminants (such as antibiotics) and kill bacteria and fungi due to enzymes present in the Zp (Kudo, 1992, Kudo and Yazawa, 1997). This demonstrates the importance of the role that the Zp plays in embryo protection.

In all vertebrates there are 2-4 major proteins that make up the Zp which are synthesised in response to E_2 stimulation in most species (Berg et al., 2004, Babin et al., 2007). However, like the Zp itself, the nomenclature for these individual proteins can vary from species to species making interpretation and comparison of scientific papers frustrating and somewhat tedious (Babin et al., 2007). For the following sections, nomenclature suggested by Spargo and Hope (2003) has been adopted for individual Zp proteins in the interest of standardisation.

To date, four distinct types of Zp proteins have been discovered in vertebrates; these include *zona pellucida a*, *b*, *c* and *x* (Zpa, Zpb, Zpc and Zpx, respectively) (Babin et al., 2007). It is now believed that an early duplication event of at least one ancient Zp gene gave rise to an ancestral form of Zpc, and the precursor for Zpa, Zpb and Zpx (Babin et al., 2007). The latter precursor underwent at least three more duplication events to produce the Zpa, Zpb and Zpx genes (Spargo and Hope, 2003). This theory is supported by Kanamori et al., (2003) who have shown close phylogenetic relationships between Zpa, Zpx and Zpb while the Zpc appears to be more distantly related. Later during the evolution of teleost of Zp genes, further duplication events occurred and now unlike higher vertebrates such as humans, teleosts express multiple forms of Zpc and Zpb (Conner and Hughes, 2003, Kanamori et al., 2003, Babin et al., 2007). The most

notable duplication event in teleosts gave rise to Zpba and Zpbb groups within the Zpb subfamily (Babin et al., 2007). In human, mouse and chicken, genes belonging to the Zpb subfamily (Zpb1 and Zpb2) are orthologous (Smith et al., 2005). However, Conner and Hughes (2003) suggest that in teleosts there may have been a duplication event involving Zpb1/Zpb2 resulting in two distinct genes that are paralogous (Zpba and Zpbb), not orthologous to their mammalian counterparts. In the medaka genome, several of these genes are linked (Kanamori et al., 2003) while in the mouse genome the Zp loci are on different chromosomes (Wassarman et al., 2004). Zpa has not been found in teleosts and Zpx has not been found in mammals; the only vertebrate in which all Zp subfamilies have been found to date is the chicken (Smith et al., 2005). There is evidence to suggest that fish once possessed a Zpa gene as the duplication event which created Zpa occurred before the divergence of fish and amphibians (Spargo and Hope, 2003). However, the gene was either lost during evolution or is yet to be discovered in fish (Spargo and Hope, 2003).

Teleost Zp genes are somewhat conserved and closely related to each other; as such, they all share some common features (Babin et al., 2007). Mammalian and teleost Zp proteins have an N-terminal signal sequence, Zp domain, a consensus furin cleavage site (CFCS) near the hydrophobic C-terminal region and a short propeptide downstream of the CFCS (Jovine et al., 2004, Wassarman et al., 2004, Darie et al., 2005) (Fig. 2.5). The Zpb protein in mammals and fish also contains a trefoil factor family domain up stream of the Zp domain, which in the past has been used for Zpb gene family identification (Wassarman et al., 2004, Fujita et al., 2008) (Fig. 2.5). In fish, Zpb and Zpc contain a proline-glutamine rich region, though this region appears to be missing in Zpx (Babin et al., 2007). All Zp proteins contain eight conserved cysteine residues which result in four intramolecular disulfides (Modig et al., 2006, Sun et al., 2010). Zpb and Zpx have an additional two cysteine residues that are conserved between mammals and teleosts (Smith et al., 2005). In addition to these ten cysteine residues, the Zpb subfamily in teleosts (not mammals) contains two additional cysteine residues bringing the total number to 12 (Smith et al., 2005).

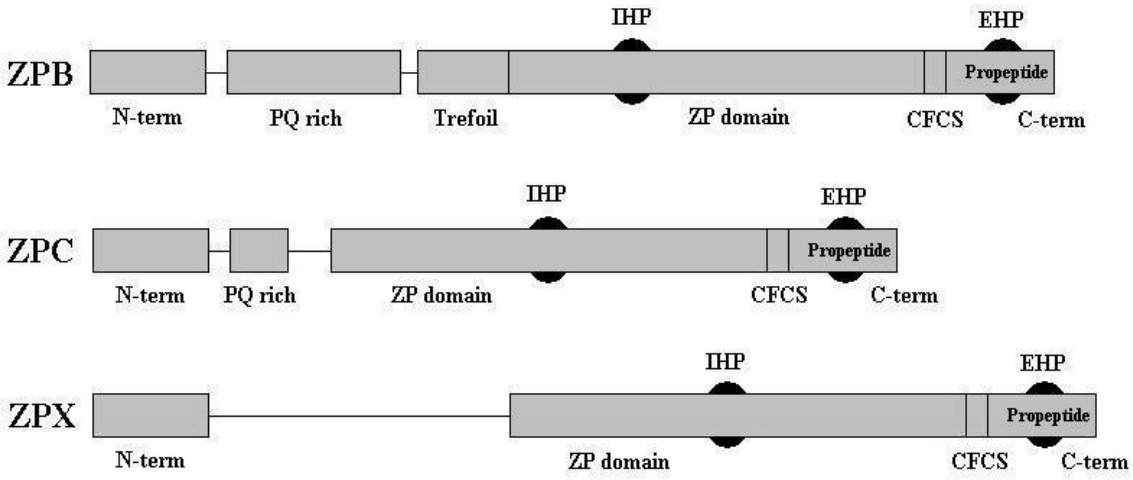

Figure 2.5 A schematic representation of teleost Zp proteins

CFCS = consensus furin cleavage site, IHP = internal hydrophobic patch and EHP = external hydrophobic patch. Adapted from Babin et al. (2007).

Due to the variety of different reproductive strategies and sites of Zp gene expression between fish species, it is quite difficult to make generalisations about the regulatory mechanisms of genes in the Zp family (Babin et al., 2007). For example, in zebrafish where Zps are exclusively expressed in the ovary, Zp expression cannot be induced by E_2 and the promoter region of these genes lack EREs (Mold et al., 2001, Liu et al., 2006). It is thought that in zebrafish, Zp expression is at least party regulated by nuclear transcription factor Y through CCAAT boxes (Mold et al., 2009) and through E-boxes (Mold et al., 2001) that bind basic helix-loop-helix proteins such as factor in germ cell α (Fig1α). In medaka (*Oryzias latipes*) and winter flounder (*Pseudopleuronectes americanus*), hepatic Zp synthesis occurs in response to E_2. The promoter region of medaka contains multiple E-boxes (Kanamori et al., 2003) (and perhaps other regulatory elements), and the promoter region of winter flounder contains imperfect EREs and CCAATT and TATAAA boxes (Lyons et al., 1993). In some salmonids, gilthead seabream (*Sparus aurata*), medaka and chicken dual synthesis in both the ovary and liver takes place (Kanamori et al., 2003, Smith et al., 2005, Modig et al., 2006). While hepatically expressed Zps are inducible by E_2 (as in Atlantic salmon), it has recently become

apparent that many factors besides E_2 could potentially be involved in the regulation of Zps (Berg et al., 2004, Knoebl et al., 2004).

Expression, synthesis and subsequent incorporation of Zp proteins into the developing Zp (zonagenesis) begins prior to vitellogenesis (Celius and Walther, 1998). Successful eggshell assembly relies on a series of sequential posttranslational events that prepares each protein for incorporation. Firstly, the endoplasmic reticulum cleaves the N-terminal region from the polypeptide then further modifies the protein by adding three or four N-linked oligosaccharides (Wassarman et al., 2004, Darie et al., 2005). Then, in the Golgi apparatus, N-linked oligosaccharides are modified to 'complex type' and O-linked oligosaccharides are added to the precursor Zp molecule (Wassarman et al., 2004). The proteins are then packed into secretory vesicles and released into the bloodstream or intercellular space, depending on the site of synthesis (Tesoriero, 1977, Jovine et al., 2002, Wassarman et al., 2004).

In mammals Zp protein assembly involves a transmembrane domain (TMD) which is at the C-terminal region of the protein (Wassarman et al., 2004). When the secretory vesicle arrives at the oocyte and fuses with the plasma membrane, the TMD anchors the protein to the egg plasma membrane to assist matrix assembly (Wassarman et al., 2004). However, teleost Zp genes lack a TMD, and assembly is believed to be dependent on two regions within the gene; the internal hydrophobic patch (IHP) and the external hydrophobic patch (EHP, Fig. 2.5) (Jovine et al., 2004). There is evidence to suggest that in the rainbow trout proteolytic cleavage at the CFCS must take place on the egg before mature Zp proteins are incorporated into the inner layer of the egg shell membrane (Darie et al., 2005). In trout, cleavage at the CFCS and consequent removal of the C-terminal propeptide (including the EHP) eliminates interactions between the EHP and IHP, leaving the IHP free to form intermolecular bridges with other peptides which subsequently creates the Zp (Darie et al., 2005). In addition, it has been found that mutations in either the IHP or EHP do not hinder Zp secretion from the site of synthesis, but completely obliterate egg shell assembly which supports the current view on formation of the Zp matrix (Jovine et al., 2004).

The structure of the Zp matrix, in terms of Zp polymers is poorly understood in teleosts. In mice, the Zp is composed of long polymers of Zpa and Zpc that are cross linked with Zpb supposedly at the trefoil domain (Wassarman et al., 2004). It is currently unclear as to how closely teleost Zp filaments follow the mammalian model, although it has been suggested that in seabream dimers of Zpc-Zpba and Zpc-Zpbb build filaments that are cross linked by parts of the Zpbb protein (reviewed in Monné et al., 2006). Additionally, it is now known that the seabream Zp contains Zp proteins in a relative ratio of 8:4:1 for Zpb: Zpc: Zpx respectively (Modig et al., 2006), and Zpbb proteins are consistently more abundant than any other isoforms (Hyllner et al., 2001). Monné et al. (2006) theorised that there are a higher number of Zp cross links in the teleost than in the mammalian extracellular matrix. This could explain why the teleost Zp is more resilient to various types of stress as suggested by Modig et al. (2008).

For some time it has been clear that the Zp of teleost species is made up of layers with varying mass and or chemical composition (Schmehl and Graham, 1987). Until recently, the detailed microscopic structure and spatial molecular composition of these layers have remained unclear. Early work showed that the Zp of salmonids is made up of an outer adhesive coating that is easily rubbed off, a thin outer layer containing pore canal plugs which continue through a thick inner layer which has an extensive network of fibrous material (Schmehl and Graham, 1987). At the start of Zp formation, proteins assemble at the base of microvilli that extend from the plasma membrane to the surrounding granulosa cells (Babin et al., 2007). Prior to ovulation, the microvilli are withdrawn from the Zp which creates pores (Schmehl and Graham, 1987). This has been shown in zebra fish and carp where the internal layers in the Zp of ovulated eggs contain continuous pore canals (Renard et al., 1990, Rawson et al., 2000) (Fig. 2.6), and in Atlantic salmon where pore canals have been observed on the outer layer of the Zp (King et al., 2003). It has been noted that the outer surface of the Zp is granular in appearance, and the grainy projections on the surface of this layer can obscure pore openings (Rawson et al., 2000, King et al., 2003) (Fig. 2.6).

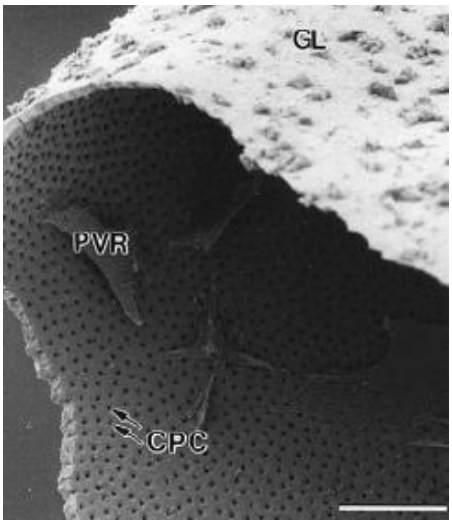

Figure 2.6 Inner and fracture surface of the zebrafish Zp

This micrograph shows pore canals (CPC) and the granular outer surface (GL) of the extracellular matrix. The PVR are remnants of the perivitelline space. Scale bar = 10 µm (Rawson et al., 2000).

The layers of the Zp have different morphological and biochemical properties (Renard et al., 1990, Schmehl and Graham, 1987) and research into the spatial distribution of the various Zp proteins within the Zp matrix is still in its infancy. Recently, Modig et al. (2008) demonstrated that in seabream pre-ovulated oocytes, the distribution of Zp isoforms are localised in distinct areas within the Zp matrix. Zpx was found exclusively in the internal part of the Zp (facing the oocyte), the middle of the Zp was made up of Zpba, Zpbb and Zpc while the outer layer contained only Zpc (Modig et al., 2008) (Fig. 2.7). Additionally Zpba was found within the oocyte and in the adjacent follicular layer. Since Zpbs were the most abundant of all isoform types, they may be important for cross-linking peptides in the matrix as previous suggested for the mammalian system. However, the inner and outer most layers of the Zp matrix were comprised of only Zpx and Zpc respectively; this suggests that these filaments may react with themselves in the absence of Zpb molecules to form polymers (Modig et al., 2008).

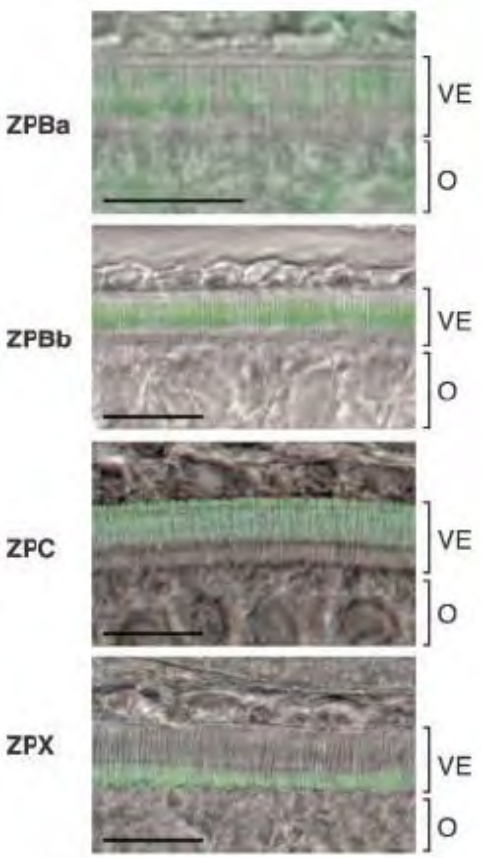

Figure 2.7 Distribution of Zp isoforms within the seabream Zp

Immunofluorescence microscopy of the Zp using isoform specific antibodies in a vitellogenic oocyte. VE = vitelline envelope = Zp, O = oocyte, scale bar = 10 µm (Modig et al., 2008).

Egg activation refers to the process where spawned eggs become metabolically active and undergo structural changes. In salmonids, activation occurs when eggs are exposed to hypotonic solutions; this causes the Zp to separate from the plasma membrane and create a perivitelline space (PS) (reviewed in Coward et al., 2002, Finn, 2007b). Then, a significant depolarisation event occurs followed by a slower, K^+ dependant hyperpolarisation (Finn, 2007b). This leads to the release of internally stored Ca^{2+} which repeatedly pulses across the egg in a series of waves (Webb and Miller, 2000), causing docking and binding of the cortical alveoli to the plasma membrane (Finn, 2007b). Thus, the plasma membrane is modified and is now referred to as the perivitelline membrane (Finn, 2007b). Formation of the perivitelline membrane results in the exocytosis of large polysialoglycoproteins, hyaline, proteases and

transglutaminases into the PS (Inoue and Inoue, 1986, Finn, 2007b). The hardening process in salmonids is thought to depend on the Ca^{2+} ions, phospholipids and enzymes present in the PS or Zp (Zotin, 1958). Upon Zp hardening, Zp proteins are polymerised yielding proteins of a higher molecular weight (Coward et al., 2002) implying that covalent crosslinks are formed between matrix proteins (Iuchi et al., 1991). This, combined with increased egg turgidity caused by water influx during hardening (Masuda et al., 1991) is thought to increase the 'toughness' of the egg making it harder, less soluble and an effective protective barrier (Iuchi et al., 1991, Coward et al., 2002).

2.1.8 Final oocyte maturation and ovulation

Developing teleost oocytes are arrested at the first meiotic prophase until they have accumulated the yolk proteins essential for oocyte survival (Lubzens et al., 2010). Once the growth phase (vitellogenesis) is complete, the oocytes are ready to resume meiosis and enter a phase called final oocyte maturation (FOM) (reviewed in Nagahama and Yamashita, 2008). This stage includes breakdown of the germinal vesicle, chromosome condensation, assembly of meiotic spindle and formation of the first polar body (Nagahama and Yamashita, 2008). FOM is believed to be regulated by 3 factors in teleosts: Lh, *maturation inducing hormone* (17α, 20β-dihydroxy-4-pregnen-3-one, MIH) (Fig. 2.4) and *maturation promoting factor* (heterodimer of cyclin B and cyclin-dependant kinase, Mpf) (Nagahama and Yamashita, 2008).

While Lh is considered to be the gonadotropin responsible for induction of FOM, it does not directly stimulate the resumption of meiotic division (reviewed in Patiño et al., 2001). Lh stimulates the biosynthesis of MIH through the conversion of intermediate precursors. Pregnenolone is converted to 17α-hydroxypregnenolone or progesterone in the outer thecal layer, and these precursors are then converted to 17α-hydroxyprogesterone which passes through the basal lamina into the granulosa layer. In the granulosa, 17α-hydroxyprogesterone is metabolised to MIH by *20β-hydroxysteroid dehydrogenase* (20β-hsd), the action of which is greatly enhanced by Lh (Nagahama and Yamashita, 2008). Once MIH is synthesised, the signal is transduced

into the oocyte cytoplasm through a G-protein-coupled receptor and as a result, formation and activation of MPF takes place (Nagahama and Yamashita, 2008). MPF then triggers the progression of meiosis and associated morphological changes in the oocyte such as GVBD, chromosome condensation and spindle formation prior to ovulation (Nagahama and Yamashita, 2008).

Ovulation refers to the process whereby oocytes are released from the surrounding follicular layer prior to fertilisation (Yorke and McMillan, 1980) (Fig. 2.2). Ovulation is thought to occur as a result of an inflammatory process wherein the follicular layer is broken down by proteolytic enzymes (Hajnik et al., 1998, Bobe et al., 2006). However, at the present time the enzymes responsible for follicular rupture have not been fully characterised (Nagahama and Yamashita, 2008).

2.2 The effect of age on reproductive physiology and gonad morphology

The effects of age on reproduction have not been widely studied in fish despite the significant part it can play in endocrine function and gonadal morphology. In snapper (*Pagrus auratus*), two year old maiden spawners had a relatively smaller gonad size than three year old repeat spawners (Cleary et al., 2000). Similarly, the ovaries of maiden striped bass (*Morone saxatilis*) contained an abnormally heterogeneous population of oocytes that were comparatively smaller than the oocytes of repeat spawning fish (Holland et al., 2000). In a study using maiden (n=2) and repeat (n=1) spawning Atlantic halibut (*Hippoglossus hippoglossus*) the dry weight of eggs from maiden fish had a significantly lower dry weight than that of repeat fish and fertilisation was 56 % versus 81 % respectively (though this study had virtually no biological replication and statistical analysis depended on multiple eggs from just three fish total) (Evans et al., 1996). Interestingly, eggs from maiden spawning fish were significantly lower in total lipid, triacylglycerol and sterol content than eggs from the repeat spawning fish (Evans et al., 1996). Eggs originating from maiden fish were also lower in two essential fatty acids: docosahexaenoic acid and arachidonic acid (Evans et al., 1996). While interesting, these results should be interpreted with caution, as this study did not allow for natural

variation that might occur within a population of fish. Similarly, maiden striped bass produced eggs that contained a few (if any) yolk granules which was markedly different to what was observed for repeat spawning fish (Holland et al., 2000). These studies indicate that maiden spawning fish may produce biochemically inferior eggs compared to repeat spawning fish (Evans et al., 1996), and the consequences of this could be biologically significant in terms of reproductive performance.

The molecular basis for variable reproductive physiology and performance in fish of various ages is for the most part guess work. However, differences in endocrine competency between fish of different ages have been investigated in some species. In striped bass, sexual maturation for the first time (pubertal development) in three year olds was associated with a 34-fold increase in *fshβ* subunit mRNA from basal levels compared to a 218-fold increase in four year olds (Hassin et al., 1999). Similarly in rainbow trout circulating levels of Fsh were significantly higher in repeat spawning fish than in maidens (Prat et al., 1996). Both of these species display the 'dummy run' phenomenon where the endocrine system does not reach full reproductive capacity until at least one reproductive season has been completed. Low *fshβ* subunit mRNA expression and subsequently low circulating levels of Fsh may account for endocrine suppression downstream at the level of the ovary. In striped bass, E_2 levels were lower during pubertal development compared to the second reproductive season (Holland et al., 2000). It was hypothesised that this could be due to insufficient gonadotropin production and would therefore also result in reduced vitellogenesis (Holland et al., 2000) and possibly other E_2 dependant processes. Lack of E_2 stimulation appears to be the basis for the lack of yolk granules in oocytes from maiden but not repeat fish from that study (Holland et al., 2000).

Saltas production data shows that survival of Atlantic salmon eggs to the eyed embryo stage from maiden (first time) spawners can be as low as 30-50 %, compared to 60-80 % for eggs from repeat spawning fish reared at ambient temperature. It is unclear as to whether the first reproductive season in Tasmanian Atlantic salmon is characterised by poor reproductive performance due to the dummy run phenomenon.

Although at this time it appears that some other endocrine dysfunction may be at play (King, H 2007, pers. comm., 6 February). None the less, the effect of age on oocyte development and endocrine function in Atlantic salmon has never been systematically investigated. As such, the underlying molecular mechanisms which regulate reproduction and therefore determine egg quality in maiden versus repeat spawning fish of this species remain unknown.

2.3 The effect of elevated temperature on reproductive physiology and egg quality

The effects of temperature on the BPG-L axis and reproductive performance in teleosts has been investigated in many species (Davies and Bromage, 2002, Mann and Peery, 2005, Watts et al., 2005, van der Meeren and Ivannikov, 2006). It is now established that shifts in water temperature of just a few degrees either above or below the optimum range can have an adverse effect on the reproductive processes in both wild and farmed species (Tveiten and Johnsen, 1999, Davies and Bromage, 2002, Mann and Peery, 2005). While photoperiod is recognised as the major variable for determining the time of maturation in salmonids, temperature is also able to delay the time of FOM and ovulation when conditions are not ideal (Taranger and Hansen, 1993, Davies and Bromage, 2002). Common wolffish (*Anarhichas lupus*, non-salmonid) are commonly found at 1-6 °C, and exposure to temperatures of 8 and 12 °C during vitellogenesis delayed FOM and consequently ovulation by 4 and 5 weeks respectively compared to the 4 °C control group (Tveiten and Johnsen, 1999). Similarly, rainbow trout that experienced temperatures of up to 20 °C had delayed and desynchronised spawning profiles compared to fish maintained between 7-10.5 °C (Davies and Bromage, 2002), and in Arctic charr (*Salvelinus alpinus*) ovulation was delayed at 8 °C and completely inhibited at 11 °C compared to fish held at 5 °C (Gillet, 1991). Interestingly, the latter study showed that when fish were transferred from 8 to 5 °C, ovulation was stimulated and synchronised. Therefore, some degree of thermal recovery was possible (Gillet, 1991). This phenomenon has also been demonstrated in Atlantic salmon where ovulation was delayed at 11 °C relative to 6 °C and completely inhibited at 16 °C until

temperature reduction to 8 °C occurred (King and Pankhurst, 2000). This suggests that in thermally challenged salmonids, temperature reduction may assist in restoring normal biological function (King et al., 2003). The biological significance of the modulating effects of temperature are unclear; however, it has been suggested that delayed ovulation may account for seasonal temperature variation from year to year by preventing oviposition at higher temperatures when food is limited (Taranger and Hansen, 1993).

Commonly used measures of reproductive fitness are absolute and relative fecundity (total number, and number of eggs relative to body weight respectively). In Atlantic cod (*Gadus morhua*), rainbow trout and common wolffish relative fecundity remained essentially unaffected by temperature manipulation; however, in common wolffish absolute fecundity was significantly lower at 12 °C compared to the 8 and 4 °C groups due to poor fish growth at elevated temperature (Tveiten and Johnsen, 1999, Davies and Bromage, 2002, van der Meeren and Ivannikov, 2006). On the other hand, temperature appears to have a varied effect on egg size. In Atlantic salmon, mean follicle diameter was smaller in fish reared at 22 °C compared to fish at 18 and 14 °C prior to and at ovulation (King et al., 2003), while common wolffish fish had significantly larger eggs at 8 °C compared to 4 and 12 °C temperature groups (Tveiten and Johnsen, 1999). In contrast, follicle diameters were essentially unaffected by thermal insult in rainbow trout at temperatures ranging between 9 and 21 °C (Pankhurst et al., 1996).

Combined with fecundity, high egg fertility and survival are the collective 'holy grail' of aquaculture hatcheries. For this reason it is unfortunate that thermal insult has a marked effect on egg fertility, survival and therefore egg viability (Davies and Bromage, 2002). In the Atlantic cod, temperatures above 9.6 °C resulted in significant reductions in egg fertility, with fertilisation rates as low as 11 % (van der Meeren and Ivannikov, 2006). Additionally, the average rate of fertilisation in Atlantic salmon was significantly reduced at 16 °C compared to 11 and 6 °C (King and Pankhurst, 2000) which is in agreement with another study on Atlantic salmon were fertility exceeded 85 % at 14 and 18 °C but was lower than 70 % at 22 °C (King et al., 2003). In Arctic charr, the

fertility of eggs from fish exposed to 8 °C then transferred to 5 °C in December was 78 %. Interestingly, if fish were transferred to 5 °C 1 month later in January, egg viability was reduced and fertility fell below 63 % (Gillet, 1991). This further outlines the potential for thermal rescue of heat challenged fish, and indicates that the timing of thermal rescue may be critical.

As mentioned, high embryo survival rates are of critical importance to the aquaculture industry. Thermally challenged rainbow trout had similar percent egg survival to the eyed stage at 9, 12 and 15 °C however no eggs survived at 18 or 21 °C (Pankhurst et al., 1996). Patterns of survival to the eyed stage were similar in Atlantic salmon where higher survival rates were seen in fish maintained at 5-7 °C compared to 8 or ~13 °C (Taranger and Hansen, 1993). In wild chinook salmon (*Oncorhynchus tshawytscha*) tagged with internal and external temperature monitors during migration from salt to fresh water, the five fish which experienced the highest temperatures (>20 °C) exhibited five of the six highest embryo mortalities (n = 15) (Mann and Peery, 2005). These studies outline the sensitivity of farmed and wild fish to variations in water temperature which occur as a result of seasonal variation and potentially climate change.

Developmental events that occur prior to ovulation can affect egg quality in a variety of ways. Under normal environmental conditions (10-15 °C) germinal vesicle migration (GVM) in white sturgeon (*Acipenser transmontanus*) is slow during autumn and winter then accelerates during spring (Webb et al., 1999). However, when fish were exposed to 18 °C the pattern of migration was altered with accelerated movement occurring during winter (Webb et al., 1999). In the same study, ovarian biopsies from thermally challenged fish showed that in females between 35-70 % of eggs had undergone GVBD however none had ovulated (Webb et al., 1999). Recently scanning electron microscopy (SEM) revealed that ovulated eggs from Atlantic salmon exposed to elevated rearing temperatures contained holes or breaks in the Zp (King et al., 2003) (Fig. 2.8). The authors suggested that the 'blister like eruptions' may provide opportunistic bacteria and fungi with an opening to invade the egg and subsequently kill

the embryo. Surprisingly, it was determined that the damage to the Zp was already present at the point of ovulation, which confirms the maternal origin of the problem and implies that zonagenesis may be adversely affected by elevated temperature (King et al., 2003). Zp damage however, is not new to salmonid aquaculture. In the British Columbian salmon industry, Zp damage is known as soft shell disease (SSD) and was found to occur in years when ambient temperature is raised (Cousins and Jensen, 1994). To investigate this phenomenon fertilised coho salmon (*Oncorhynchus kisutch*) eggs were incubated at either 8 or 13 °C and Zp integrity was analysed via SEM (Cousins and Jensen, 1994). Incubation at 13 °C significantly raised the hydrostatic pressure and consequently the surface tension of the eggs (Cousins and Jensen, 1994). As a result, the central pore canal plugs were pushed out instead of being loosely fitted inside the canal as was the case at 8 °C (Cousins and Jensen, 1994). The authors concluded that this could provide bacteria and fungi with an entry point into the inner membrane of the Zp, and suggested that the problem of reduced egg survival is therefore not due to a specific pathogen, but is associated with Zp integrity (Cousins and Jensen, 1994). While the situation in Atlantic salmon appears to be different, and fertilised eggs are routinely incubated at 8 °C in the Atlantic salmon industry, this does outline the importance of Zp integrity and the sensitivity of all stages of smolt production to thermal insult. Especially given that salmonids in general are susceptible to SSD (Cousins and Jensen, 1994).

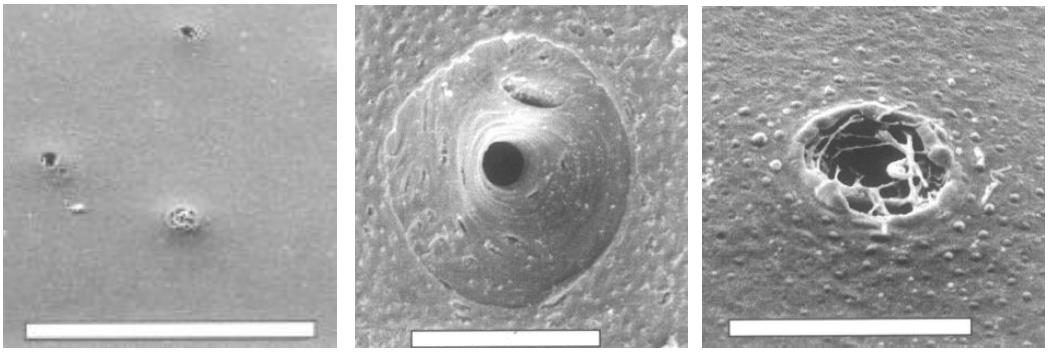

Figure 2.8 Scanning electron micrographs of damaged Atlantic salmon ova

The Zp of the eggs in these micrographs contain abnormal 'blister like' eruptions (scale bars = 150, 15 and 15 µm respectively), reproduced from King et al., (2003).

The effects of thermal challenge can be seen in eggs post fertilisation during early development. In the cold water fish Atlantic cod, increases in rearing temperature above 9.6 °C resulted an increased incidence of abnormal egg development after fertilisation (van der Meeren and Ivannikov, 2006). Specifically, with increased temperature the incidence of asymmetric cell cleavage, blastodisc abnormalities and fragmentation or disintegration of cells increased (Fig. 2.9) (van der Meeren and Ivannikov, 2006). Changes such as this will undoubtedly have an effect on egg mortality (van der Meeren and Ivannikov, 2006).

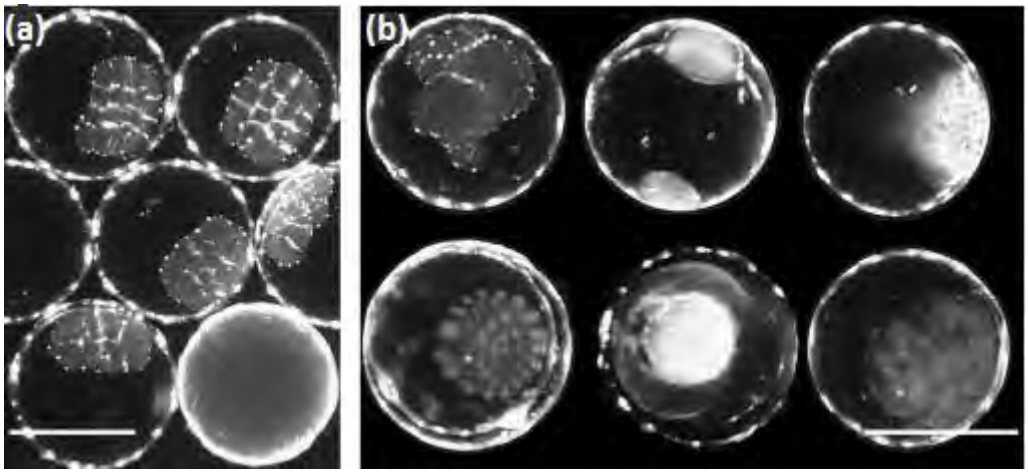

Figure 2.9 Normal versus abnormal development in fertilised Atlantic cod eggs
(a) An unfertilised egg (bottom right) and normal development versus (b) abnormal development: asymmetric cell cleavage (upper left), small blastodisc with a second opaque spot (upper middle), opaque blastodisc (upper right and lower middle) and fragmentation or disintegration of cells (lower left and right) (scale bar = 1 mm) (van der Meeren and Ivannikov, 2006).

It is now established that thermal challenge can have a marked effect on many reproductive performance indicators such as fertility, survival and post fertilisation development (Gillet, 1991, Pankhurst et al., 1996, van der Meeren and Ivannikov, 2006). However, the exact mechanism(s) by which temperature exerts its effects is far less certain, and this is likely to vary between species and with severity of thermal exposure. It is known however, that the effects of thermal challenge come about through the impairment of endocrine function at a variety of levels (Pankhurst et al., 1996, King and

Pankhurst, 2000, King and Pankhurst, 2004a). In the pituitary of red seabream (*Pagrus major*), maintenance at 24 °C caused a reduction in pituitary *gonadotropin-releasing hormone receptor* (*gnrhr*) gene expression levels relative to fish reared at 17 °C for 10 and 19 days (Okuzawa et al., 2003). Additionally, exposure to elevated temperature resulted in the down-regulation of *follicle stimulating hormone receptor* (Fshr) in Japanese flounder (*Paralichthys olivaceus*) during sexual differentiation (Yamaguchi et al., 2007), and adult female pejerrey (*Odontesthes bonariensis*) during vitellogenesis (Soria et al., 2008). In the pejerrey study, thermally induced endocrine suppression resulted in an increased incidence of ovarian atresia and spawning was completely inhibited (Soria et al., 2008). In addition to the aforementioned impairment, the ovaries of thermally challenged fish may also be affected indirectly by the action of cortisol (F). When exposed to elevated temperature, a fish's primary response may be the production of 'stress proteins' such as F and intracellular heat stress proteins (Hsps) (reviewed in Schreck et al., 2001, Iwama et al., 1998). In Arctic charr, it has been shown that F antagonises the induction of Vtg by E_2 while it potentiates expression of Zp proteins (Berg et al., 2004). In Atlantic salmon, F levels were generally higher at elevated temperatures and a weak but significant negative correlation was found between F and circulating Vtg during summer (King et al., 2003). In contrast, a study using fathead minnow (*Pimephales promelas*) showed that treatment of F and 17α-ethinylestradiol (EE_2) in combination potentiated expression of *vtg* in a dose dependant manner compared to treatment with EE_2 alone (Brodeur et al., 2005). Collectively these results indicate that exposure to high temperature is likely to generate a general stress response in terms of cortisol production (King et al., 2003), and the effects of this may be species dependant.

In teleosts, a critical role of E_2 is to stimulate transcription of Vtg and Zp genes during sexual development. In rainbow trout, exposure to elevated temperature had no significant effect on plasma levels of T or E_2 (Pankhurst et al., 1996). The authors suggested that the mechanisms controlling Vtg synthesis were in turn unaffected, and the poor reproductive performance of fish at elevated temperatures may be due to

endocrine impairment during FOM or ovulation (Pankhurst et al., 1996). In contrast, E_2 was generally lower in Atlantic salmon reared at 16 °C relative to 6 and 11 °C (King and Pankhurst, 2000) and generally lower in fish reared at 22 °C compared to 14 °C (King et al., 2003). This suggests that the two salmonoid species may respond differently to thermal challenge. In the same study by King et al., (2003), T levels followed a different pattern to that of E_2 and were higher in fish reared at 22 °C at 3 of 5 sampling points. The comparatively high levels of T and low amounts of E_2 in the high temperature groups suggest that conversion of T to E_2 was impaired, perhaps due to inhibition of Cyp19a1a activity (King et al., 2003). This in turn could account for the relatively low concentration of plasma Vtg measured in thermally exposed fish at some sampling points (King et al., 2003). As E_2 also triggers the synthesis of Zp proteins in salmonids, this also raises doubt as to whether zonagenesis in these animals was 'normal', especially in light of the observed Zp abnormalities (King et al., 2003). The effect of thermal stress on E_2 synthesis was also investigated in ovarian follicles taken from Atlantic salmon maintained at 14, 18 or 22 °C for one month (Watts et al., 2004). Follicles from fish reared at all temperatures produced T in response to human chorionic gonadotropin and various gonadal steroid precursors (Watts et al., 2004). However, E_2 production was impaired only in the 22 °C group which further supports the theory of impaired Cyp19a1a synthesis or activity at high temperature (Watts et al., 2004). While no direct evidence has been put forward to support Cyp19a1a inhibition in adult salmon, the thermal sensitivity of Cyp19a1a has been demonstrated in several non-salmonid species. Nineteen month old red seabream showed suppression of *cyp19a1a* gene expression at 25 and 20 °C relative to 15 °C after 4 and 8 weeks respectively (Lim et al., 2003). Similarly, *cyp19a1a* gene expression was reduced in Atlantic halibut larvae reared at 13 °C relative to 7 °C (van Nes and Andersen, 2006). In light of the demonstrated thermal sensitivity of *cyp19a1a* in juvenile fish, research into the effects of elevated temperature on *cyp19a1a* gene expression and/or activity in female Atlantic salmon is justified. Verification of the relationship between temperature, *cyp19a1a* and

plasma E_2 will assist in understanding the molecular basis for reduced egg quality in female Atlantic salmon at high temperature.

Once E_2 is synthesised, it must bind to Ers in order to initiate or maintain transcription. In order to determine if maintenance at elevated temperature impairs Atlantic salmon E_2 signal transduction (and therefore reducing the biological potency of E_2 in terms of downstream protein synthesis), the binding affinities of Ers were determined in ovarian follicles taken from fish exposed to various temperatures during vitellogenesis (Watts et al., 2005). This study revealed that Er binding affinity was significantly lower in follicles originating from fish exposed to 22 °C in February compared to 14, 18 and 22 °C at other times during development (Watts et al., 2005). While high affinity receptors were present in the 22 °C in February, they only represented 20 % of the total receptor population (Watts et al., 2005). The authors of this study suggested that a critical period of temperature sensitivity may occur before or during February (Watts et al., 2005).

As mentioned, multiple studies have eluded to a potential 'window of sensitivity' in terms of thermal insult in Atlantic salmon (Watts et al., 2004, Watts et al., 2005, King et al., 2007); meaning that the effects of thermal challenge may be exacerbated at certain stages of sexual development (Watts et al., 2004). To investigate the effects of thermal challenge on reproductive performance at various stages of vitellogenesis, Atlantic salmon were maintained at 14 °C continuously or at 22 °C for 4, 6 or 12 weeks during the austral summer or autumn (King et al., 2007). Endocrine suppression was evident in fish exposed to 22 °C after just 3 days of thermal exposure compared to the 14 °C group (King et al., 2007). In these fish, plasma E_2 and Vtg concentrations were approximately half of the levels observed in fish from the 14 °C control treatment (King et al., 2007). The fertility and survival of ova from fish continuously maintained at 14 °C were greater than 85 and 70 % respectively while a 6 or 12 week exposure to 22 °C caused a reduction in fertility (<70 and <45 %) and survival (40 and 13 %) respectively (King et al., 2007). However, despite significant endocrine effects observed throughout vitellogenesis, a 4 week exposure to elevated temperature caused significant reductions

in fertility (<65 %) and survival (30 %) only when the exposure occurred between mid-February and mid-March (King et al., 2007). The authors from that study found that short term thermal challenge can be just as harmful of prolonged exposure, and corroborated the findings from previous work that suggested the presence of a period of heightened thermal sensitivity (Watts et al., 2005). This is quite concerning as temperature spikes that exceed 20 °C are sometimes recorded in farming areas (Watts et al., 2005, Battaglene et al., 2008), and if these spikes occur at a critical time of reproduction the effects on reproductive performance could be devastating (King et al., 2007).

In an effort to overcome the negative impacts of elevated temperature on endocrine function and reproductive performance, the usefulness of a *luteinizing hormone releasing hormone* analogue (Lhrha, 25 $\mu g.kg^{-1}$ body weight) implant was investigated in Atlantic salmon exposed to elevated temperature (King and Pankhurst, 2004a). Tasmanian Atlantic salmon were housed at 6, 11 or 16 °C from early April (late vitellogenesis) through to the preovulatory period in early June (King and Pankhurst, 2004a). Approximately one month prior to spawning fish were treated with Lhrha and reproductive performance indicators as well as plasma levels of T, E_2, and MIH were analysed to determine if normal preovulatory changes in gonadal steroids had occurred (King and Pankhurst, 2004a). In untreated fish, ovulation was delayed at 11 °C relative to 6 °C, and delayed at 16 °C until temperature reduction to 8 °C (King and Pankhurst, 2004a). Ovulation was advanced in the 6 and 11 °C groups following Lhrha treatment while no effect was seen in treated fish at 16 °C (King and Pankhurst, 2004a). In general, preovulatory changes in gonadal steroids are characterised by declining E_2 and a transitory peak in T, followed by a significant increase in MIH (Slater et al., 1994, King and Pankhurst, 2004b). In the untreated controls, a preovulatory rise in MIH was only observed in the 6 °C group (King and Pankhurst, 2004a). However, preovulatory peaks in MIH were observed in the 6 and 11 °C groups in response to Lhrha (King and Pankhurst, 2004a). In contrast, there was little production of MIH in the 16 °C group with or without Lhrha (King and Pankhurst, 2004a). While Lhrha did promote MIH

production and advance ovulation in the 6 and 11 °C groups, egg fertility and survival was not different to the controls (King and Pankhurst, 2004a). Additionally, broodstock in the 16 °C group that were treated with Lhrha either produced non-viable ova or died prior to ovulation (King and Pankhurst, 2004a). Interestingly, when fish reared at 16 °C were given an Lhrha implant in combination with a temperature ramp down to 11 °C prior to ovulation, egg quality was fully restored which indicates that a combination of thermal and hormonal therapy may be required at elevated temperature (King and Pankhurst, 2004b). At the present time, this treatment protocol has not been tested at the higher temperatures typically encountered at Tasmanian farms.

To date, gene expression profiles of key proteins (such as Vtg, Zps, Erα and Cyp19a1a) that are thought to play a role in egg quality in maiden or repeat spawning Atlantic salmon have not been characterised over an entire reproductive season, let alone under different thermal regimes. Additionally, the expression of different Zp gene subgroups has never been analysed to explore the molecular mechanisms underlying Zp damage in thermally challenged maiden or repeat spawning fish. A general lack of understanding has made it difficult to pinpoint the extent of endocrine dysfunction. For example, does impairment occur at the transcriptional level for a variety of critical reproductive genes, including those encoding hormones (or receptors) that play a role in reproductive regulation? In the case of the Zp matrix, a defect may even occur at the level of egg shell assembly after gene transcription and protein synthesis. Due to this lack of knowledge, the salmon industry currently lacks the ability to develop new industry practices and reach desired fertility outcomes at high temperature which could threaten the sustainability of the industry.

2.4 General research aims

The economic viability of farming Atlantic salmon is strongly influenced by the cost of producing smolts. Understanding the molecular mechanisms that contribute to, or cause reproductive failure in maiden spawning fish is an essential component of reducing industry production costs, and at a broader level, ensuring that there are

sufficient smolts produced each year to maintain industry production. Therefore, the overall aim of the research is to investigate the effect of temperature on reproductive development in maiden and repeat spawning farmed Atlantic salmon, and to understand the molecular basis for improved egg quality and survival. To do this, the research will firstly develop the molecular tools required to measure the expression levels of genes important for successful reproduction, and then use these tools to assist in understanding the molecular basis of normal reproductive and egg shell development in maiden and repeat spawning salmon reared under improved (cooled) conditions. The molecular mechanisms underlying optimal egg development in these fish will then be compared to those in fish reared under conditions frequently encountered in the Tasmanian aquaculture industry (thermally challenged). Accordingly, the relationship between stock age, rearing temperature and egg quality will be established and correlated to specific endocrine blockages where applicable. With this information in hand, broodstock management strategies can then be formulated to target specific endocrine defects and therefore improve egg quality in Atlantic salmon that experience thermally induced endocrine suppression.

Reducing the reliance on repeat spawning fish for egg production has been identified as an industry priority. Therefore, in an effort to improve the reproductive performance of maiden fish, we aim to overcome endocrine suppression using hormonal and thermal manipulation techniques. As such, the methodology required to achieve desired fertility outcomes in maiden fish will be explored and passed on to the salmon industry. The research will therefore help to protect and enhance the egg producing capacity and sustainability of the Atlantic salmon industry by assisting in the development of maiden broodstock management protocols. This research is being completed in close consultation with our industry partner Saltas, and has the support of the Tasmanian Atlantic salmon industry as a whole.

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Chapter 3- Hepatic reference gene selection in adult and juvenile female Atlantic salmon at normal and elevated temperatures

3.1 Abstract

The use of quantitative real-time polymerase chain reaction (qPCR) has become widespread due to its specificity, sensitivity and apparent ease of use. However, experimental error can be introduced at many stages during sample processing and analysis, and for this reason qPCR data are often normalised to an internal reference gene. The present study used three freely available algorithms (GeNorm, NormFinder and BestKeeper) to assess the stability of hepatically expressed candidate reference genes (*hprt1*, *tbp*, *ef1α* and *β-tubulin*) in two experiments. In the first, female Atlantic salmon (*Salmo salar*) broodstock of different ages were reared at either 14 or 22 °C for an entire reproductive season. Therefore, a reference gene that does not respond to thermal challenge or reproductive condition was sought. In the second, estrogen treated juvenile salmon were maintained at the same temperatures for fourteen days and a reference gene that does not respond to temperature or estrogen was required. Additionally, we performed independent statistical analysis to validate the outputs obtained from the program based analysis. Based on the independent statistical analysis performed the stability of the genes tested was *tbp* > *ef1α* > *hprt1* > *β-tubulin* for the temperature/reproductive development experiment and *ef1α* > *hprt1* > *tbp* for the estrogen administration experiment (*β-tubulin* was not analysed). Results from the algorithms tested were quite ambiguous for both experiments; however all programs consistently identified the least stable candidate gene. BestKeeper provided rankings that were consistent with the independent analysis for both experiments. When an inappropriate candidate reference gene was used to normalise the expression of a hepatically expressed target gene, the ability to detect treatment-dependent changes in target gene expression was lost for multiple groups in both experiments. We have

highlighted the need to independently validate the results of reference gene selection programs. In addition, we have provided a reference point for those wishing to study the effects of thermal challenge and/or hormonal treatment on gene stability in Atlantic salmon and other teleost species.

3.2 Introduction

The use of quantitative real-time polymerase chain reaction (qPCR) has become widespread due to its specificity, sensitivity, broad dynamic range, cost effectiveness, high throughput capability and the need to measure exact levels of gene transcription (Huggett et al., 2005, Bustin et al., 2009). Despite its apparent ease of use, there are many stages in between initial sampling and performance of the qPCR that can introduce variability, and essentially affect the quality and reliability of the data produced.

There are a few common standardisation techniques that are used to compensate for introduced variability; for example, data can be normalised to sample size (tissue weight or number of cells), quantity of RNA extracted, or a stably expressed reference or 'housekeeping' gene (Huggett et al., 2005). However, there are potentially significant drawbacks associated with each of these methods. RNA extractions are routinely carried out on tissue samples that may contain various cell types. For this reason, ensuring that the cellular make-up of dissected tissue is consistent between animals of different disease or developmental state can be quite difficult (Huggett et al., 2005), and the specific contribution of each cell type to the total amount of RNA extracted could be disproportionate between samples. Subsequently, qPCR results acquired may reflect changes in cell composition and not a response to experimental conditions. While ensuring that the input amount of total RNA is the same between samples for cDNA synthesis is essential, this should not be used as a complete standardisation strategy. Standard RNA quantification methods mostly measure the ribosomal RNA (rRNA) fraction that can account for ~80 % of total RNA. Therefore, this

method relies on the assumption that the ratio of messenger RNA (mRNA) to rRNA does not change as a result of the experimental treatment or condition (Suzuki et al., 2000, Vandesompele et al., 2002). However, previous studies have demonstrated that stability of the rRNA fraction cannot be taken for granted (Hansen et al., 2001, Solanas et al., 2001). Additionally, this method of standardisation does not control for pipetting error during cDNA synthesis, inhibitory factors contained in the tissue, or error introduced during the reverse transcription and qPCR phases of sample processing and analysis respectively (Huggett et al., 2005). Normalisation to one or several internal control (reference) genes is by far the most common method used to manage technical or other variation when estimating gene expression levels. Its inclusion in qPCR studies is preferred over other methods of normalisation since the reference mRNA template is present at all stages of processing and analysis, and will therefore reflect the cumulative change in sample dynamics (Pfaffl et al., 2004). However, inappropriate use of a single or multiple reference genes for normalisation can limit one's ability to detect small changes in mRNA abundance, or alter the fundamental findings and conclusions of a study (Dheda et al., 2005).

In the past, many studies have assumed the stability of a small group of 'classic' reference genes without proper validation (Vandesompele et al., 2002). However, guidelines for reference gene selection are becoming more stringent, and there are now various prerequisites that a candidate reference gene must fulfill before it can be considered appropriate for normalising experimental error. For instance, the reference gene transcript level should not change as a result of experimental treatment (Vandesompele et al., 2002), it should be expressed at a level that is similar to that of the target gene (Bustin and Nolan, 2004, Huggett et al., 2005) and amplification should be RNA specific with the absence of pseudogenes and contaminating genomic DNA (gDNA) (Kreuzer et al., 1999). In fish species, it is now apparent that the expression of many commonly used reference genes may vary on the basis of gender (Filby and Tyler, 2007), tissue type (Olsvik et al., 2005), developmental stage (Tang et al., 2007) and experimental conditions such as type and length of exposure to exogenous chemicals

(Arukwe, 2006). Therefore, indiscriminate use of reference genes without stringent testing and validation could lead to incorrect expression profiling and interpretation of results (Dheda et al., 2005, Filby and Tyler, 2007).

For many studies, a question remains as to what is the best way to determine whether candidate reference genes are appropriate for normalising non-biological sample variation. At the present time, there are three popular algorithms which have been specifically designed to determine the most suitable reference gene or combination of genes from a panel of candidates, namely BestKeeper (Pfaffl et al., 2004), GeNorm (Vandesompele et al., 2002) and NormFinder (Andersen et al., 2004). Recently it has been demonstrated that discrepancy can sometimes occur between results obtained from these software packages, and it has been suggested that additional external evaluation is necessary to independently confirm the validity of genes selected by computer programs (Hibbeler et al., 2008, Mane et al., 2008, Setiawan and Lokman, 2010).

Salmonids are the most widely studied group of teleosts. However, studies investigating the usefulness of reference genes in qPCR have mainly focused on immature salmon (Arukwe, 2006, Ingerslev et al., 2006, Jorgensen et al., 2006), and no data are available for the expression of candidate reference genes for adult fish over an entire reproductive season. In addition to this, there are fundamental knowledge gaps concerning whether the age of an adult fish, or rearing temperatures outside of the optimum range modulate the expression of reference genes as demonstrated for other target genes during reproductive development (Ch 4: Pankhurst et al., 2011). In order to be able to assess the effects of temperature on reproduction, a reference gene is required whose expression is stable throughout reproductive development and across temperature ranges. In the present chapter, we have assessed the expression stability of *elongation factor 1 alpha* (*ef1α*), *hypoxanthine phosphoribosyltransferase 1* (*hprt1*), *tata binding protein* (*tbp*) and *β-tubulin*, all of which are routinely used reference genes in the field of teleost reproductive physiology. In Experiment 1, gene expression was measured in hepatic tissue from 2+ (maiden) and 3+ (repeat) year old female Atlantic

salmon reared at either 14 or 22 °C during a reproductive season (for a full experimental justification see Ch 4: Pankhurst et al., 2011 and Ch 5: Anderson et al., 2012). Reference gene stability was then determined using the freely available BestKeeper, GeNorm and NormFinder algorithms, and methods recommended elsewhere in the literature (Setiawan and Lokman, 2010). Then in a second experiment, we determined whether the same reference gene candidates (except β-tubulin) were suitable normalising genes in a study where juvenile Atlantic salmon were given either a blank or 17β-estradiol (E_2) implant, and maintained at either 14 or 22 °C for 14 days (for a full experimental justification see Ch 7). Studies such as these could become even more critical in years to come as wild and farmed fish species are affected by climate change, and we explore novel methods such as hormonal therapy to improve reproductive performance under thermal challenge.

3.3 Methods

3.3.1 Sampling

For Experiment 1, data from the maiden and repeat spawning fish used in Chapter 4 (Pankhurst et al., 2011) were used. Therefore, information regarding fish husbandry, experimental conditions and the sampling timeline and protocol and be found in Chapter 4 (Pankhurst et al., 2011). This research activity was undertaken with approval from the Animal Ethics Committees of the University of the Sunshine Coast and Griffith University (approval numbers AN/A/07/35 and EAS/02/07/AEC respectively).

For Experiment 2, data from the juvenile fish used in Chapter 7 were used in this chapter. Details for fish husbandry, experimental conditions, implant synthesis and sampling can be found in Chapter 7. This experiment was conducted under approval from the Animal Ethics Committee of the University of the Sunshine Coast (approval number AN/A/07/35).

3.3.2 RNA isolation, cDNA synthesis and qPCRs

For both experiments in this chapter, hepatic RNA was isolated, and cDNA synthesised according to the protocol outlined in Chapter 4 (Pankhurst et al., 2011).

Details for the gene specific primers (used to amplify *hprt1*, *ef1α*, *β-tubulin* and *tbp* transcripts), qPCR reaction components and cycling conditions used in this chapter are available in Chapter 4 (Pankhurst et al., 2011).

3.3.3 Candidate reference gene analysis

Raw C_q values were transformed to relative expression levels using the '$\triangle C_q$' method and the equation $Q = E^{(\text{minCq - sampleCq})}$ where Q = relative quantities, E = efficiency (+ 1) and minC_q refers to the C_q of the sample with the highest expression level (lowest C_q) in the data set for any given gene. Input for GeNorm and NormFinder were data transformed to relative expression levels, stability was assessed according to the program's instructions. Sub-group identifiers were included for NormFinder analysis. For BestKeeper, data were entered without modification as raw C_q according to the program's instructions.

Previous authors have recommended that statistical differences in qPCR be detected non-parametrically (Thellin et al., 1999, Setiawan and Lokman, 2010). Therefore, statistical differences between groups on a monthly (for Experiment 1) or daily basis (for Experiment 2) were analysed using the Kruskal-Wallis test coupled with Bonferroni's Correction to reduce the risk of type 1 error using SPSS (version 17.0). The P value for all analyses was initially set at 0.05 before adjustment by Bonferroni's correction. Kendall's tau non-parametric correlation analysis was performed to assess whether a significant relationship existed between raw C_q value and sampling point for each gene in both experiments. The R^2 method was used for interpretation of the correlation results where the correlation coefficient is squared, then multiplied by 100 to give the percent of variation in C_q accounted for by time. Target gene (*vitellogenin*)

expression was calculated and inter-assay variability was accounted for using Relative Expression Software Tool (REST©) (Pfaffl et al., 2002).

3.4 Results

3.4.1 Stability analysis of candidate reference genes

In Experiment 1, *ef1α* consistently had the highest transcript abundance, *hprt1* and *β-tubulin* had intermediate transcript abundance, and *tbp* had the lowest (Fig. 3.0). When considering the standard error of the average quantification cycle (C_q) for all groups of fish studied, *tbp* had the lowest transcript abundance variance followed by *ef1α*, *hprt1* and *β-tubulin* (Fig. 3.0). When Kruskal-Wallis analysis coupled with Bonferroni's correction was performed on a month-by-month basis, no significant differences in transcript abundance were found for *tbp* during the reproductive season (Fig. 3.0). For *ef1α*, significant differences in transcript abundance were found for two sets of statistical comparison, for *hprt1* four sets of comparison were statistically significant and for *β-tubulin* five sets of comparison were statistically significant (Fig. 3.0). When Kendall's tau correlation analysis was performed between C_q value and sample point, significant C_q-time relationships were found for all genes at $p \leq 0.01$ except *β-tubulin*. For *tbp*, sample point accounted for 7.2 % of C_q variation, for *ef1α* time of sampling accounted for 9.3 % of C_q and for *hprt1* time accounted for 29.37 % of C_q variation.

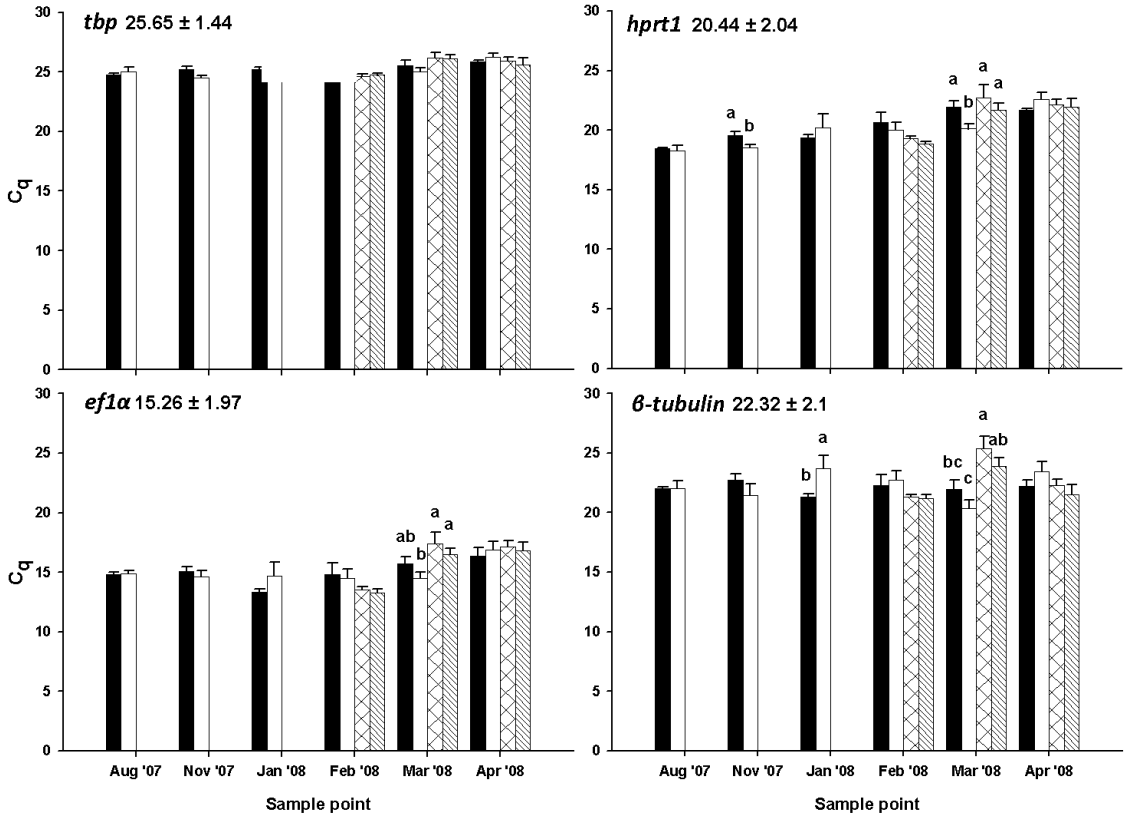

Figure 3.0 Abundance of hepatic mRNA transcripts for *tbp*, *hprt1*, *ef1α* and *β-tubulin* in female maiden spawning Atlantic salmon reared at either 14 (black bars) or 22 °C (cross-hatched bars) and repeat spawning salmon reared at 14 (open bars) or 22 °C (diagonal lines) during the reproductive season (Experiment 1). The average quantification cycle (C_q) of all groups analysed, and the standard error is shown at the top left of the graph. Bars show the average C_q (+SEM) for each group of fish (n = 7). Different superscripts denote statistical significance between groups at a given sample point at $p \leq 0.05$.

The BestKeeper algorithm ranked Tbp as the most appropriate reference gene while GeNorm ranked Tbp and Hprt1 as the most suitable pair (Table 3.0). This partly agreed with the results from NormFinder where Tbp was ranked third and Hprt1 was ranked first. Hprt1 was given a rank of three by BestKeeper which was not in agreement with any other algorithm. Ef1α was given a rank of two by BestKeeper and NormFinder and three by GeNorm. However, all three algorithms were in agreement when β-tubulin was assigned a rank of four, and was named as the least appropriate candidate gene for use in qPCR normalisation. When the hepatic expression of the target gene *vitellogenin* (egg yolk protein) was normalised using *β-tubulin*, then compared to the data

normalised by *tbp*, the ability to detect treatment-dependent changes in target gene expression was lost during January and March (Fig. 3.1).

Table 3.0 Ranking of candidate reference genes

	Experiment 1			Experiment 2		
Gene name	BK	NF	GN*	BK	NF	GN*
Tbp	1	3	1	3	3	3
Ef1α	2	2	3	1	2	1
Hprt1	3	1	1	2	1	1
β-tubulin	4	4	4	-	-	-

BestKeeper (BK), Norm Finder (NF) and GeNorm (GN)

- *β-tubulin* was not analysed in Experiment 2

* GeNorm selects the best pair of candidate genes, not a single gene

In Experiment 2 which utilised juvenile salmon, *ef1α* had the highest transcript abundance followed by *hprt1* then *tbp* (Fig. 3.2). This order is consistent with results from the Experiment 1 and in fact, the average C_q values for each gene between the different studies were very similar. *ef1α* had the lowest transcript abundance variance as demonstrated by the low standard error for this gene, *hprt1* had an intermediate level of variance and *tbp* had the highest (Fig. 3.2). The standard error for the three genes examined in this experiment ranged between 0.68 - 1.4 cycles which is lower than 1.44 - 2.04 cycles that was observed for the same genes (not including *β-tubulin*) in Experiment 1. Kruskal-Wallis analysis followed by Bonferroni's correction revealed that no significant differences in C_q existed between experimental groups within a given sample point for *ef1α* (Fig. 3.2). The same could not be said for *hprt1* where one significantly different comparison was found or for *tbp* where six sets of comparisons were statistically significant. Kendall's tau correlation analysis revealed a small but significant relationship between *ef1α* C_q and sample point, where sample point accounted for 3.61 % of C_q variation ($p \leq 0.05$). No significant relationship between time and expression of *tbp* or *hprt1* was found.

Unlike Experiment 1 where two out of three programs listed Tbp as an acceptable reference gene, all three algorithms ranked Tbp as the least stable reference gene candidate in Experiment 2 (Table 3.0). GeNorm selected Ef1α and Hprt1 as the most appropriate pair of candidates, BestKeeper selected Ef1α and NormFinder selected Hprt1 as the best candidate. It is interesting that NormFinder listed Hprt1 as the most stable single gene, though listed Ef1α and Tbp as the best combination of two genes to use for target gene normalisation. Similarly, addition of the least stably expressed gene (Tbp) into a panel of Hprt1 and Ef1α made a significant contribution (0.24) to the normalisation factor calculated by GeNorm despite its lower ranking.

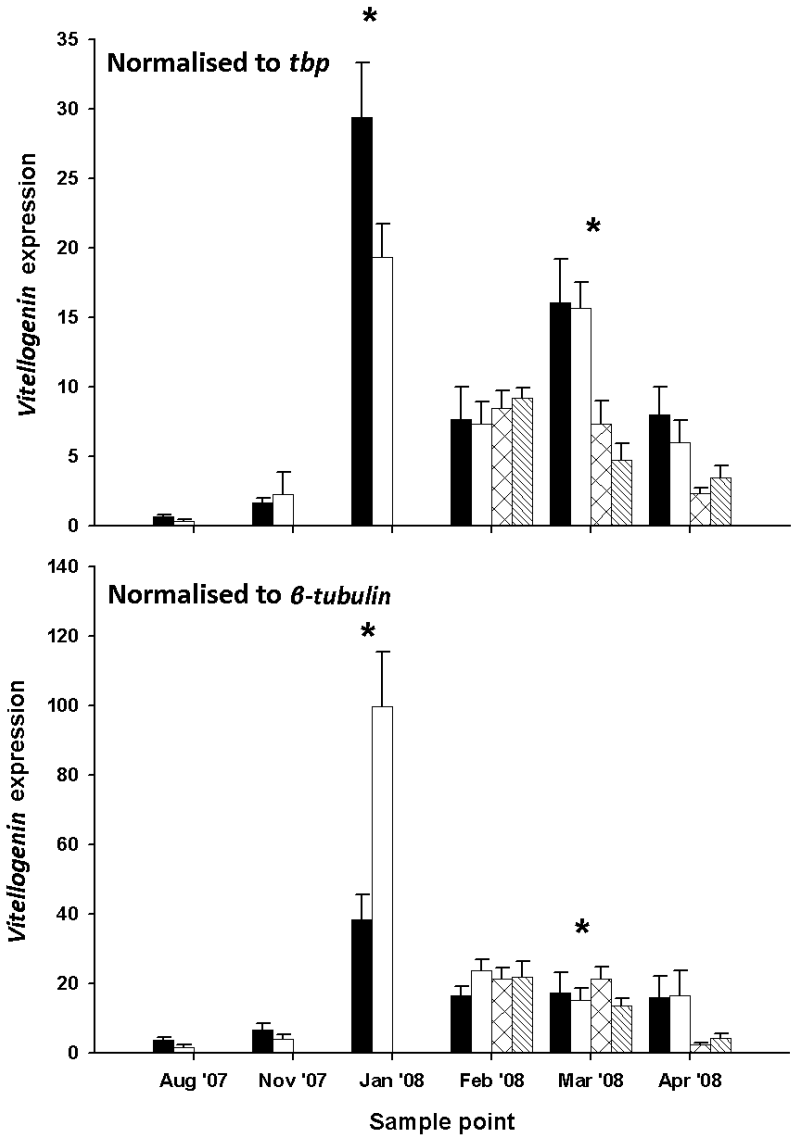

Figure 3.1 *Vitellogenin* gene expression normalised to either *tbp* or *β-tubulin* in female maiden spawning Atlantic salmon reared at either 14 (black bars) or 22 °C (cross-hatched bars) and repeat spawning salmon reared at 14 (open bars) or 22 °C (diagonal lines) during the reproductive season (Experiment 1). The mean (+ SEM) gene expression levels for each group of fish (n = 7) are displayed. The asterisk is placed above sample points where different target gene expression results were obtained after normalisation.

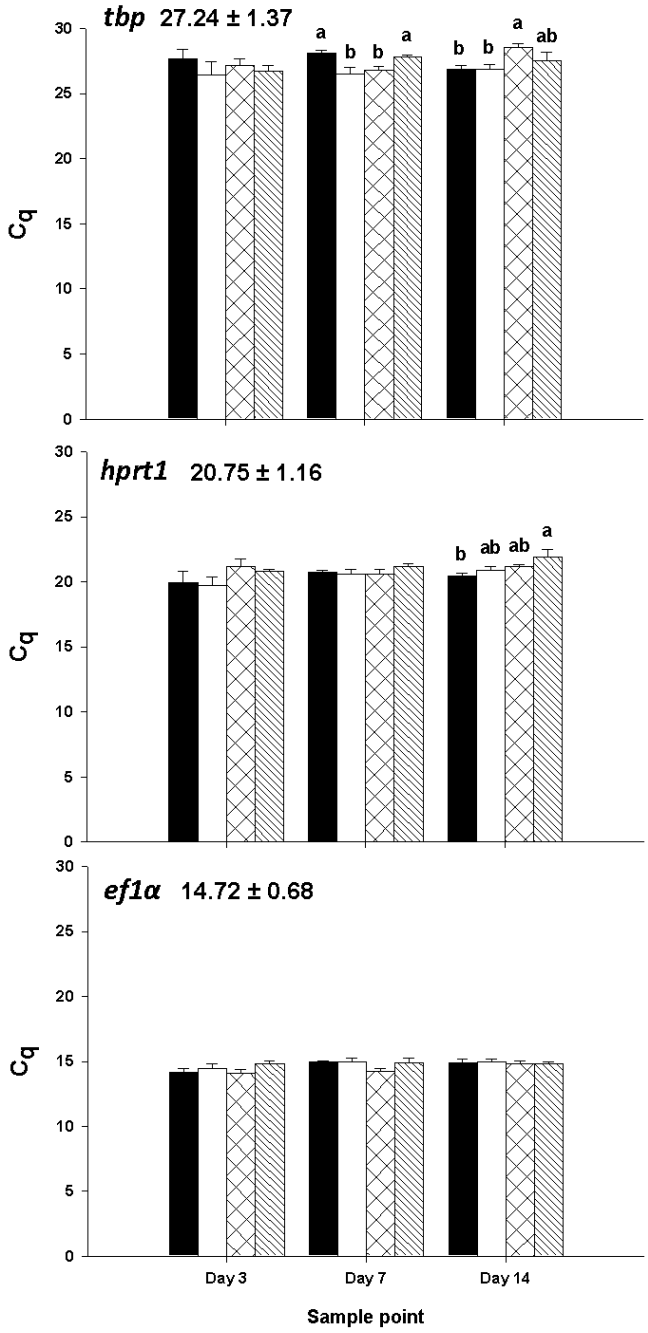

Figure 3.2 Abundance of hepatic mRNA transcripts for *tbp*, *hprt1* and *ef1α* in juvenile Atlantic salmon (Experiment 2) given a blank silastic implant at 14 (black bars) or 22 °C (cross-hatched bars), or an E_2 implant at 14 (open bars) or 22 °C (diagonal lines). The average quantification cycle (C_q) of all groups analysed, and the standard error is shown at the top left of the graph. Bars show the average C_q (+ SEM) for each group of fish (n = 7). Different superscripts denote statistical significance between groups at a given sample point at $p \leq 0.05$.

3.5 Discussion

We have reported on the stability of candidate reference genes for the entire reproductive season of maiden and repeat spawning female Atlantic salmon reared under cool (14 °C) and warm (22 °C) conditions in Tasmania (Experiment 1). Statistical analysis of *tbp* C_q value revealed that no significant differences were present between experimental groups of fish within a given sampling point for the entire reproductive season. *tbp* also had the lowest transcript abundance variance over the entire eight month period which suggests that it is the most stably expressed gene of the candidate panel tested. However, correlation analysis revealed that 7.2 % of the variation in transcript abundance could be accounted for by sampling point. This may indicate that *tbp* gene expression is down-regulated (has a higher C_q) to some extent over time because it is linked to reproductive status. For this reason, a degree of caution should be exercised when directly comparing the normalised expression levels of target genes from fish sampled in different months, particularly between the start and end of the reproductive season (i.e. August ’07 versus April ’08) as normalisation could introduce some error.

Two statistically significant comparisons were found for the C_q value of *ef1α* during March; in November and March a total of four significant comparisons were found for *hprt1* and five were found for *β-tubulin*. Therefore, it is not surprising that *ef1α* had the second lowest level of C_q variance followed by *hprt1* then *β-tubulin*. The presence of statistically significant differences in C_q value indicates that these genes may not be suitable candidates for target gene normalisation during certain months of the year. In fact, normalisation of a hepatically expressed target gene (*vitellogenin*) to *β-tubulin* resulted in a loss of the ability to detect treatment-dependent changes in target gene expression during January and March when compared to target gene expression data normalised by *tbp* (Fig. 3.1). These results confirm the work of previous authors where inappropriate use of a reference gene significantly altered the interpretation of qPCR results (Radoni et al., 2004, Dheda et al., 2005).

In a similar fashion to *tbp*, a significant correlation between sample point and C_q was found for *ef1α* that accounted for 9.3 % of C_q variation. Again, it may be at the discretion of the researcher to decide whether comparisons of qPCR data are of critical importance between months at the start and end of the reproductive season, or whether within month assessment of gene expression levels will suffice. However, the strongest relationship between sample point and transcript abundance was found for *hprt1* as ~30 % of C_q variance could be attributed to the month of sampling. The apparent connection between gene expression and developmental state, the number of statistically significant comparisons found and high standard error compared to other candidate genes made *hprt1* a poor choice for qPCR data normalisation in the present study. Even though no significant correlation was found between *β-tubulin* and sample point, the use of a *β-tubulin* to normalise target gene expression variance resulted in experimental bias due to a combination of its high standard error and high within-month C_q variation (Fig. 3.1). Based on the statistical analysis performed, the usefulness of candidate reference genes for normalisation is as follows: *tbp* > *ef1α* > *hprt1* > *β-tubulin*.

The BestKeeper algorithm provided rankings that were in agreement with the ranking obtained (above) via independent statistical analysis. GeNorm also selected Tbp as an ideal reference gene, however this gene was chosen in combination with Hprt1 which showed the largest relationship between C_q and sample point, and four statistically significant comparisons. This is possibly because the design of GeNorm does not take into account complex experimental design, so variation in gene expression within months and over time was not taken into account. However, GeNorm successfully identified β-tubulin as the least stably expressed candidate gene. This is most likely because GeNorm uses standard deviation of log transformed expression ratios to calculate stability (Vandesompele et al., 2002), and β-tubulin had the greatest overall C_q standard error and within month variance according to independent statistical analysis. NormFinder also ranked β-tubulin as the least suitable gene for target gene normalisation. However, unlike the other algorithms, NormFinder assigned a rank of

three to Tbp which is surprising given the lower standard error, within month variance and C_q-time correlation of *tbp* compared to the rest of the candidate panel. Based on our data, it appears that all three reference gene selection programs are useful for eliminating the least stably expressed gene from a panel of candidates. However, ranking of the remaining genes between algorithms was ambiguous which indicates that the output of such programs should not be used complacently. External validation is also warranted to account for conflict between algorithms, program assumptions and limitations when complex experimental designs are employed.

In addition to Experiment 1 where adult fish were used, we also determined the suitability of the same candidate reference genes (except β-tubulin) for use in a study where juvenile salmon were given a blank or E_2 pellet and reared at either 14 °C or 22 °C for fourteen days (Experiment 2). In Experiment 2, the gene expression of *ef1α* did not significantly change as a result of experimental treatment within each sampling point. However, a significant C_q- sample point correlation was found for this gene that accounted for 3.61 % of C_q variance. While this is a statistically significant relationship ($p \leq 0.05$), it is probably not of major concern due to its magnitude. One significant comparison was detected for *hprt1* gene expression at day fourteen, and a total of six sets of comparison were significant for *tbp*. *ef1α* also had the lowest transcript abundance variance, followed by *hprt1* then *tbp*. This strongly suggests that stability of candidate reference genes in this experiment is as follows: *ef1α* > *hprt1* > *tbp*.

All three reference gene programs were in agreement when assigning a rank of three to Tbp which was the same result achieved through external statistical analysis (above). Through this analysis it has become apparent that freely available algorithms are consistently able to identify the least stable gene from a panel of candidates. BestKeeper found Ef1α to be the most stable candidate followed by Hprt1 while the opposite was found using NormFinder. In a similar fashion to the Experiment 1, the BestKeeper algorithm gave the same rankings to the reference gene candidates as independent statistical analysis and therefore has been the most reliable algorithm in our studies. GeNorm selected Ef1α and Hprt1 as the most suitable pair of candidates;

although the addition of Tbp to the gene panel significantly improved overall stability value (by 0.24) despite its apparent lower stability. Therefore the results from GeNorm suggest that all three reference genes should be used for accurate normalisation of qPCR data. In addition, the expression of a hepatically expressed gene was normalised to *tbp* alone or in combination with *hprt1* and *ef1α*, significantly different results were achieved for two groups of fish (data not shown). Additionally, variance in the target gene expression data, and therefore noise, was reduced when all three genes were used for normalisation instead of *tbp* alone (data not shown).

In a broad sense, our data agree with previous studies that outline the need to validate reference genes on an experiment-to-experiment basis (Arukwe, 2006, Filby and Tyler, 2007). For example, in Experiment 1 there is strong evidence to suggest that Tbp was the best candidate for target gene normalisation, while in Experiment 2 Tbp was ranked third but may still prove useful as a reference gene in combination with other candidates. Based on our data, we recommend Tbp and Ef1α as a starting point when selecting candidate reference gene for research using female Atlantic salmon broodstock where hepatic gene expression will be measured during reproductive development. Furthermore, *ef1α* and *hprt1* appear to be quite stable in juvenile fish treated with E_2 under thermal challenge. As a final point, all three algorithms correctly identified the least stable candidates in both of our experiments and can therefore prove useful to initially screen data and eliminate the most undesirable genes.

In recent years it has become clear that no single gene is stably expressed under all experimental conditions for any given tissue or species. Our study further highlights the need to evaluate reference gene stability separately for every experiment as it is likely that no one gene will be consistently stable across experiments. For those who are interested in algorithm/program based assessment of candidate gene stability, we recommend that external statistical validation be carried out in light of the ambiguous results that the various programs can yield. Furthermore, full justification for the reference genes selected should be provided in any publications containing relative qPCR data.

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Chapter 4- Thermal impairment is differentially expressed in maiden and repeat spawning Atlantic salmon

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Thermal impairment of reproduction is differentially expressed in maiden and repeat spawning Atlantic salmon

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ABSTRACT

Groups of maiden or repeat spawning Atlantic salmon were maintained during vitellogenesis in austral autumn at either 14 °C or 22 °C through until April when all fish were transferred to a spawning temperature of 8 °C. There was no difference in body weight within groups for maidens and repeats, with repeats being consistently larger than maidens, no difference in condition factor amongst groups, but consistently higher gonad weight in repeats than maidens. Gonadosomatic index (GSI) and follicle diameter were suppressed in both maidens and repeats at 22 °C, with the effect being most marked in repeat spawners. Relative fecundity (egg kg^{-1}) determined from ovarian tissue samples also showed depression in repeats at 22 °C. Fish from both age classes held at 22 °C had a higher proportion of atretic follicles. Plasma levels of estradiol-17β (E_2) were strongly depressed in both maidens and repeats exposed to 22 °C throughout autumn but there was some evidence of recovery amongst maiden fish by late April. A similar effect was seen on plasma testosterone (T) levels. Plasma cortisol levels were generally low and typical of levels in unstressed fish indicating that stress did not account for the inhibitory effects observed. Hepatic *zona pellucida* protein gene expression was significantly inhibited in both maiden and repeat spawning fish reared at 22 °C, but with some evidence of recovery after temperature reduction to 8 °C. Hepatic vitellogenin (Vtg) gene expression was also lower in both maiden and repeat spawning fish exposed to 22 °C and this was accompanied by reduced plasma Vtg levels in maidens, but not repeats at 22 °C. Maidens at 14 °C began ovulating first followed by repeats at 14 °C, then repeats at 22 °C followed by maidens at 22 °C. There was reduced fertility in maidens at 22 °C relative to both maidens and repeats at 14 °C, whereas repeats at 22 °C showed intermediate fertility between 14 °C fish and 22 °C maidens. Survival to the eyed egg stage was highest in maidens at 14 °C, significantly suppressed at 22 °C in maidens, and at intermediate levels in repeats at both temperatures. This suggests that repeat spawning Atlantic salmon may be more robust in the face of thermal insult which combined with their larger size and egg production, could make their use desirable under production situations where there was any threat of exposure to higher than normal temperature.

1. Introduction

It is increasingly clear that climate change will strongly affect aquatic poikilotherms including fish, and this will be variously expressed through changes in community composition and structure, changes in species range, local and perhaps widespread extinctions of some species (reviewed in Graham and Harrod, 2009). A reasonable expectation is that salmonids will be particularly susceptible due to their distributional preferences for cooler water (e.g. Reddin et al., 2000); Welch et al., 1998a,b), and the fact that the complex anadromous life histories of many salmonid populations potentially expose them to thermal stress at a succession of critical life history stages (reviewed by Jonsson and Jonsson, 2009). Increases in temperature above optimal levels can have inhibitory effects on a range of biological processes including feeding and growth, behaviour, smoltification, disease resistance and reproduction (Battaglene et al., 2008; Graham and Harrod, 2009; Jonsson and Jonsson, 2009; Pankhurst and King, 2010; Steinum et al., 2008). A point of particular sensitivity appears to be reproductive development in females whereby inappropriately elevated temperatures impair or retard ovarian steroidogenesis – particularly 17β-estradiol (E_2) synthesis – and the subsequent hepatic synthesis of vitellogenin (Vtg), oocyte growth and development, oocyte maturation and ovulation, and egg fertility and survival (reviewed in Pankhurst and King, 2010).

Management strategies in culture situations are currently limited to thermal protection of broodstock during critical stages of vitellogenesis (Pankhurst and King, 2010); however, this requires significant infrastructure for thermal management and is not always

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possible in some aquaculture operations. A limited set of studies has examined the scope for endocrine therapy as a protectant during or after exposure to high temperature, mainly examining the value of treatment with synthetic analogues of gonadotropin releasing hormone (GnRHa). Treatment of an Australian cultured stock of Atlantic salmon (*Salmo salar*) with GnRHa was ineffective at maintaining fertility at high temperature but did restore fertility when applied in combination with a graded reduction in temperature (King and Pankhurst, 2004a). Similar experiments using longer release profile formulations of GnRHa in northern hemisphere stocks did result in maintenance of ovulation at elevated temperatures, but still with reduced egg survival at high temperatures (Vikingstad et al., 2008). The implication is that hormone therapy is only likely to be partially effective at offsetting the damaging effects of exposure to high temperature, although the more direct effects of gonadotropin or steroid therapy remain to be investigated (Pankhurst and King, 2010).

An unexplored area is the effect of thermal stress on different age classes of fish, with the majority of farmed salmon being from maiden or first-spawning broodstock. The first cycle of reproduction in maiden spawning Atlantic salmon covers the initial period of reproductive maturation or puberty. Studies on other species suggest that puberty is associated with an increase in hypothalamic GnRH mRNA and this is followed by FSH expression and secretion and increased levels of steroid hormones (Campbell et al., 2006; Okuzawa, 2002). In addition, the recently discovered G-coupled protein receptor GPR54 and its ligand the Kisspeptin have been shown to have an important role in initiating GnRH secretion (reviewed in Taranger et al., 2010). Blockades in the endocrine cascade prior to puberty have been identified at the levels of GnRH and FSH synthesis, and gonadotropin receptor binding at the gonadal level in a variety of species including salmonids (Gur et al., 2000; Nocillado et al., 2007; Okuzawa, 2002). The main effect of this appears to be the occurrence of a 'dummy run' with only partial endocrine activation and gonadal response, in the season before the first spawning period occurs, with the phenomenon being described in striped bass (*Morone saxatilis*) (Holland et al., 2000), masu salmon (*Oncorhynchus masou*) (Amano et al., 1992) and rainbow trout (*Oncorhynchus mykiss*) (Prat et al., 1996). In other species full 'endocrine maturity' may also not occur until the second spawning season. In both snapper (*Pagrus auratus*) (Cleary et al., 2000) and striped bass (Holland et al., 2000) relative ovarian size and plasma levels of gonadal steroids are lower in the first than the second spawning seasons. Cultured Atlantic salmon stocks in Tasmania are farmed towards the upper limit of their thermal tolerance range with the result that growth occurs at a faster rate, and developmental milestones are reached at a younger age than in their northern hemisphere counterparts, with most fish maturing as grilse after a single winter at sea (at 3 years of age) compared with 2 sea-winters in the northern hemisphere (King and Pankhurst, 2003). Rapidly maturing southern hemisphere Atlantic salmon does not appear to display the dummy run phenomenon. However, part of the inhibitory effect of elevated temperature could arise from its effect on grilse showing endocrine immaturity of the type described above for snapper and striped bass.

A key step in the maturation process is the production of E_2 by the developing ovarian follicle. E_2 is transported in the bloodstream to the liver where it binds to estrogen receptors (ER) in the hepatocyte cytoplasm. The E_2–ER complex in turn acts as a promoter for expression of the gene or genes coding for Vtg (reviewed in Watts et al., 2003), which is then sequestered into the developing oocyte through a process of receptor-mediated endocytosis (Tyler et al., 2000). The second important effect of E_2 is to stimulate hepatic synthesis of precursors of three structural proteins (collectively termed ZP) that will form the *zona pellucida* of the developing oocyte, and subsequently the chorion of the mature egg (Tyler et al., 2000). The genes coding for ZP are highly sensitive to stimulation by E_2 and ZP appear rapidly in the plasma soon after hepatic exposure to estrogens (Berg et al., 2004; Celius et al., 2000; Fujita et al., 2004). Disruption of E_2 synthesis and subsequent E_2–ER binding in the Tasmanian stock of Atlantic salmon is accompanied by chorionic abnormality, poor fertility and reduced embryonic survival (Pankhurst and King, 2010), suggesting that part of the effect may result from disruption of the expression of important E_2-inducible genes.

The present study examined whether the effect of thermal insult is differentially expressed in different age classes of Atlantic salmon. Maiden (3 year old) and repeat (4 year old) spawners were exposed to temperatures previously shown to inhibit reproductive development in this stock, and effects on ovarian growth and plasma levels of gonadal steroids and hepatic synthesis of Vtg were assessed. In addition, the E_2-dependent expression of genes coding for Vtg and *zona pellucida* (ZP) proteins was also measured.

2. Materials and methods

2.1. Fish husbandry and maintenance

Maiden (first spawning 2+year old fish) and repeat (second spawning 3+year old fish) cultured adult females were held at the SALTAS Wayatinah Hatchery (Tasmania, Australia) at ambient temperature and photoperiod in either 200 (maidens) or 50 (repeats) m^3 circular tanks at stocking densities of 12–18, and 24–36 kg m^{-3} for maidens and repeats, respectively until early January 2008. In January, fish were divided into treatment groups (n = 28 per group) and transferred to temperature-controlled 4 m^3 tanks (14 fish per tank) under simulated ambient photoperiod. Fish were not fed from the time of transfer to the temperature controlled systems in January consistent with hatchery practice for management of this experimental stock of fish.

Treatment groups:

1. Maidens held at 14 °C;
2. Repeats held at 14 °C;
3. Maidens held at 22 °C;
4. Repeats held at 22 °C.

All fish were maintained at the nominated temperature (14 or 22 °C) until early April when all fish were exposed to a temperature ramp down over 11 days to 8 °C to induce final oocyte maturation and ovulation (King and Pankhurst, 2000). Temperature profiles for the two temperature regimes are shown in Fig. 1.

2.2. Sampling protocol

Fish from both maiden and repeat groups were sampled on the 31st August and 2nd November 2007, and 7th January 2008 to cover the initiation of vitellogenesis for each age class (Samples 1 to 3), and after introduction to the controlled temperature regimes on the 14th February 2008 (Sample 4), 28th March (Sample 5) and 25th April (Sample 6). Seven fish were sampled from each group at each sample time, leaving 7 fish from each treatment to proceed through to ovulation and stripping, after the final destructive sample in April.

For sampling, fish were netted from the holding tanks, terminally anaesthetised in Aqi-S ™ (Crop & Food, New Zealand), weighed, measured and then blood sampled by caudal puncture using pre-heparinised syringes fitted with 22 G needles. Blood plasma was centrifuged at 12,000 *g* for 3 mins, and stored frozen at −20 °C for later measurement of plasma hormones. Ovaries were excised, weighed and portions allocated to 50 mL-pots containing teleost saline or 10% neutral buffered formalin for fecundity estimation and follicle measurement, and histology, respectively. Segments of liver were transferred to 1–2 mL of RNA Later ™ (Qiagen, Germany) to stabilise mRNA for later measurement of gene expression. Samples were held overnight at 4 °C, then stored at −20 °C.

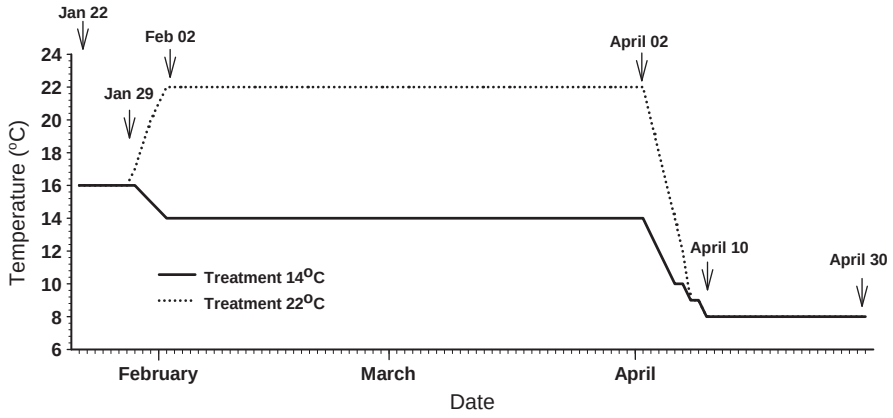

Fig. 1. Thermal treatment regimes for Atlantic salmon maiden and repeat spawners in 14 °C and 22 °C treatments.

Gonadosomatic indices (GSI) were calculated as (gonad weight/total body weight) × 100, and condition factor (CF) as (body weight/length^3) × 100. Fecundity was determined by dispersing all ovarian follicles from an ovarian segment of ~5 g in teleost saline using 22 G needles, and counting all vitellogenic (opaque) follicles present in the sample. Total fecundity was determined by correction for total ovarian weight and expressed as relative fecundity kg^{-1} body weight. Follicle size distributions were determined by measuring the horizontal diameter of 50 follicles from each fish, using an eye piece micrometre fitted to a dissecting microscope. Fixed tissue for histology was dehydrated in an ethanol series, embedded in paraffin wax, sectioned at 5 μm and stained with hematoxylin and eosin. At ovulation, ova were stripped and fertilised as described in King et al. (2003) for measurement of egg size, fertility and survival to the eyed stage at 250 degree-days of incubation. All animal experiments were conducted in accordance with Australian law under ethical approval EAS/02/07/AEC issued by the Griffith University Animal Ethics Committee.

2.3. Plasma steroid and vitellogenin measurement

Plasma levels of E_2, testosterone (T) and cortisol were measured by radioimmunoassay in 100 μL plasma extracted with 1 mL ethyl acetate using the protocol and reagents for E_2 and T as described in Pankhurst and Carragher (1992), and for cortisol as in Pankhurst et al. (2008). Extraction efficiency was determined by recovery of ^{3}H-labelled steroid from replicates of a plasma pool and was 78, 79 and 74% for E_2, T, and cortisol, respectively. Assay values were corrected accordingly for extraction losses. Interassay variability was determined by repeat measurement of a pooled internal standard and was (% CV) 7.4, 13.9 and 12.3 (n = 2), for E_2, T and cortisol, respectively.

Plasma Vtg levels were measured by enzyme linked immunosorbent assay using the reagents and protocol described in Watts et al. (2003). Plasma samples were diluted at 1:1000 in assay buffer for measurement. Interassay was assessed by repeat measurement of a Vtg standard from the central part of the assay curve and was (% CV) 13.1 (n = 6). Pooled internal standards were not used here due to the tendency of Vtg to denature following repeated freeze-thaw cycles.

2.4. Measurement of hepatic gene expression

2.4.1. RNA isolation and cDNA synthesis

Total RNA was isolated from hepatic tissue using the Illustra RNAspin Mini kit (GE Healthcare, United Kingdom) according to the manufacturer's protocol. Four hundred nanograms of liver-derived RNA was used to synthesise cDNA for use in polymerase chain reactions (PCRs), and real-time/quantitative PCR (qPCR) using the QuantiTect® reverse transcription kit (Qiagen, Germany). This kit includes a DNA elimination step to remove potential contamination of PCRs by genomic DNA. Following synthesis, cDNAs were diluted 5-fold with diethylprocarbonate (DEPC)-treated water and stored at −20 °C until use.

2.4.2. Partial isolation of ZPC gene

To amplify a fragment of the *zona pellucida* C (ZPC, equivalent to ZP3, ZPγ and choriogenin L) gene from *S. salar*, the ZPC mRNA nucleotide sequences from rainbow trout (GenBank accession number AF231708), masou salmon (*O. masou*) (EU042126) and Java medaka (*Oryzias javanicus*) (AY913760) were aligned using the ClustalW2 sequence alignment tool (http://www.ebi.ac.uk/) and degenerate primers were designed from conserved regions (ZPC F1 and ZPCR1, Table 1). The 25 μL PCR reaction contained 17.9 μL PCR grade water, 2.5 μL 10× PCR buffer, 0.5 μL 10 mM dNTPs, 2 μL 25 mM $MgCl_2$, 500 nM forward primer, 500 nM reverse primer, 0.1 μL TAQ (Fisher Biotec, Australia) and 1 μL liver derived cDNA template. Thermal cycling consisted of an initial denaturation step at 94 °C for 1 min; followed by 34 cycles of 94 °C for 30 s, 49 °C for 30 s and 72 °C for 4 min; with a final 10 min incubation at 72 °C. The PCR product was purified using the QIAquick® PCR purification kit (Qiagen) and cloned using the pGEM®-T easy vector system (Promega, Madison, WI) according to the manufacturer's instructions. Clones were selected via blue/white screening and successful transformation was confirmed via colony PCRs using M13 primers designed by Promega. PCRs which yielded a product of the expected size were purified (as above) then sequenced by the Australian Genome Research Facility before being submitted to GenBank (accession number GU075906).

2.4.3. qPCR primer design

Gene specific primers (GSPs) for vitellogenin A (Vtg), *zona pellucida* B (ZPB, designed to detect all known ZPB genes), ZPC, and the potential qPCR reference genes: hypoxanthine phosphoribosyltransferase 1 (HPRT1), TATA box binding protein (TBP), elongation factor 1 alpha (EF1α) and beta tubulin (β-tubulin) were designed from species-specific mRNA sequences using Primer3 software (http://frodo.wi.mit.edu/primer3/, Table 2). All primers were designed to have an optimum

Table 1
Degenerate PCR primers used for ZPC isolation.

Gene name	Primer name	Sequence (5′→3′)	Product size
ZPC	ZPCF1	ACT CCC TSR TCT ACA YCT TCA C	491 bp
	ZPCR1	CTG GAA CCT GAA RGC YTC CA	

bp = base pairs, Degenerate base codes: S = GC, R = AG, Y = CT and K = GT.

Table 2
qPCR primers.

Gene name	Primer name	Sequence (5′→3′)	Prod. size	E*	Source sequence
Vtg	VtgF4	AAC TTT GCC CCT GAA TTT GC	95 bp	0.984	DQ834857
	VtgR4	GCT CTA GCC AGA CCC TCC GC			
ZPB	ZPBF1	GTTT CCA GGG ATG CCA CTC T	113 bp	0.937	AJ000664, AJ000665
	ZPBR1	TGG TAG ATG GCA AAG GCA GA			
ZPC	ZPCF5	GTC CCC CTG CGT ATC TTT GT	121 bp	0.969	GU075906
	ZPCR4	AAC CTG TCA CTT TGG CAT CG			
HPRT1	HPRT1F1	GAT GAT GAG CAG GGA TATGAC	165 bp	0.963	BT043501
	HPRT1R1	GCA GAG AGC CAC GAT ATG G			
TBP	TBPF1	TCC CCA ACC TGT GAC GAA CA	117 bp	0.981	BT059217
	TBPR1	GTC TGT CCT GAG CCC CCT GA			
EF1α	EF1αF2	GCA CCA CGA GAC CCT GGA AT	94 bp	0.969	AF321836
	EF1αR2	CAC GTT GCC ACG ACG GAT AT			
β-tubulin	βTubF1	CCG TGC TTG TCG ACT TGG AG	144 bp	0.975	DQ367888
	βTubR2	CAG CGC CCT CTG TGT AGT GG			

E = efficiency, bp = base pairs, and TBC = to be confirmed.

annealing temperature of 60 °C and were supplied by GeneWorks (Australia).

2.4.4. qPCR validations

qPCRs were conducted on a Rotor-gene 6000 series thermal cycler (Qiagen) using Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen) as the fluorescent label according to the manufacturer's instructions. Validation curves were carried out in triplicate using serially diluted cDNA as the template and the following cycling conditions: 50 °C for 2 min; 95 °C for 2 min; 40 cycles of 95 °C for 15 s; 60 °C for 15 s, and 72 °C for 20 s (acquiring). At the end of cycle 40, all primers were tested for specificity via melt curve analysis which consisted of a 90 s preconditioning step at 72 °C, followed by a temperature gradient up to 95 °C at 1 °C per 5 s. Following amplification, the size of all qPCR products was determined by running 4 μL of the product on a 2% agarose gel; gene identity was then confirmed through sequencing. In each qPCR run, negative reverse transcription and no-template controls were analysed to ensure the absence of genomic DNA and other contamination. Reaction efficiencies (Table 2) were automatically calculated by Rotor-gene software version 1.7.87 using the equation: $E = [10^{(-1/M)}] - 1$, where E is equal to efficiency and M is equal to slope.

2.4.5. qPCR protocol

To determine the relative expression of target genes, the 10 μL qPCR reaction contained 5 μL SYBR, 200 nM forward primer, 200 nM reverse primer, 1.6 μL PCR grade water and 3 μL of diluted cDNA template. Cycling parameters and melt curve analysis were as described above. In each qPCR run and for every gene analysed, negative no-template controls and a calibrator sample were included to detect possible contamination, and control for in-between run variability, respectively. The reference gene selected for expression normalisation was TBP as it showed consistently high stability under the experimental conditions, and did not change across time or developmental stage. The software REST© 2008, V2.0.7 (Pfaffl et al., 2002) was used to calculate expression of key genes relative to TBP expression for each sample.

2.5. Statistical analysis

Pairwise comparison of means of morphometric and plasma hormone data (Samples 1 to 3) was made using an independent samples *t*-test, and multiple comparisons (subsequent sample times) using one-way ANOVA with comparison of means by Tukeys-b using the SPSS (version 17.0) statistical package. Differences in relative gene expression levels were detected non-parametrically using the Kruskal–Wallis test coupled with Bonferroni's Correction to reduce the risk of type 1 error. The P value for significance was set at 0.05 for all analyses.

3. Results

There was no difference in body weight within groups for maidens and repeats, with repeats being consistently larger than maidens (maidens 2.2–4 kg, repeats 4–9 kg over the sampling period; data not shown). Condition factor was lower in the repeats than the maidens at Samples 1 and 2 but these fish had recovered by Sample 3 and there was no difference in CF thereafter (data not shown). Gonad size as measured by GSI was low in both age classes at Samples 1 and 2 (and higher in repeats than maidens at Sample 1 only), then increased rapidly from Sample 3 onwards (Fig. 2). GSI was depressed in maidens

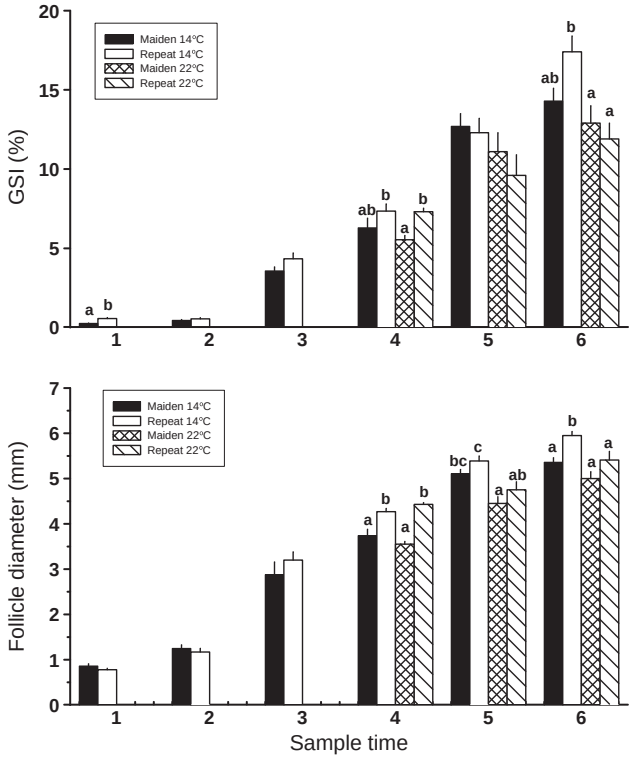

Fig. 2. Gonadosomatic index and follicle diameters (mean + SE [n = 7]) of; maiden spawners held at 14 °C (filled bars); repeat spawners held at 14 °C (open bars) maidens at 22 °C (cross-hatched bars), and repeats at 22 °C (hatched bars) during autumn (sample times are given in Materials and methods). Different superscripts within sample times denote significant differences (P<0.05).

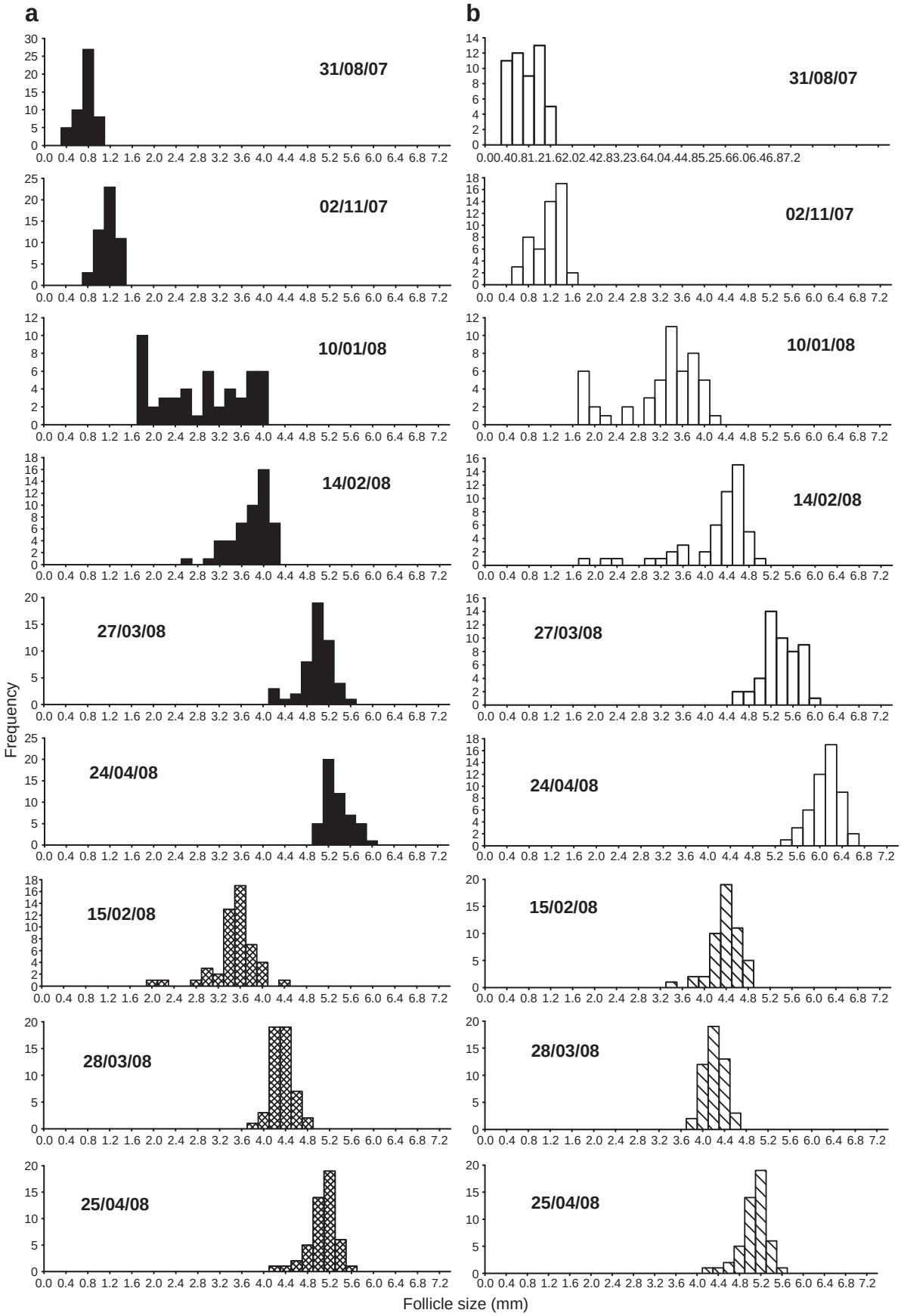

Fig. 3. Follicle size distribution for individual maiden and repeat spawners exposed to a) 14 °C or b) 22 °C. Fish shown have mean follicle diameters that approximate the group mean for that sample time. Other details as for Fig. 2.

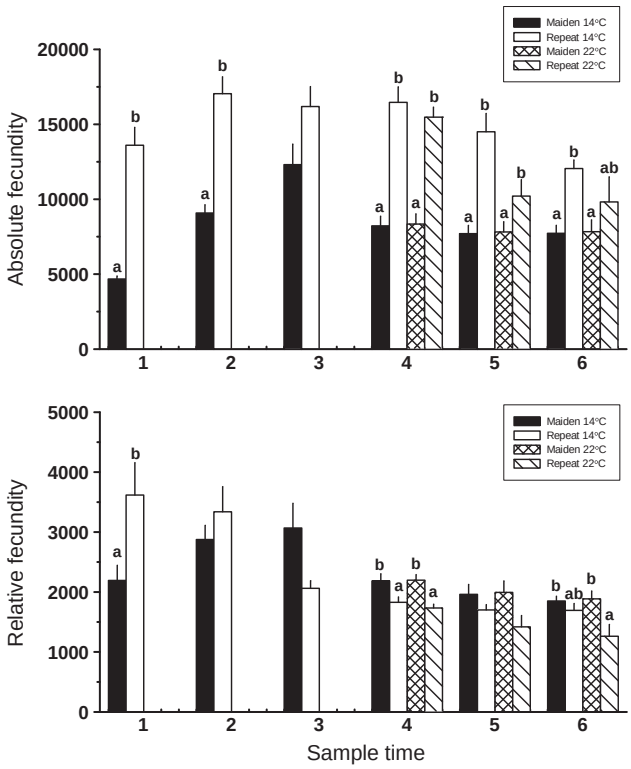

Fig. 4. Absolute and relative fecundity of maiden and repeat spawners exposed to 14 °C or 22 °C during autumn. Other details as for Fig. 2.

at 22 °C with respect to repeats at both temperatures at Sample 4, not different amongst groups at Sample 5 and depressed in both age classes at 22 °C relative to repeats at 14 °C at Sample 6 (Fig. 2). The mean diameter of vitellogenic follicles was around 1 mm in both age classes at Samples 1 and 2 but increased rapidly in concert with gonad size, from Sample 3 onwards. Follicle diameters were not different between age classes for the first three samples, larger in repeats than maidens at both temperatures at Sample 4, smaller in both age classes at 22 °C than their respective 14 °C counterparts at Sample 5, and lower in all groups relative to 14 °C repeats at Sample 6 (Fig. 2). Representative follicle size distributions (chosen from fish where the modal follicle size class was close to the group mean for that sample time) showed a progression of modal size with time that mirrored increases in mean follicle diameter (Fig. 3). There was a high level of size synchrony in both maidens and repeats at Samples 1 and 2, but this disappeared in January when there was a wide spread of size classes present in association with recruitment of follicles into the major growth phase of vitellogenesis. This trend was maintained in repeats at Sample 4 in February. Thereafter, both groups showed increasing size synchrony as follicle size increased. There are no obvious effects of exposure to 22 °C on follicle size distribution, with the pattern of development being very similar in all groups (Fig. 3). Absolute fecundity was higher in repeats at all times except in fish

Table 3
Proportion of fish with atretic follicles present in histological sections of ovary.

	Sample					
Group[1]	1	2	3	4	5	6
1	0	0	0	0	2/7	2/7
2	6/7[2]	6/7[2]	4/7[2]	5/7[2]	2/7	2/7
3	–	–	–	2/7	3/7	5/7
4	–	–	–	2/7	5/7	5/7

[1] Groups: 1 = maidens at 14 °C, 2 = repeats at 14 °C, 3 = maidens at 22 °C, 4 = repeats at 22 °C.
[2] Fish with atretic remnants from the previous cycle of vitellogenesis.

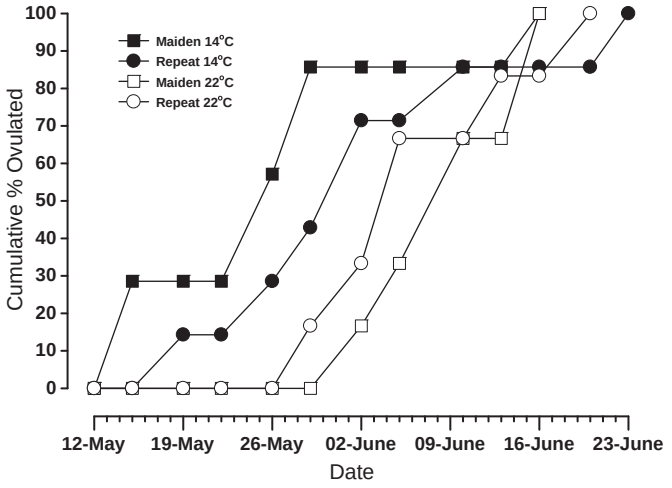

Fig. 5. Cumulative ovulation in maidens at 14 °C (filled squares); repeats at 14 °C (filled circles); maidens at 22 °C (open squares), and repeats at 22 °C (open circles).

held at 22 °C at Sample 6 (Fig. 4). Relative fecundity (eggs kg^{-1}) was variable but again showed depression in repeats at 22 °C by Sample 6 (Fig. 4).

Histological assessment of ovarian condition showed a gradual transition in gonadal stage in both maiden and repeat spawners from gonads which had a mixture of small pre-vitellogenic and early stage vitellogenic follicles at Sample 1, to ovaries in which larger vitellogenic follicles predominated by Sample 3. Ovaries of maidens

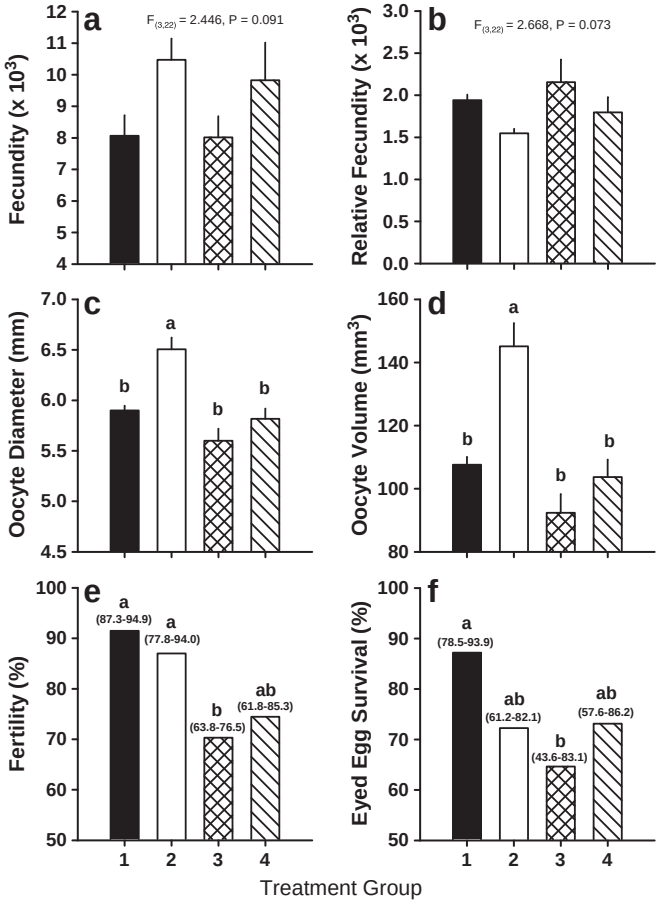

Fig. 6. Post-ovulatory fecundity, fertility and survival to eyed stage from maidens and repeat spawners exposed to 14 °C or 22 °C during autumn. Other details as for Fig. 2.

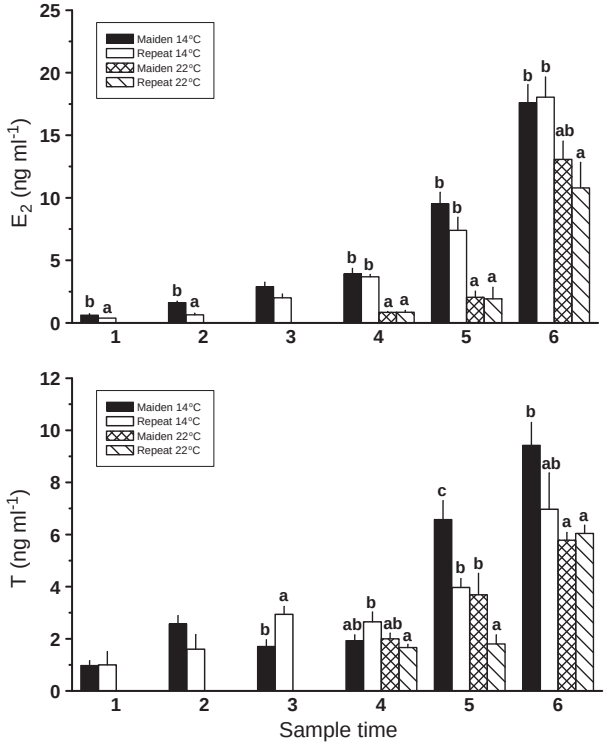

Fig. 7. Plasma levels of testosterone (T) and estradiol (E_2) amongst maiden and repeat spawners exposed to 14 °C or 22 °C during autumn. Other details as for Fig. 2.

and repeats were structurally similar but with the exception that the gonads of repeats retained remnants of atretic vitellogenic mature follicles that had not ovulated in the previous spawning season (Table 3). Generally, only the thick, highly refractile egg membrane (*zona pellucida*) of these atretic follicles remained. No atretic follicles were observed in the ovaries of maiden fish. By Sample 4, ovaries of repeat and maiden spawning fish from both temperature treatments were characterised by the predominance of large vitellogenic follicles, with atretic previous-season follicles (based on *zona pellucida* thickness) still present in the ovaries of some repeat spawners. At Sample 5, irrespective of thermal regime, gonadal tissue was characterised by enlargement of vitellogenic follicles, consistent with the changes in follicle size-class distribution reported above. The ovaries of fish from both 14 °C and 22 °C treatments showed atresia within the current season's follicles. At 14 °C, 2 out of 7 fish in both maiden and repeat groups had atretic follicles, but the prevalence of these within the sample of tissue sectioned was low (usually 1–2 follicles). In contrast, 3 out of 7 of the maidens, and 5 out of 7 of the repeats held at 22 °C showed follicular atresia, and the prevalence of atresia was high, with multiple follicles within the sample tissue affected (Table 3). The pattern was maintained at Sample 6.

Ovulations were complete in all groups at approximately the same time; however, maidens at 14 °C began ovulating first followed by repeats at 14 °C, then repeats at 22 °C followed by maidens at 22 °C (Fig. 5). There were no statistical differences in post-ovulatory fecundity amongst groups, although repeats tended to have higher egg production than maidens (Fig. 6). Relative fecundity was also not different between groups. Egg diameter and volume were both larger in repeats than maidens at 14 °C, however, this difference disappeared in repeats exposed to 22 °C. There was markedly reduced fertility in maidens at 22 °C relative to both maidens and repeats at 14 °C (Fig. 7). Repeats at 22 °C showed intermediate fertility between 14 °C repeats and 22 °C maidens. Survival to the eyed egg stage was highest in maidens at 14 °C, significantly suppressed at 22 °C in maidens, and at intermediate levels in repeats at both temperatures (Fig. 6).

Plasma levels of E_2 were higher in maidens than repeats at Samples 1 and 2, but not different at sample 3 (Fig. 7). At Samples 4 and 5, E_2 levels in both maidens and repeats were suppressed at 22 °C relative to 14 °C, but by Sample 6, only repeats showed lower E_2 levels at 22 °C than 14 °C. Plasma T levels were not different at Samples 1 and 2 but higher in repeats at Sample 3. At Sample 4, plasma T levels in repeats were lower at 22 than at 14 °C, and at Sample 5 plasma T levels were lower in both maidens and repeats at 22 than at 14 °C (Fig. 7). By Sample 6, T levels were lower in maidens at 22 °C than at 14 °C but there was no difference between temperatures in repeats. Plasma cortisol levels were elevated in maiden fish relative to repeats at Samples 1–3 (means of up to 20 ng mL^{-1} compared with <5 ng mL^{-1} in repeats; data not shown). This was thought to relate to the crowding process required to catch maidens from the larger volume 200 m^3 tanks. At Samples 4–6, plasma cortisol levels were below 10 ng mL^{-1} in all groups and there was no effect of holding temperature (data not shown).

Plasma Vtg levels were elevated in maidens compared with repeats at the first 3 sampling times (Fig. 8). This pattern was still present at Sample 4, and maidens but not repeats at 22 °C showed suppression relative to the 14 °C treatment. There were no differences amongst treatments at Sample 5, but plasma Vtg levels of repeats at 22 °C were elevated over those of repeats at 14 °C at Sample 6 (Fig. 8). Relative levels of hepatic Vtg gene expression were low in both maidens and repeats at Samples 1 and 2, but increased markedly at Sample 3, with levels being higher in maidens than in repeats (Fig. 8). There was no difference between groups at Sample 4, but expression was suppressed at 22 °C in both maidens and repeats at Sample 5. At Sample 6, expression was suppressed in maidens, but not repeats at 22 °C, relative to fish at 14 °C (Fig. 8).

ZPB gene expression was low in both age classes at Samples 1 and 2, but higher in maidens than repeats at sample 1 (Fig. 9). As with Vtg expression, there was a large increase at Sample 3, with maidens again showing higher expression than repeats. At Samples 4–6, ZPB gene expression was suppressed in both maidens and repeats at 22, relative

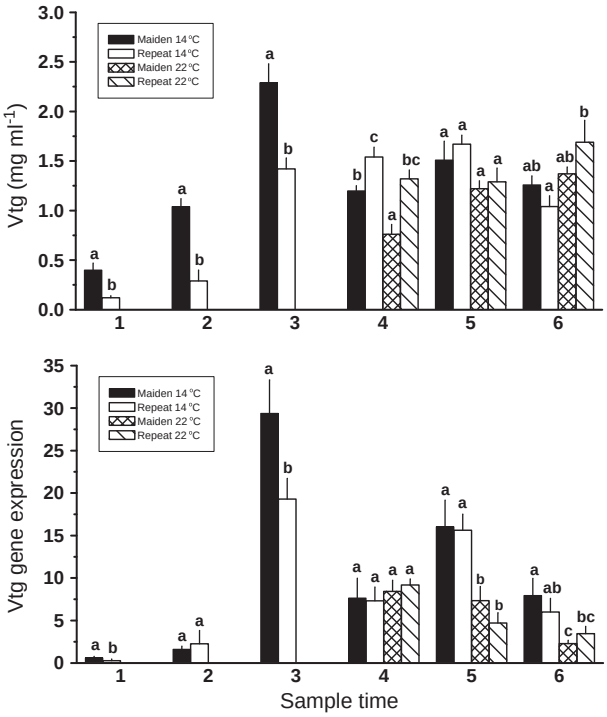

Fig. 8. Plasma levels of Vitellogenin (Vtg) and relative Vtg gene expression amongst maiden and repeat spawners exposed to 14 °C or 22 °C during autumn. Other details as for Fig. 2.

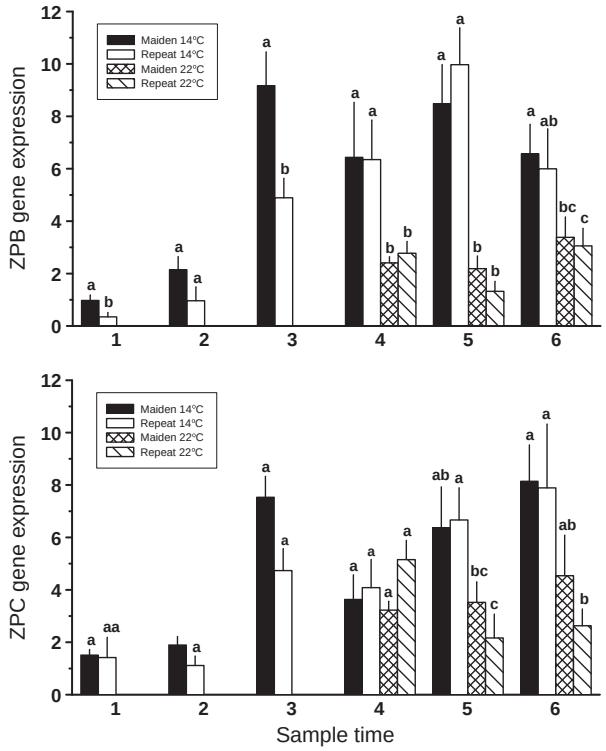

Fig. 9. Relative *zona pellucida* B (ZPB) and C (ZPC) gene expression amongst maiden and repeat spawners exposed to 14 °C or 22 °C during autumn. Other details as for Fig. 2.

to 14 °C. ZPC gene expression followed a similar pattern with low levels of expression at Samples 1 and 2 and an increase at Sample 3, but here with no differences between maidens and repeats. At Sample 4, expression was not different amongst groups, whereas at Samples 5 and 6, there was suppression of expression at 22 relative to 14 °C in repeats but not maidens (Fig. 9).

4. Discussion

Developmental trajectories of maiden Atlantic salmon held at 14 °C in the present study were similar to those previously described for this population (King and Pankhurst, 2003), with vitellogenesis occurring in late austral spring and accelerating through summer and autumn, prior to the peak of ovulation in late May and early June. The temporal pattern of repeat spawners held at 14 °C was very similar to that of maidens, with the minor difference that repeats initially had lower somatic condition factors than maidens, reflecting recovery from the energetic demands of the previous spawning season. This difference had disappeared by January. Both age classes showed similar patterns of oocyte size distribution, with a period of relatively unsynchronised growth of the oocyte clutch in January and February. At later sample times this resolved into much tighter size distribution of the maturing oocyte clutch. A similar pattern of oocyte growth has been previously described for this population (King and Pankhurst, 2003), and also for rainbow trout (Tyler et al., 1990).

Despite very similar developmental trajectories, repeat spawners in the present study were slightly slower to ovulate, with equivalent levels of cumulative ovulation being reached about a week later in repeats than in maidens. Plasma profiles of T and E_2 were generally similar in maidens and repeats at 14 °C, and consistent with previously described steroid profiles for this stock (King and Pankhurst, 2003; King et al., 2003). However, plasma E_2 levels were lower in repeats than maidens during the initial stages of ovarian development (August and November), and this was accompanied by lower plasma Vtg levels and at some sample times Vtg and ZPB gene expression during the spring and early summer. This suggests that despite the similar follicle growth trajectories, increases in E_2 synthesis and the E_2-dependent hepatic expression of Vtg and ZP genes are delayed in repeats relative to maidens, and that the slightly slower progression to ovulation is a reflection of this. The pattern appears not be universal amongst salmonids with maiden and repeat spawning rainbow trout showing very similar temporal patterns of increase in plasma levels of T and E_2. Maiden spawners did; however, show a biphasic spring increase in plasma Vtg that was absent in repeats (Scott and Sumpter, 1983; Sumpter et al., 1984).

Exposure of Atlantic salmon broodstock in the present study to 22 °C had no detectable impact on fish size or condition, but did result in suppression of relative gonad size and follicle diameter in repeats but not maidens. Exposure to high temperature did not noticeably affect follicle size distribution or fecundity, suggesting that the dynamics of oocyte recruitment were unaffected at higher temperature. Oogenesis and recruitment of follicles into vitellogenesis in salmonids and other species are closely associated with increases with expression of follicle stimulating hormone (FSH), its ovarian receptor (FSH-R), and plasma E_2 levels (Campbell et al., 2006; Lubzens et al., 2010). Both E_2 synthesis and FSH and FSH-R expression have been demonstrated to be suppressed by exposure to elevated temperatures (Pankhurst and King, 2010; Soria et al., 2008); however, the critical period for this process appears to be early in development, and in the present study this would have occurred during austral spring before the imposition of thermal challenge, suggesting that oocyte recruitment is relatively robust once it is established.

Maintenance at 22 °C in the present study did generate a higher incidence of ovarian atresia in both age classes, and progress to ovulation was also retarded with the effects being most marked in maidens. This was accompanied by significant reductions in egg fertility and survival in maiden fish but not repeats. The inhibitory effects of exposure to elevated temperature on follicle size, ovulation and egg fertility and survival are consistent with earlier studies on this stock (King and Pankhurst, 2000; 2004a,b; King et al., 2003; 2007; Watts et al., 2004;), and also northern hemisphere populations of Atlantic salmon (Taranger and Hansen, 1993; Taranger et al., 2003; Vikingstad et al., 2008), Arctic charr (*Salvelinus alpinus*) (Gillet, 1991), brook trout (*S. fontinalis*) (Hokanson et al., 1973), and southern hemisphere stocks of rainbow trout (Pankhurst and Thomas, 1998; Pankhurst et al., 1996). The present study is the first to present evidence that the age class of the reproductive stock can influence the response to thermal challenge, and in particular, that repeat spawners may be more robust in terms of their reproductive response to exposure to high temperature, despite the fact that inhibition of follicle growth and related endocrine processes (see following discussion) still occurs.

Consistent with earlier studies (reviewed in Pankhurst and King, 2010), plasma levels of E_2 were depressed in the present study in both age classes of fish held at 22 °C, in February and March, and also amongst repeats in April. This supports the view that a key effect of exposure to higher than normal temperature is inhibition of P_{450} aromatase (arom) activity, with subsequent reductions in E_2 synthesis and release to the plasma (Watts et al., 2004). There is increasing evidence that thermal inhibition of arom activity occurs across a taxonomically diverse range of teleosts (Guiguen et al., 2010). However, plasma T levels were also suppressed in fish of both age classes held at 22 °C in the present study, and this effect has also been observed previously in this stock of Atlantic salmon (King et al., 2003; 2007). This indicates that the processes higher in the endocrine pathway were also affected by exposure to elevated temperature. *In vitro* studies have shown that ovarian steroidogenesis upstream of E_2 production is strongly maintained in the face of thermal challenge (Watts et al., 2004), suggesting that effects on plasma T levels were generated higher in the endocrine cascade than at the level of gonadal

steroidogenesis. Candidates include the transcription and synthesis of gonadotropin releasing hormone and its pituitary receptor, and luteinising β-subunit (LH-β) (Okuzawa et al., 2003; Soria et al., 2008).

Plasma cortisol measurements in the present study suggested that suppression of plasma T and E_2 levels were not the result of stress suppression of gonadal steroidogenesis of the type described for a wide range of teleosts (reviewed by Schreck, 2010). Plasma cortisol was only elevated above levels typical of unstressed adult fish from this stock (Thomas et al., 1999), in maiden fish sampled with crowding from a large stock tank, early in the experiment. At all other times, cortisol levels were low, and not different between treatments or age classes, indicating that the experimental conditions themselves were not perceived as stressful.

Hepatic Vtg gene expression and subsequent synthesis is strongly E_2-dependent (reviewed by Babin et al., 2007), and this is reflected in the present study by the increase in Vtg expression through Samples 1–3 concomitant with increasing plasma E_2 concentrations. However, there was not a consistent subsequent relationship between E_2 and Vtg expression, with suppression in plasma E_2 at 22 °C in February and March, but reduced Vtg gene expression in March only. This suggests that either there is a time lag between reductions of plasma E_2 and subsequent effects on Vtg expression, or that beyond a certain level of stimulation, plasma E_2 levels and E_2-dependent gene activation become to an extent, uncoupled. The relatively short latency between increases in plasma E_2 and Vtg production (Sun et al., 2003), and the occurrence of weak relationships between absolute levels of E_2 and Vtg or ovarian growth in a number of species (reviewed in Pankhurst, 2008) suggest that the second explanation is more likely.

A similar effect was observed in plasma Vtg levels where despite reductions in both plasma E_2 and Vtg gene transcription, only maidens held at 22 °C showed significant suppression of plasma Vtg levels, and only at the February sample. Suppression of plasma Vtg levels at higher temperatures has previously been described for maidens of this stock (King et al., 2003; Watts et al., 2004) with the most marked effects occurring during February and March. A subsequent study confirmed that the most thermally sensitive period was February–March where maintenance at 22 °C for discrete 28 day periods suppressed plasma Vtg (King et al., 2007). In all three studies there was some recovery in plasma Vtg levels amongst fish held at high temperature by April, and the same effect was found in the present study.

ZPB and ZPC gene expression showed similar patterns of increase with ovarian growth and development as plasma E_2 and Vtg with maidens again showing earlier increases than repeats. The suppression of ZPB gene expression in both age classes at 22 °C, and the suppression of ZPC gene expression amongst repeats is generally consistent with the role of E_2 in stimulating both ZPB and ZPC gene expression (reviewed in Modig et al., 2007), with suppression of ZPB gene expression being correlated with reductions in plasma E_2 in the present study. The effect on ZPC gene expression was less marked and this may relate to the fact that ZP expression can be modulated by other endocrine factors including cortisol and androgens (Modig et al., 2007).

Both age classes in the present study showed endocrine suppression in the face of extended exposure to maintenance at 22 °C, but only maiden fish showed significant reductions in subsequent egg fertility and survival relative to 14 °C controls. Repeats showed some apparent reduction in fertility and survival, albeit not to levels that were significantly different from fish held at 14 °C. There are multiple determinants of egg quality (reviewed in Lubzens et al., 2010); however, a notable difference between maidens and repeats in the present study appears to be the suppression of plasma Vtg levels in maidens only. Critically, this effect occurred in February, the time which our previous studies have identified as a period of high sensitivity to thermal insult (King et al., 2007). This period also coincides with the major period of oocyte growth and when the ovary shows the highest level of asynchrony of growth of the developing oocyte clutch (King and Pankhurst, 2003; this study). The genesis of the differential age class response is not clear, particularly given that both the age classes showed thermal suppression of plasma E_2, Vtg and ZPB gene expression, but may relate to the size of the broodstock fish. Repeats held at 14 °C generally had larger follicle diameters than maidens, and this may indicate that they have greater capacity to sequester resources into growing oocytes than younger (smaller) fish. This size effect may provide some protective advantage to repeats in terms of their capacity to recover from thermal stress. A study by Bromage et al. (1992) showed that egg size in rainbow trout was not a strong predictor of egg quality; however, more recent investigations in a range of salmonid species have confirmed the role of maternal investment in the determination of egg size, and that egg size tends to be positively correlated with subsequent larval survival (Janhunen et al., 2010; Pakkasmaa et al., 2001). This does not preclude the possibility that repeats are also more efficient at mobilising and sequestering other factors that enhance egg viability. Possible candidates here include vitamins A and E, and low density lipoproteins accumulated independently of Vtg sequestration (reviewed in Lubzens et al., 2010).

A critical stage of egg formation involves the deposition and structural organisation of ZP proteins that will ultimately compose the egg chorion (Modig et al., 2007). In turn, integrity of the chorion is strongly implicated in egg viability in both Atlantic salmon (King et al., 2003) and chinook salmon (*Oncorhynchus tshawytscha*) (Barnes et al., 2003). There were no differences between age classes in ZPB gene expression in thermally challenged fish in the present study, and ZPC gene expression was reduced only in repeats where there was not significant suppression of fertility or survival. Acknowledging that gene transcription, and subsequent protein synthesis and chorion assembly are not synonymous, the results of the present study do suggest that the basis for age class differences identified here may not reside in differences in chorion assembly and integrity.

The most effective way to protect Atlantic salmon broodstock from thermal insult remains the isolation of fish from temperature change during the critical early autumn period of vitellogenesis (Pankhurst and King, 2010). The results of the present study suggest that in the absence of the capacity to do this, the use of repeat spawning stock may partially offset the effects of exposure to elevated temperature. This may offer a management alternative for culture situations where large-scale temperature control is not a practical option. The phenomenon described here may also have some implications for egg survival amongst natural stocks exposed to elevated temperatures during vitellogenesis. Natural spawning runs of Atlantic salmon contain variable numbers of repeat spawners, accounting for example, in UK rivers, from <1–34% of spawning fish (Phillips and Rix, 1988). Increased egg survival from these fish at a similar level over maidens under conditions of thermal stress to that seen in the present study means that repeat spawners could contribute disproportionately to egg survival. For example, in a spawning run containing 34% repeat spawners, egg survival and fecundity data from the present study (fecundity is on average 1.25 times higher in repeats than in maidens, egg survival for repeats at 22 °C is 1.2 times that of maidens) might predict that the viable egg contribution from repeat spawners could be as high as 43% of the total. However, capture data from Scottish coastal net fisheries monitored since the mid 1960s shows that the capture of repeat spawners entering Scottish waters is generally much lower (1% or less) (Julian Maclean, Marine Scotland Science, pers. comm.) suggesting that any impacts would be more modest. The present study does further emphasise the complexity of quantifying the effects of climate change on reproduction in natural stocks of fish.

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Chapter 5- Effect of thermal challenge on plasma gonadotropin levels and ovarian steroidogenesis in female Atlantic salmon

5.1 Abstract

Exposure of female Atlantic salmon to elevated temperature can result in a dramatic reduction in egg fertility and embryo survival. Reductions in plasma 17β-estradiol (E_2) levels are associated with much of the observed reduction in reproductive performance; however, the molecular basis for reduced E_2 levels remains unknown. This study examined gene expression of ovarian steroidogenic enzymes and plasma levels of gonadotropins in maiden and repeat spawning Atlantic salmon exposed to higher than normal temperatures. Circulating levels of *follicle stimulating hormone* were significantly elevated in both maiden and repeat spawning fish maintained at 22 °C compared to 14 °C during vitellogenesis, but plasma luteinising hormone levels were mostly unaffected. In contrast, gene expression of the ovarian *p450 aromatase a* and *cholesterol side chain cleavage protein* were depressed at 22 °C compared to 14 °C. Hepatic gene expression of *estrogen receptor alpha* did not change with thermal challenge. The results show that the ovarian response to Fsh is inhibited at 22 °C, at least partly as a result of reduced expression of genes coding for steroidogenic enzymes.

5.2 Introduction

Reproductive development in fish is controlled by hypothalamic *gonadotropin-releasing hormone* (Gnrh) and subsequent production and release of *follicle stimulating hormone* (Fsh) from the pituitary (Oppen-Berntsen et al., 1994, Dickey and Swanson, 2000). In response to Fsh, cholesterol is metabolised in a stepwise fashion to produce the gonadal steroids testosterone (T) and 17β-estradiol (E_2); the latter then stimulates the hepatic synthesis of *vitellogenin* (Vtg) and *zona pellucida* proteins (Zp) which are essential for oocyte development (reviewed in Lubzens et al., 2010). Exposure to higher-than-

optimal temperatures can adversely affect the endocrine system and consequently impair reproductive development, as demonstrated for a variety of species (Tveiten and Johnsen, 1999, Pankhurst and King, 2010). In female pejerrey (*Odontesthes bonariensis)*, short exposure to 23 or 27 °C resulted in a significantly lower level of gonadal Fsh receptor (Fshr) mRNA and circulating E_2 relative to the 19 °C control group (Soria et al., 2008). The authors of the pejerrey study concluded that circulating E_2 was depressed as a result of thermal inhibition at the level of the gonad and pituitary (Soria et al., 2008). Furthermore, in an *in vitro* study, Watts et al. (2004) showed that conversion of T to E_2 in the gonad was inhibited at elevated temperature, and suggested this occurred through impairment of the steroidogenic enzyme *p450 aromatase a* (Cyp19a1a). As described in Chapter 4 (Pankhurst et al., 2011), we also found that plasma T levels were depressed at high temperature in Atlantic salmon (*Salmo salar*) suggesting that endocrine blockages also occur upstream of E_2 production. Temperature impairment of *cyp19a1a* during sexual differentiation has been observed in other fish species including Japanese flounder (*Paralichthys olivaceus*) and Atlantic halibut (*Hippoglossus hippoglossus*) (Kitano et al., 1999, van Nes and Andersen, 2006). Additionally, in a further *in vitro* study, Watts et al. (2005) also showed a decrease in the number of high affinity estrogen receptors (Ers) in cultured hepatic tissue from Atlantic salmon reared at 22 °C relative to 18 and 14 °C. It is thought that the combination of depressed E_2 levels and reduced hepatic Er binding efficiency explains the impact on downstream processes such as vitellogenesis and zonagenesis described in thermally challenged fish (Watts et al., 2005, Ch 4: Pankhurst et al., 2011). Collectively, these studies suggest that thermal impairment is likely to occur at a range of levels in the reproductive endocrine cascade during reproductive development.

In farmed Tasmanian Atlantic salmon, high ambient water temperature accelerates growth rate and shortens production time relative to their Canadian ancestors (Jungalwalla, 1991). However, temperatures experienced by broodstock during summer can reach 22 °C (Battaglene et al., 2008) which is high enough to induce the endocrine anomalies described previously (King et al., 2003, Ch 4: Pankhurst et al.,

2011). Regardless of the mechanisms by which E_2 and subsequent vitello- and zonagenesis are depressed in thermally exposed fish, the collective result of endocrine suppression is a reduction in reproductive performance. Fertility in Tasmanian Atlantic salmon was lower in fish reared at 22 °C during vitellogenesis compared to 14 and 18 °C (King et al., 2003). Other studies on Atlantic salmon and rainbow trout maintained at elevated temperature for a period of time prior to spawning have also shown subsequent reductions in embryo survival (Taranger and Hansen, 1993, Pankhurst et al., 1996). The majority of eggs produced by the Australian Atlantic salmon industry come from first-spawning (maiden, 2+ years old) fish (Jungalwalla, 1991). However, a recent study confirmed industry observations that maiden Atlantic salmon were more susceptible to exposure to high temperature than second-spawning (repeat, 3+ years old) in terms of egg fertility and embryo survival (Ch 4: Pankhurst et al., 2011). Because Tasmanian Atlantic salmon are routinely reared close to their upper limit of thermal tolerance, any further increase in temperature that occurs as a result of normal seasonal fluctuation or climate change will result in major production challenges (Pankhurst and King, 2010). In an attempt to minimise exposure of broodstock to elevated temperature, the salmon industry in Australia is moving towards re-circulating systems that allow complete thermal regulation (Battaglene et al., 2008, Pankhurst and King, 2010). The apparently lower susceptibility of repeat spawning fish to thermal challenge may offer an additional option for the management of thermal insult, but assessment of this possibility is hindered by significant knowledge gaps in relation to pituitary and ovarian function in thermally challenged fish.

The aim of the present study was to investigate whether thermal challenge inhibits reproductive processes at the level of the pituitary (circulating Fsh and Lh levels), ovary (expression of genes coding for steroidogenic enzymes) or liver (*estrogen receptor alpha*, *erα* gene expression) in maiden and repeat spawning Atlantic salmon.

5.3 Methods

5.3.1 Fish maintenance

The maiden and repeat spawning fish used in Chapter 4 (Pankhurst et al., 2011) were also used to generate data for the present chapter. As such, detailed information regarding fish, fish husbandry, experimental conditions and sampling procedures can be found in Chapter 4 (Pankhurst et al., 2011). In addition, ovary tissue was dissected and stored in RNA Later™ as described previously for liver tissue. This research activity was undertaken with approval from the Animal Ethics Committees of the University of the Sunshine Coast and Griffith University (approval numbers AN/A/07/35 and EAS/02/07/AEC respectively).

5.3.2 Plasma Fsh and Lh measurement

Plasma Fsh (all sampling points) and Lh (March and April sampling only) measurements were performed using an RIA developed for coho salmon (*Oncorhynchus kisutch*) by Swanson et al. (1989) with some modifications. Briefly, the assay utilised rabbit antisera specific to the coho salmon Fsh or Lh beta subunit (lots #8621 and #38.5.92 respectively), and highly purified coho Fsh and Lh as the standards (Swanson et al., 1991). In these assays phosphate buffered saline (pH 7.4) was used instead of barbital and 500 µl polyethylene glycol (4 %) was included on day 4. All assays for a specific hormone were analysed in a single batch, and therefore inter-assay variation was not calculated. The cross reactivity of Fsh in the Lh assay and Lh in the Fsh assay was approximately 4.4 and 6 %, respectively (Swanson et al., 1989). ANCOVA was performed to determine whether parallelism was present between Atlantic salmon plasma and the respective purified coho standard that was serially diluted in PBS-BSA (triplicates) for Fsh and Lh. The detection limit (LOD) of the Fsh and Lh assays was approximately 0.6 and 0.5 $ng.ml^{-1}$ respectively.

5.3.3 Measurement of steroidogenic enzyme and erα gene expression

Total RNA was isolated from 15 mg of hepatic tissue using the Illustra RNAspin Mini kit (GE Healthcare), and from 120 mg of ovarian tissue using TRIZOL® reagent (Invitrogen) according to each of the manufacturer's protocols. RNA yield and 260/280 purity ratio was determined using the NanoDrop 2000 (Thermo Scientific). An RNA integrity number (RIN) was determined for a random sample of hepatic and ovarian RNA (n=48) using the 2100 Bioanalyzer to establish RNA quality (Agilent). All RNA had a RIN value that was higher than 8 which is considered to be ideal for down-stream molecular applications such as qPCR (Fleige and Pfaffl 2006).

One microgram of ovary-derived and 0.4 µg of liver-derived RNA was used to synthesise cDNA for use in real-time quantitative PCR (qPCR) using the QuantiTect® reverse transcription kit (Qiagen). This kit includes a DNA elimination step to remove potential contamination of qPCRs by genomic DNA. Following synthesis, cDNA was stored at -20 °C until use.

qPCRs for *cyp19a1a*, *cholesterol side chain cleavage protein* (*cyp11a1*) and *erα* were conducted using the qPCR cycler, cycling conditions and controls outlines in Chapter 4 (Pankhurst et al., 2011). For *erα* and *cyp19a1a*, the 10 µl qPCR reaction contained 5 µl SYBR mix, 200 nM each primer, 3.6 µl PCR grade water and 1 µl cDNA template. For *cyp11a1*, the reaction components were the same except only 2.6 µl of PCR grade water was added and the final concentration of $MgCl_2$ was 4 instead of 3 mM.

Gene specific primers (GSPs) were designed from the Atlantic salmon Cyp19a1a, Cyp11a1 and Erα mRNA sequences available on GenBank to have an optimum annealing temperature of 60 °C using Primer3 software (http://frodo.wi.mit.edu/primer3/, Table 5.0). These qPCR assays were developed and validated using the technique outlined in Chapter 4 (Pankhurst et al., 2011) and the reaction efficiencies are shown in Table 5.0. *Tata binding protein* (*tbp*, reference gene) qPCRs were performed using the primers cycling conditions described in Chapter 4 (Pankhurst et al., 2011). Hepatically expressed *tbp* showed consistently high stability under the experimental conditions and was

therefore used as a reference gene for normalisation of *erα* (Ch 3, Anderson and Elizur 2012). Similarly, the suitability of using gonadally expressed *tbp* to normalise the expression of Cyp genes was confirmed through statistical analysis using the method described in Chapter 3 (Anderson and Elizur 2012). In short, transcript abundance was similar between groups within each sample point, and a significant relationship did not exist between transcript abundance and sample point as revealed by Kendall's tau non-parametric correlation analysis (data not shown). The software Rest© 2008, V2.0.7 (Pfaffl et al., 2002) was used to normalise the data, adjust for inter-assay variability and calculate expression of key genes for each sample relative to the calibrator.

Table 5.0 qPCR primers and sequence information

Gene name	Primer name	Sequence (5'→3')	Prod. size	E*	Source seq.
Cyp19a1a	Cyp19F1	TTC CAT CCC GTG GTG GAC TT	106 bp	0.982	AF436885
	Cyp19R1	TGC GGC CCA TGT TCA GAA T			
Cyp11a1	Cyp11F1	GGC GTT CCA ACA GGG TGA TT	106 bp	0.949	DQ361039
	Cyp11R1	ACT CGG GCC ACA AAG TCC TG			
Erα	ErαF1	AAG CAT GCC GCC TCA GAA AG	150 bp	1.003	X89959
	ErαR2	TCC TGT GCT CCA GGT CAC CA			

*E = efficiency, bp = base pairs

5.3.4 Statistical analysis

Circulating hormone levels were compared using one-way ANOVA coupled with Tukeys-b for post-hoc analysis and a P value of 0.05. Values for fish with a non-detectable plasma concentration of Fsh were recorded as the lower limit of detection for the assay (0.6 $ng.ml^{-1}$) for statistical analysis. Differences in relative gene expression levels were detected non-parametrically as outlined in Chapter 4 (Pankhurst et al., 2011). All analysis was performed using SPSS version 17.0.

5.4 Results

5.4.1 Plasma Fsh and Lh

Serial dilutions of Atlantic salmon plasma were parallel to the dilution curves obtained for purified coho salmon standards for Fsh and Lh as determined by ANCOVA (where a P value below 0.05 indicates a deviation from parallelism, the P values for Fsh and Lh were 0.62 and 0.34 respectively, Fig. 5.0). Circulating levels of Fsh were significantly higher in maiden than repeat spawning fish maintained at natural temperature in August and November but by January, the difference in Fsh plasma concentration had disappeared (Fig. 5.1). From August until February, the plasma concentration of Fsh did not exceed 4 $ng.ml^{-1}$ in any fish. In February, there were no statistical differences in plasma Fsh levels between temperatures within age classes, but levels were higher in repeat spawning fish at 22 °C compared to maiden spawning fish at 14 °C. In March and April fish held at 22 °C had significantly higher levels of Fsh than fish at 14 °C irrespective of age. In April, repeat spawning fish at 22 °C had significantly higher plasma levels of Fsh than maidens at 22 °C, even though the standard error for repeat spawning fish at 22 °C was quite large due to a high level of variation between all fish.

In March and April the mean (± SD) plasma levels of Lh for all fish were 0.97 ± 0.21 $ng.ml^{-1}$ and 0.94 ± 0.25 $ng.ml^{-1}$ respectively (Fig. 5.2). In March, Lh levels were significantly lower in maiden spawning fish at 14 °C and repeat spawning fish at 22 °C compared to maiden fish at 22 °C while repeat fish at 14 °C exhibited an intermediate concentration. In April, there was no significant difference in Lh concentration among the groups.

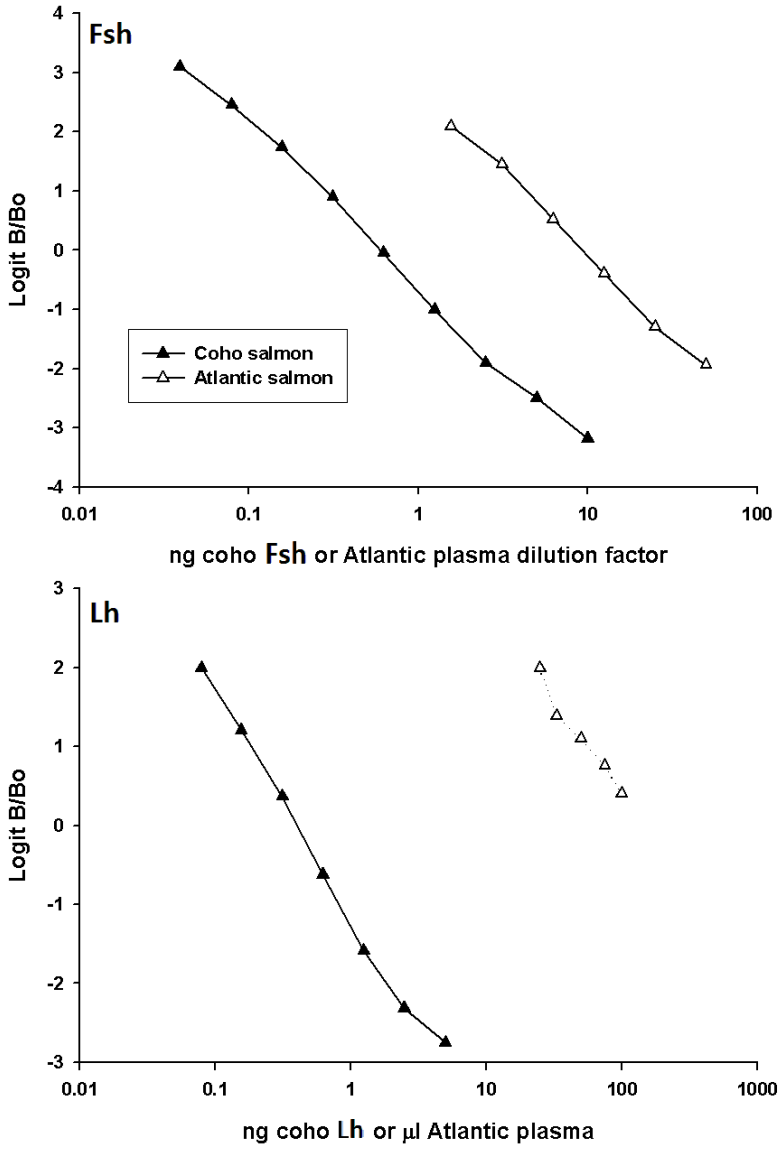

Fig. 5.0 Parallelism between serially diluted purified coho salmon standards, and Atlantic salmon plasma diluted in PBS-BSA for *follicle stimulating hormone* (Fsh) or *luteinizing hormone* (Lh).

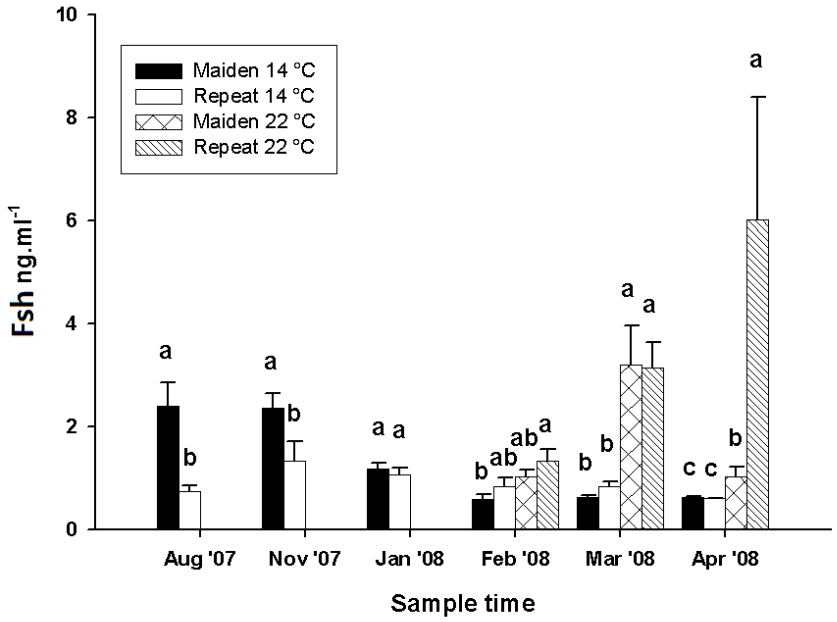

Figure 5.1 Plasma levels (mean + SE, n=7) of *follicle stimulating hormone* (Fsh) in maiden and repeat spawning Atlantic salmon held at either 14 or 22 °C. Fsh levels below the LOD are displayed as 0.6 ng.ml^{-1}. Different superscripts between groups at each sampling point denote significantly different means ($p \leq 0.05$).

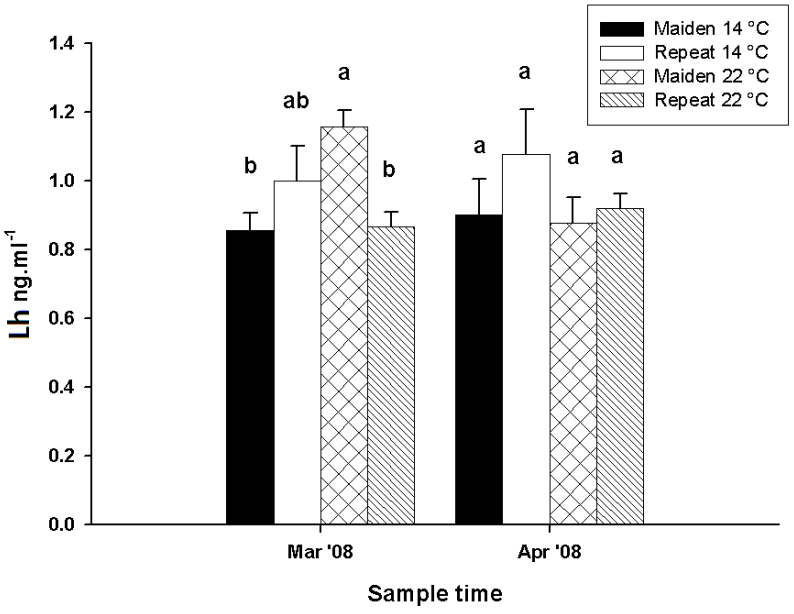

Figure 5.2 Plasma levels (mean + SE, n = 7) of *luteinizing hormone* (Lh) in maiden and repeat spawning Atlantic salmon held at either 14 or 22 °C. Different superscripts between groups at each sampling point denote significantly different means ($p \leq 0.05$).

5.4.2 Relative expression of cyp genes and erα

Gene expression profiles for *cyp19a1a* and *cyp11a1* followed a similar trend throughout the experiment (Fig. 5.3). In August, the relative level of expression of *cyp19a1a* was significantly higher in maiden than repeat spawning fish. From November until January *cyp19a1a* gene expression steadily increased and there were no differences between maiden and repeat spawning fish. In February, expression was suppressed in 22 °C repeat spawning fish relative to 14 °C maiden spawners, but there were no differences between temperatures within age classes. By March, thermal inhibition of *cyp19a1a* was present in repeat spawning fish at 22 °C relative to 14 °C repeat spawners, and in April, expression was suppressed in both age classes at 22 °C relative to 14 °C.

In a similar fashion to *cyp19a1a*, *cyp11a1* gene expression was significantly higher in August in maiden spawning fish compared to repeats spawners (Fig. 5.3). From November until February, *cyp11a1* gene expression level increased in a time-dependent manner and was not significantly different between treatment groups, or as a result of thermal exposure (in February). In March *cyp11a1* gene expression was suppressed in repeats spawning fish at 22 °C compared to repeats spawning fish at 14 °C, and in April, both age classes showed reduced expression at 22 °C.

In August, relative *erα* gene expression was significantly elevated in maiden fish compared to repeat spawning fish reared at natural temperature (Fig. 5.4). By November, the difference in *erα* gene expression between maiden and repeat spawning fish had disappeared and by January, the level of gene expression had risen by approximately 2.5 fold in both groups. For the remaining months (February, March and April ‘08), there was no significant difference in *erα* gene expression level as a result of thermal manipulation or broodstock age.

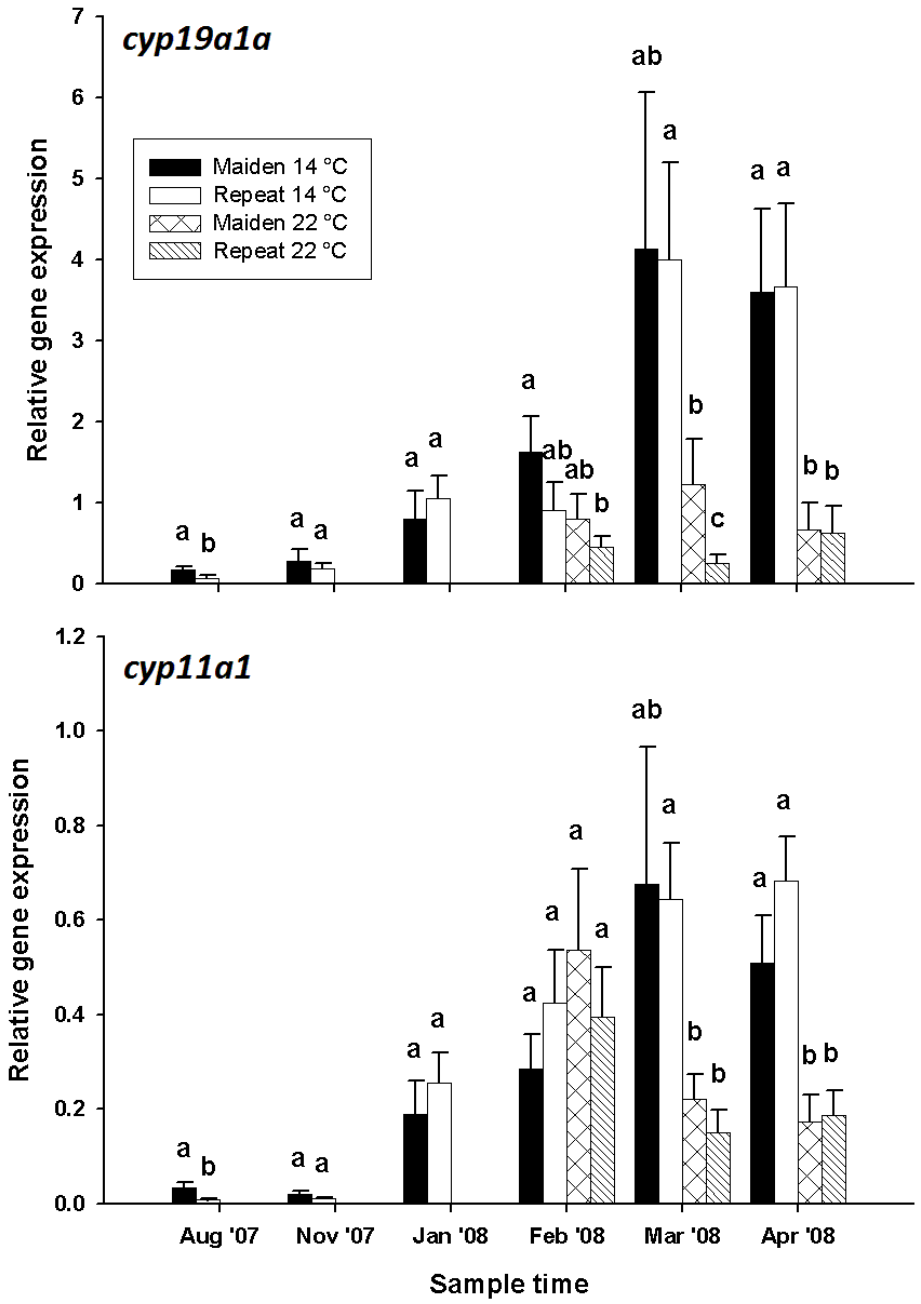

Figure 5.3 Relative gene expression levels (mean + SE, n = 7) of gonadal *p450 aromatase a* (*cyp19a1a*) and gonadal *cholesterol side chain cleavage protein* (*cyp11a1*) in maiden or repeat spawning Atlantic salmon held at 14 °C or 22 °C. Gene expression levels were normalised against *tata binding protein* expression. Different superscripts between groups at each sampling point denote significantly different means ($p \leq 0.05$).

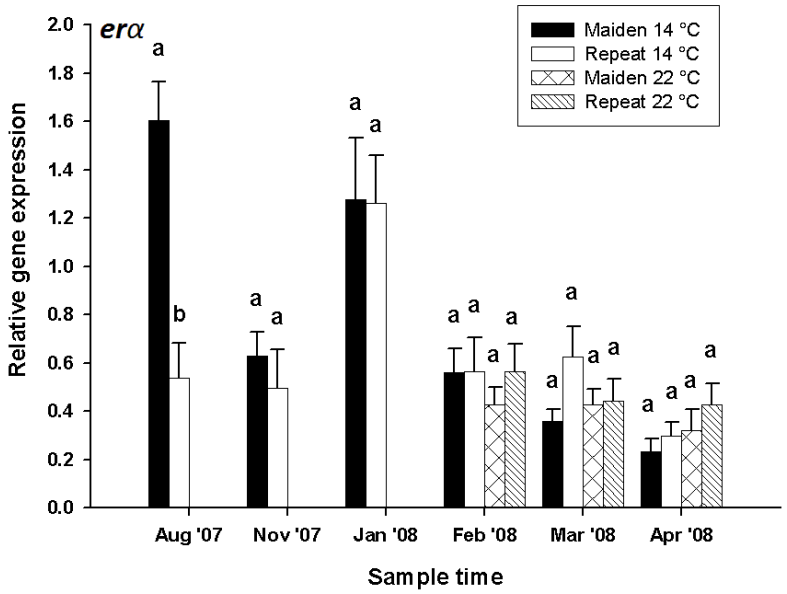

Figure 5.4 Relative hepatic gene expression levels (mean + SE, n = 7) of *estrogen receptor alpha* (*erα*) in maiden or repeat spawning Atlantic salmon held at 14 °C or 22 °C. Gene expression levels were normalised against *tata binding protein* expression. Different superscripts between groups at each sampling point denote significantly different means ($p \leq 0.05$).

5.5 Discussion

In the present study, RIAs originally developed to quantify coho salmon Fsh and Lh (Swanson et al., 1989) were used to assess gonadotropin levels in Atlantic salmon. Parallelism of assay binding curves for serially diluted Atlantic salmon plasma and purified coho salmon standards allowed measurement of gonadotropin-like immunoreactive material in the plasma of Atlantic salmon. This assay has also been used to quantify putative plasma gonadotropins in a northern hemisphere stock of Atlantic salmon (Olsen and Walther, 1993, Oppen-Berntsen et al., 1994). With the proviso that this is a heterologous assay, the immunoreactive constituents in Atlantic salmon plasma are hereafter termed Fsh and Lh, respectively. Circulating levels of Fsh were significantly lower in repeat compared to maiden spawning fish in August and November which corresponds to the initial period of oocyte development in Tasmanian Atlantic salmon (King and Pankhurst, 2003). By January there was no significant difference in the circulating levels of Fsh as a result of stock age. As described in

Chapter 4 (Pankhurst et al., 2011) for the same fish used in this chapter, somatic condition factor was lower in repeat than maiden spawning fish at sampling points prior to January reflecting the fact that repeat spawning fish were recovering from the energetic demands of the previous reproductive season. It appears that this recovery may also be reflected in lower plasma Fsh levels.

From August until February, the circulating concentration of Fsh in the present study did not exceed 4.0 $ng.ml^{-1}$ which is similar to levels previously reported for Northern Hemisphere Atlantic salmon during the corresponding phase of reproductive development (Oppen-Berntsen et al., 1994). However, in contrast to the Northern Hemisphere stock where plasma Fsh levels increased from mid-vitellogenesis and remained elevated until just prior to spawning, plasma Fsh levels in the present study did not increase as development progressed despite plasma E_2 levels being similar in the two stocks (Oppen-Berntsen et al., 1994, Ch 4: Pankhurst et al., 2011). Inter-annual differences in circulating Fsh levels have been observed for other species of salmonids. Slater et al. (1994) measured circulating Fsh levels in wild female spring chinook salmon (*Oncorhynchus tshawytscha*) during migration in 2 successive years. In 1989, circulating levels of Fsh were low during reproductive development and never exceeded 5 $ng.ml^{-1}$; the following year, Fsh levels reached a maximum of approximately 30 $ng.ml^{-1}$ in late summer. The substantial difference in Fsh concentration between years for this species could not be explained by the authors, although it is interesting that fish sampled during 1989 had higher circulating E_2 levels at 4 of 9 sampling points compared to fish from 1990 (Slater et al., 1994).

Plasma levels of Fsh were significantly elevated during autumn in fish reared at 22 °C compared to 14 °C with the effect being more prolonged in repeat spawning fish. Plasma E_2 levels in the same fish (Ch 4: Pankhurst et al., 2011) were significantly reduced in thermally challenged fish during March and April. E_2 is a known regulator of gonadotropin secretion and can exert either positive or negative feedback on Fsh at different developmental stages (Larsen and Swanson, 1997, Levavi-Sivan et al., 2006). In vitellogenic rainbow trout and 3 year old Mediterranean Sea bass (*Dicentrarchus*

labrax), E_2-treatment resulted in lower plasma levels of Fsh (Saligaut et al., 1998, Mateos et al., 2002). Since fish exposed to high temperature in the present study had reduced levels of circulating E_2, it is possible that reduced negative feedback on Fsh by E_2 at high temperature was at least partly responsible for the elevated plasma levels of Fsh observed. Because increases in temperature also increase metabolic rate (Johnston and Dunn, 1987), increased plasma Fsh levels are also consistent with the broad effects of temperature on metabolism. Either or both mechanisms may have operated here.

Lh plays a primary role in controlling the final maturation of oocytes reviewed by Nagahama and Yamashita (2008), and as such, is only found circulating at significant levels during the peri-ovulatory period in salmonids (Oppen-Berntsen et al., 1994, Breton et al., 1998). In the present study, plasma levels of Lh were unaffected by temperature and did not exceed 1.5 $ng.ml^{-1}$ which is consistent with previous observations for Atlantic salmon at the corresponding phase of reproductive development (Oppen-Berntsen et al., 1994). In rainbow trout, plasma Lh levels began to increase approximately 15 days prior to ovulation, and peaked 3 days before ovulation (Breton et al., 1998), emphasizing the importance of the timing of sampling with respect to ovulation in order to detect preovulatory rises in Lh. Oppen-Berntsen et al. (1994) recorded variable plasma Lh levels in Atlantic salmon towards the end of maturation and attributed the variation in plasma Lh to the fixed time of sampling relative to the variable time of ovulation for individual fish. The April sampling point in the present study was approximately 20, 24, 38 and 31 days prior to the commencement of ovulation for maiden and repeat spawning fish reared at 14 °C, and maiden and repeat spawning fish reared at 22 °C respectively (Ch 4: Pankhurst et al., 2011). If the preovulatory pattern of circulatory Lh concentration in rainbow trout is similar to that of Atlantic salmon, our April sample point was too early to detect increases in Lh associated with oocyte maturation and ovulation. With that proviso, there was no evidence in the present study of a thermal effect on plasma Lh levels during the late stages of vitellogenesis.

Gonadal levels of *cyp19a1a* and *cyp11a1* gene expression were significantly lower in repeat compared to maiden spawning fish in August. However, by November the relative expression levels in maiden and repeat spawning fish were similar for both gonadal genes. In salmonids, Fsh is the regulating factor that promotes the synthesis of E_2 via an enzyme mediated steroidogenic pathway in the gonad (Suzuki et al., 1988, Oppen-Berntsen et al., 1994). It would appear that repeat spawning fish in the present study did not have sufficient stimulation by Fsh to produce the *cyp19a1a* and *cyp11a1* transcript levels observed in maiden fish early in reproductive development. As noted in Chapter 4 (Pankhurst et al., 2011) for the same fish used in the present chapter, condition factor, circulating E_2 levels and the gene expression levels of *vtg* and *zona pellucida b* were also lower in repeat spawning fish during the initial stages of oocyte recruitment and development. As noted previously, this may reflect the fact that repeat spawning fish were still recovering from the energetic demands of the previous spawning season.

By February, there was evidence that temperature was down-regulating *cyp19a1a* gene expression. For the same groups of fish, we found that E_2 levels were lower at 22 °C than 14 °C while plasma T levels remained unaffected during February (Ch 4: Pankhurst et al., 2011). The modest reduction in *cyp19a1a* gene expression at high temperature could have contributed to those lower E_2 levels. In March and April, after exposure to differential thermal regimes for approximately 2 and 3 months respectively, gene expression was down-regulated for *cyp19a1a* in fish reared at 22 °C regardless of age. The thermal sensitivity of *cyp19a1a* has been confirmed in a range of other species. Nineteen month old red seabream (*Pagrus major*) showed suppression of *cyp19a1a* gene expression at 25 and 20 °C relative to 15 °C after 4 and 8 weeks respectively (Lim et al., 2003). Similarly, *cyp19a1a* gene expression was reduced in Atlantic halibut larvae reared at 13 °C relative to 7 °C (van Nes and Andersen, 2006). Most studies involving the thermal inhibition of *aromatase* address issues surrounding sex change/reversal in juvenile fish (reviewed in Devlin and Nagahama, 2002). However, we have shown here that adult fish also show *aromatase* inhibition as a result of

thermal challenge, as was suggested previously for Atlantic salmon (Watts et al., 2004, Ch 4: Pankhurst et al., 2011).

The molecular mechanism(s) by which gonadal steroidogenic enzymes are affected by elevated temperature are not properly understood, especially in adult fish. Various studies have shown that *forkhead transcription factor* (Foxl2) is involved in transcriptional regulation of *cyp19a1a* in female fish (Nakamoto et al., 2006, Wang et al., 2007) and is sensitive to thermal exposure. Yamaguchi et al. (2007) showed that high water temperature had a negative influence on *foxl2* gene expression in Japanese flounder during sexual differentiation. The Foxl2 gene is not only expressed in juvenile fish; Nakamoto et al. (2006) and Zhou et al. (2007) found that *foxl2* was expressed in both previtellogenic and vitellogenic follicles of adult medaka (*Oryzias latipes*). Therefore, thermal inhibition of gonadal *foxl2* gene expression may have occurred in the present study, with the result that subsequent regulation of *cyp19a1a* did not take place normally. If this is the case, other gonadal steroidogenic enzymes may also have been affected, as Foxl2 has been implicated as a regulator of *17α-hydroxylase/C17,20-lyase* (Cyp17a) in medaka (Zhou et al., 2007). We previously found that T levels were lower in thermally exposed fish during March (maiden and repeat spawning fish) and April (maiden spawning fish only) suggesting that the same effect was present (Ch 4: Pankhurst et al., 2011). Another possibility is that Fsh signal transduction, and subsequent steroidogenesis was partially inhibited because *follicle stimulating hormone receptor* (*fshr*) gene expression was thermally impaired. The fact that the higher level of circulating Fsh observed during March and April in the present study did not stimulate increased *cyp11a1* and *cyp19a1a* gene expression at 22 °C is consistent with this possibility. In female adult pejerrey, 8 days of exposure to high temperature caused a significant reduction in *fshr* gene expression during vitellogenesis (Soria et al., 2008). In a similar fashion, maintenance at high temperature resulted in lower *fshr* gene expression levels in Japanese flounder during sex differentiation (Yamaguchi et al., 2007). A change in *fshr* gene expression is likely to affect downstream Cyp19a1a activation through its role in transducing modulation of intracellular cAMP levels (Planas

et al., 1997, Montserrat et al., 2004). The Cyp19a1a gene contains cAMP responsive elements (CREs) in several species (Wong et al., 2006, Kazeto et al., 2001), and cAMP has been shown to stimulate gonadal *cyp19a1a* gene expression *in vitro* (Yamaguchi et al., 2007).

In the present study, *cyp11a1* gene expression was suppressed in thermally challenged repeat spawning fish during March and in both maiden and repeat spawning fish in April. Cyp11a1 catalyses the side chain cleavage of cholesterol to pregnenolone and is considered to be the first rate-limiting step in the gonadal biosynthesis of T, and subsequently E_2 (reviewed in Payne and Hales, 2004). We reported that for the same fish used in the present study, plasma T levels were generally reduced at 22 °C in maiden and repeat spawners during mid to late vitellogenesis (Ch 4: Pankhurst et al., 2011). It therefore seems likely that inhibition of *cyp11a1* gene expression was contributing to the lower level of circulating T reported. There is little information on Cyp11a1 regulation in teleosts; however, a recent *in vitro* study by Luckenbach et al. (2011) found that exposure of coho salmon ovarian follicles to Fsh caused a moderate increase in *cyp11a1* gene expression. In mammals, stimulation by Fsh causes an increase in cAMP which up-regulates the synthesis of steroidogenic cytochrome p450 enzymes through CREs (Payne and Hales, 2004) as has been shown previously for rodent and bovine Cyp11a1 (Waterman, 1994, Sher et al., 2007). This may suggest that thermally suppressed *fshr* gene expression and subsequent reduction in Fsh signal transduction is partly responsible for the suppressed *cyp11a1* gene expression observed in the present study and the lower plasma T levels (Ch 4: Pankhurst et al., 2011) among fish held at 22 °C. Regardless of the molecular basis for reduced *cyp19a1a* and *cyp11a1* gene expression, the collective result of reduced steroidogenic enzyme gene expression is significantly lower circulating levels of E_2 in fish reared at 22 °C (Ch 4: Pankhurst et al., 2011).

In the present study hepatic *erα* gene expression was 3-times higher in maidens than repeat spawning fish during August. As noted previously, the onset of vitellogenesis is retarded in repeat spawning fish (Ch 4: Pankhurst et al., 2011) and the

lower expression of *erα* may contribute to this effect. After August, there was no difference in relative *erα* gene expression between any groups at any sampling point for the duration of the experiment. Similarly, in a study by Pawlowski et al. (2000), hepatic tissue from rainbow trout was maintained *in vitro* at either 14 or 18 °C, and after 12 and 24 hours of exposure to elevated temperature no difference in *er* gene expression was observed. In Atlantic halibut reared at 7, 10 or 13 °C during sexual differentiation, there was no clear temperature related pattern in *er* gene expression during 120 days of thermal management (van Nes and Andersen, 2006). While it appears that temperature does not modify the expression of *erα* in adult female Atlantic salmon, an *in vitro* study by Watts et al. (2005) revealed that Ers in liver tissue from Atlantic salmon reared at 22 °C during vitellogenesis had a significant reduction in E_2 binding affinity compared to Ers from fish maintained at 14 or 18 °C. Reduced Er binding affinity due to thermal challenge has also been reported for other teleost species such as tilapia (*Oreochromis aureus*) (Tan et al., 1999). While we did not determine whether Er affinity was impaired in the present study, diminished Er binding affinity could have potentiated the effects of lower circulating E_2, and therefore could have contributed to the lower expression of hepatic genes described in Chapter 4 (Pankhurst et al., 2011).

Information regarding the effects of abnormally raised temperature on the circulating levels of Fsh and Lh, and the gene expression levels of *cyp11a1*, *cyp19a1a* and *erα* for maiden and repeat spawning female Atlantic salmon was not previously available. Here, for the first time, we have demonstrated that thermal challenge results in the inhibition *cyp11a1* and *cyp19a1a* gene transcription which is likely to have impaired T, E_2, *vtg*, Vtg and *zp* production as described in Chapter 4 (Pankhurst et al., 2011). However it is currently unclear as to whether *cyp11a1* and *cyp19a1a* transcription was affected directly, or whether events up-stream were impaired which had a flow-on affect. We have also demonstrated that Fsh levels are elevated from mid-vitellogenesis in fish held at high temperature, possibly due to a lack of negative feedback by E_2 in combination with a general increase in metabolism. However, the relatively high levels of Fsh were unable to stimulate steroidogenesis, perhaps due to

other thermal impairments at multiple levels of the endocrine cascade controlling reproductive development. During the later stages of vitellogenesis, we have provided evidence that Lh levels are mostly unaffected by temperature up to 1 month prior to spawning. For circulating Fsh and Lh, and expression of the genes studied, the effects of thermal challenge do not appear to discriminate between fish of different ages. Therefore it is unlikely that the basis for increased thermal resilience of repeat spawning fish lies at the level of gonadal steroidogenesis, instead it may be a function of increased egg size, and increased ability of repeat spawning fish to sequester factors that promote egg viability as suggested previously (Ch 4: Pankhurst et al., 2011).

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Chapter 6- The effect of Gnrh treatment during vitellogenesis on the reproductive physiology of thermally challenged female Atlantic salmon

6.1 Abstract

Tasmanian Atlantic salmon broodstock can experience temperatures above 20 °C which is high enough to impair reproductive development and inhibit ovulation. In thermally challenged fish, plasma levels of 17β-estradiol (E_2) are typically low which impairs vitellogenesis and zonagenesis relative to fish maintained at cooler temperatures. The present study investigated the prolonged use of gonadotropin-releasing hormone analogue (Gnrha) during vitellogenesis as a means of maintaining endocrine function and promoting egg quality at elevated temperature in maiden and repeat spawning Atlantic salmon. Gnrha-treatment during vitellogenesis did not compensate for the negative effects of thermal challenge on the timing of ovulation, egg size, egg fertility or embryo survival in any fish maintained at 22 °C relative to 14 °C. The lack of effectiveness was reflected by the endocrine data as plasma *follicle stimulating hormone* (Fsh) and *luteinising hormone* (Lh) levels were not different between Gnrha-treated and untreated groups at 22 °C. Furthermore, plasma testosterone (T) and E_2 levels were unchanged in Gnrha-treated fish at 22 °C, and plasma levels were generally lower in both groups maintained at 22 °C relative to 14 °C. Transcription of *vitellogenin* (*vtg*), *zona pellucida b* (*zpb*) and *zona pellucida c* (*zpc*) was not enhanced in Gnrha-treated fish relative to untreated fish at 22 °C, presumably due to suppression of plasma E_2. These results indicate that thermal impairment of reproduction is likely to occur at multiple levels, and is difficult to overcome via hormonal manipulation. Co-treatment with Gnrha and a dopamine inhibitor, or direct administration of E_2 may warrant investigation in the future as development of new management strategies are essential for maintaining egg quality at high temperature without the added cost of intensive temperature control.

6.2 Introduction

Increases in ambient water temperature that occur as a result of normal seasonal fluctuation or anthropogenic climate change can fundamentally affect the physiology of farmed Atlantic salmon, *Salmo salar* (Battaglene et al., 2008). This is especially relevant for Tasmanian Atlantic salmon that are reared towards their upper-limit of thermal tolerance, and experience reproductive dysfunction as temperature increases above optimum levels (reviewed in Pankhurst and King, 2010). As a result of exposure to elevated temperature, a marked reduction in circulating levels of plasma 17β-estradiol (E_2) typically occurs in females (King et al., 2007, Ch 4: Pankhurst et al., 2011). As E_2 is the primary regulator of vitellogenesis and zonagenesis, gene expression levels of *vitellogenin* (*vtg*), *zona pellucida b* (*zpb*) and *zona pellucida c* (*zpc*), and plasma levels of Vtg were suppressed in fish reared under thermal challenge (King et al., 2007, Ch 4: Pankhurst et al., 2011). Watts et al. (2005) showed that the number of high affinity E_2 receptors (Er) in cultured hepatocytes *in vitro* was lower when the tissue originated from fish maintained at 22 °C relative to 18 and 14 °C, suggesting that the physiological effects of reduced E_2 tone may have been exacerbated by decreased efficiency of E_2 receptor binding.

The collective effect of suppressed plasma E_2 and associated hepatic gene expression at high temperature is a reduction in reproductive performance. Egg fertility was greatly reduced in Tasmanian Atlantic salmon maintained at 22 °C relative to those reared at 18 and 14 °C (King et al., 2003). Similarly, survival of eggs to the eyed stage decreased in Atlantic salmon and rainbow trout (*Oncorhynchus mykiss*) maintained at elevated temperature prior to spawning (Taranger and Hansen, 1993, Pankhurst et al., 1996). However, in our recent study we showed that egg quality was reduced to greater extent in first-time (maidens, 2+ years old) than in second-time (repeats, 3+ years old) spawning Atlantic salmon reared at 22 °C compared to their respective controls at 14 °C (Ch 4: Pankhurst et al., 2011). The increased susceptibility of smaller maiden fish to thermal challenge may have been a function of their smaller egg size, and reduced ability to sequester factors that promote egg quality during vitellogenesis (Ch 4:

Pankhurst et al., 2011). In addition, maiden spawning fish also exhibited lower plasma Vtg levels than repeat spawning fish during late austral summer (February); February has been recognised as a critical month for oocyte development, and appears to be a particularly sensitive period for thermal inhibition (King et al., 2007, Ch 4: Pankhurst et al., 2011). Therefore, the use of repeat spawning fish as opposed to maidens may partially offset the negative impacts of elevated temperature on egg quality in Tasmanian Atlantic salmon. However, it is not viewed as a viable option due to the higher cost and risk associated with rearing large fish intensively for an additional year. As an alternative, hormonal therapy may maintain endocrine status and subsequent egg quality through critical periods at elevated temperature. However, the potential of such management strategies remains to be explored.

Gonadotropin-releasing hormone (Gnrh) has been used since the 1970s to synchronise and induce ovulation by stimulating the secretion of endogenous *luteinizing hormone* (Lh) in a wide range of species (reviewed in Zohar and Mylonas, 2001). Its effectiveness at promoting gonadal maturation and maintaining egg quality in salmon reared at elevated temperature has also been assessed. In Atlantic salmon maintained at 16 °C from late vitellogenesis, treatment with a Gnrh analogue (Gnrha) in combination with a temperature ramp down to 8 °C advanced ovulation and enhanced egg fertility relative to sham treated fish (King and Pankhurst, 2004b). In addition to the usual role of inducing final oocyte maturation (FOM), treatment with Gnrha also resulted in an increase in *follicle stimulating hormone beta* (*fshβ*) subunit gene expression, and a subsequent increase in culture medium Fsh levels in a coho salmon (*Oncorhynchus kisutch*) pituitary cell culture (Dickey and Swanson, 2000). *In vivo* Gnrha implantation during vitellogenesis also resulted in a premature rise in plasma testosterone (T) and E_2, and promoted egg development relative to untreated female Atlantic salmon (Crim et al., 1986). The salmon in the two latter studies were vitellogenic at the time of Gnrh-treatment, which indicates pituitary responsiveness to Gnrh during the earlier stages of reproductive development in terms of Fsh production. In a more recent study on Atlantic salmon, treatment with Gnrh resulted in an increase

in plasma E_2 prior to an increase in plasma T (King and Pankhurst, 2007). This suggests that in addition to stimulating the Lh-mediated increase in maturational steroids late in oocyte development, Gnrh may also elevate plasma T and E_2 levels earlier in development, either through the action of Fsh or Lh.

Little information is available on the effects of prolonged Gnrh-treatment on the endocrine system and oocyte development during vitellogenesis. However, in non-salmonids, Gnrh is capable of stimulating the complete cycle of oocyte development, growth and maturation (Morehead et al., 1998). It is also unclear as to whether maiden and repeat spawning broodstock will respond in the same way to hormonal therapy under thermal challenge given their differential response to elevated temperature in terms of egg quality. Gnrh is able to induce the synthesis and subsequent release of Fsh, and promote E_2 production and egg development during vitellogenesis in several species. Therefore, it is possible that treatment with Gnrha will maintain endocrine function in female Atlantic salmon broodstock, provided that pituitary and/or gonadal responsiveness is maintained at elevated temperatures. As such, the aim of the present study was to investigate whether prolonged Gnrha therapy during reproductive development could offset the inhibitory effects of thermal challenge in terms of endocrine function, egg fertility and embryo survival in maiden and repeat spawning Atlantic salmon broodstock. To determine the effects of Gnrha on maiden and repeat spawning fish at the cellular and molecular level, plasma levels of pituitary hormones, gonadal steroids and Vtg, as well as E_2-dependent hepatic gene expression of *vtg*, *zpb* and *zpc* were measured throughout reproductive development.

6.3 Methods

6.3.1 Fish husbandry and maintenance

Maiden and repeat spawning adult females (with an mean ± SE body weight of 4.01 ± 0.07 and 7.03 ± 0.15 kg respectively) from the SALTAS spawning stock were held in 200 (maidens) or 50 (repeats) m^3 circular tanks at ambient photoperiod and

temperature under standard conditions of husbandry at the SALTAS Wayatinah hatchery until January 2009. In mid-January, fish were transferred to replicate temperature-controlled 4 m^3 (2 x 2 x 1 m) Rathbun tanks (14 fish per tank) under simulated ambient photoperiod according to 6 treatment groups (see below). Fourteen and 22 °C represent typical cool, and warm Tasmanian summers respectively. Fish were not fed from the time of transfer to the temperature controlled systems in January consistent with hatchery practice for management of this experimental stock of fish. All fish were maintained at the nominated temperature (14 or 22 °C) until late March when all fish were exposed to a gradual temperature ramp down to 8 °C to induce final oocyte maturation and ovulation as in King and Pankhurst (2000). Temperature profiles for the two temperature regimes are shown in Fig. 6.0.

Treatment groups:

(1) Maiden 14 °C

(2) Repeat 14 °C

(3) Maiden 22 °C

(4) Maiden Gnrh implant 22 °C

(5) Repeat 22 °C

(6) Repeat Gnrh implant 22 °C

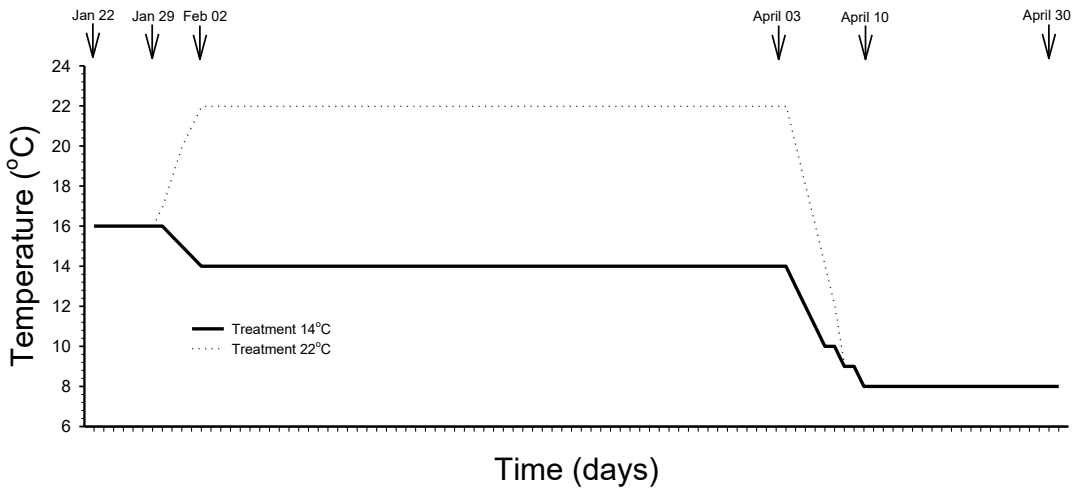

Figure 6.0 Thermal adjustment and hormone implantation timeline for untreated and Gnrha-treated maiden and repeat spawning female Atlantic salmon broodstock maintained at 14 or 22 °C during vitellogenesis.

6.3.2 Sampling protocol

Maiden and repeat spawning fish reared at ambient temperature were sampled in late October 2008 to establish an early-vitellogenic reference point for each group (Sample 1), and on the 22nd January 2009 at the same time that temperature transfer to 14 or 22 °C occurred for the remaining fish (Sample 2). Subsequent samples were taken from all 6 experimental groups during February 25-27 (Sample 3), March 26-27 (Sample 4), and April 15-16 (Sample 5). Six to 7 fish were sampled from each group at each sample time (subject to the availability of stock), leaving 7 fish from each treatment to proceed through to ovulation and stripping, after the final destructive Sample in April. Fish from treatment groups 4 (maidens at 22 °C) and 6 (repeats at 22 °C) were implanted (abdominal) with an Ovaplant™ (Syndel, Canada) cholesterol pellet containing 37 µg of the D-Arg^{6},Pro^{9}NEt analogue of salmon Gnrh on 22nd January (during temperature transfer), and again over February 26-27 to give repeat doses of approximately 7-12 µg.kg^{-1}. The dose chosen was designed to stimulate or maintain pituitary secretion of Fsh, but remain below the threshold likely to stimulate an Lh surge and possible premature stimulation of oocyte maturation and ovulation (King and Pankhurst, 2004a).

At each sample point, fish were caught from both replicate tanks (approximately half from each tank) then humanely sacrificed as described in Chapter 4 (Pankhurst et al., 2011). Blood was collected and centrifuged at 12000 ×g for 5 min at room temperature as described in Chapter 4 (Pankhurst et al., 2011). Tissues were then harvested, and morphometric data gathered according to procedure outlined in Chapter 4 (Pankhurst et al., 2011). For fish that were left to ovulate, ova were stripped and fertilised as described in King et al. (2003) for measurement of egg size, fertility and survival to the eyed stage at 250 degree-days of incubation. All animal experiments were conducted in accordance with Australian law under ethical approval issued by Griffith University and University of the Sunshine Coast Animal Ethics Committees (ENV/25/08AEC and AN/A/09/44 respectively).

6.3.3 Plasma hormone and vitellogenin measurement

Plasma Fsh (all sampling points) and Lh (March and April samples only) measurements were performed using an RIA developed for coho salmon by Swanson et al. (1989) with some modifications according to Chapter 5 (Anderson et al., 2012).

Plasma levels of E_2, T and cortisol were determined by radioimmunoassay using the reagents and procedure for E_2 and T described in Pankhurst and Carragher (1992), and for cortisol as in Pankhurst et al. (2008). Further details for these assays are available in Chapter 4 (Pankhurst et al., 2011).

Plasma Vtg levels were measured by enzyme linked immunosorbent assay using the reagents and protocol as described in Watts et al. (2003). Further details for this assay are available in Chapter 4 (Pankhurst et al., 2011).

6.3.4 RNA extraction, cDNA synthesis and qPCRs

Total hepatic RNA was isolated and used to synthesis cDNA using the procedure outlined in Chapter 4 (Pankhurst et al., 2011). In addition, RNA quality was determined

using the method described in Chapter 5 (Anderson et al., 2012). qPCRs were conducted using the cycler, cycling conditions, reaction components and controls outlined in Chapter 4 (Pankhurst et al., 2011). Gene specific primers (GSPs) detailed in Chapter 4 (Pankhurst et al., 2011) were used to amplify hepatic target (*vtg*, *zpc*, *zpb*) and candidate reference gene (*tata binding protein* (*tbp*), *hypoxanthine phosphoribosyltransferase 1* (*hprt1*), *beta-tubulin* (β-tubulin) and *elongation factor 1 alpha* (*ef1α*) transcripts. The suitability of using Tbp, Hprt1 and Ef1α as reference genes for normalisation was assessed using the method outlined in Chapter 3 (Anderson and Elizur 2012). As a result of this analysis (data not shown), expression data from *tbp* was used for accurate target gene normalisation using the Rest© software 2008 package, V2.0.7 (Pfaffl et al., 2002). This package was also used to calculate the level of target gene expression relative to a calibrator sample analysed in every run, and adjust the level of target gene expression to account for inter-assay variability which was negligible (the %CV was extremely low: < 0.85% for all qPCR assays).

6.3.5 Statistical analysis

Morphometric, plasma hormone and gene expression data was statistically analysed as in Chapter 4 (Pankhurst et al., 2011).

6.4 Results

6.4.1 Morphometric data

The mean (± SE) body weight of maiden spawners (4.01 ± 0.07 kg) was consistently lower than that of repeat spawners (7.03 ± 0.15 kg) during the course of the experiment (data not shown). CF was lower in repeat than maiden spawning fish at Sample 1; however, CF was not different among fish from all groups thereafter (data not shown). There was a general trend of increasing GSI and follicle diameter with sample time across all treatment groups (Fig. 6.1). The GSI of fish from all treatments was similar

after introduction to the temperature-controlled tanks and hormonal treatment; however, follicle diameter was lower in all fish reared at 22 °C relative to their respective 14 °C control at Sample 5, and did not change as a result of treatment with Gnrh (Fig. 6.1). Total fecundity (during development) was highest in repeat spawners but there was no consistent difference in relative fecundity between repeat and maiden spawning fish, and no discernible effect of temperature regime or hormonal treatment (Fig. 6.2).

6.4.2 Ovulation, egg fertility and embryo survival

Fish from group 1 (maidens at 14 °C) ovulated first, followed by a cluster of group 3, group 4 (untreated and treated maidens at 22 °C respectively), group 5 (repeats at 22 °C), and then group 2 (repeats at 14 °C) (Fig. 6.3). Group 6 (repeats at 22 °C treated with Gnrh) showed markedly delayed ovulation relative to all other groups. Fecundity and relative fecundity at ovulation was unaffected by temperature or hormonal treatment (Fig. 6.4). At 22 °C, repeat spawning fish had slightly higher absolute, but not relative fecundity. Maiden and repeat spawning fish at 22 °C had markedly smaller post-ovulatory egg diameters and volumes (Fig. 6.4) than their respective controls at 14 °C. Egg size was smaller in maiden spawning fish than in repeat spawning fish at 14 °C, although egg size was similar between maiden and repeat spawning fish at 22 °C. Fertility and eyed egg survival was higher in fish reared at 14 °C than at 22 °C; however, the reduction in embryo survival was less marked in repeat than maiden spawning fish compared to the respective 14 °C control group (Fig. 6.4). Treatment with Gnrh did not significantly affect egg fertility or survival in maiden or repeat spawning fish relative to the appropriate control at 22 °C.

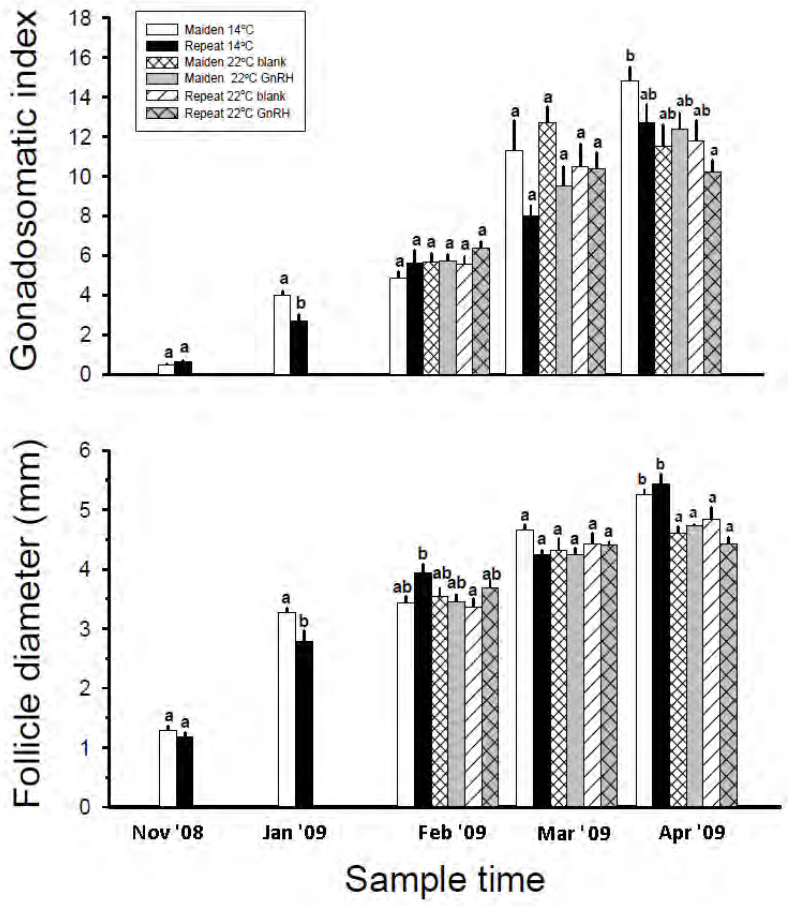

Figure 6.1 Mean + SEM (n = 7) gonadosomatic index and follicle diameter of maiden and repeat spawners. Sample 1 and 2: fish were exposed to ambient photoperiod and temperature. Samples 3-4: fish sampled that were exposed to either 14 °C or 22 °C and implanted with a Gnrha or blank pellet. Fish received pellet implants at Samples times 2 and 3. The temperature was ramped down to 8 °C for all groups between Samples 4 and 5. Different superscripts among sample times denote significant differences ($p \leq 0.05$).

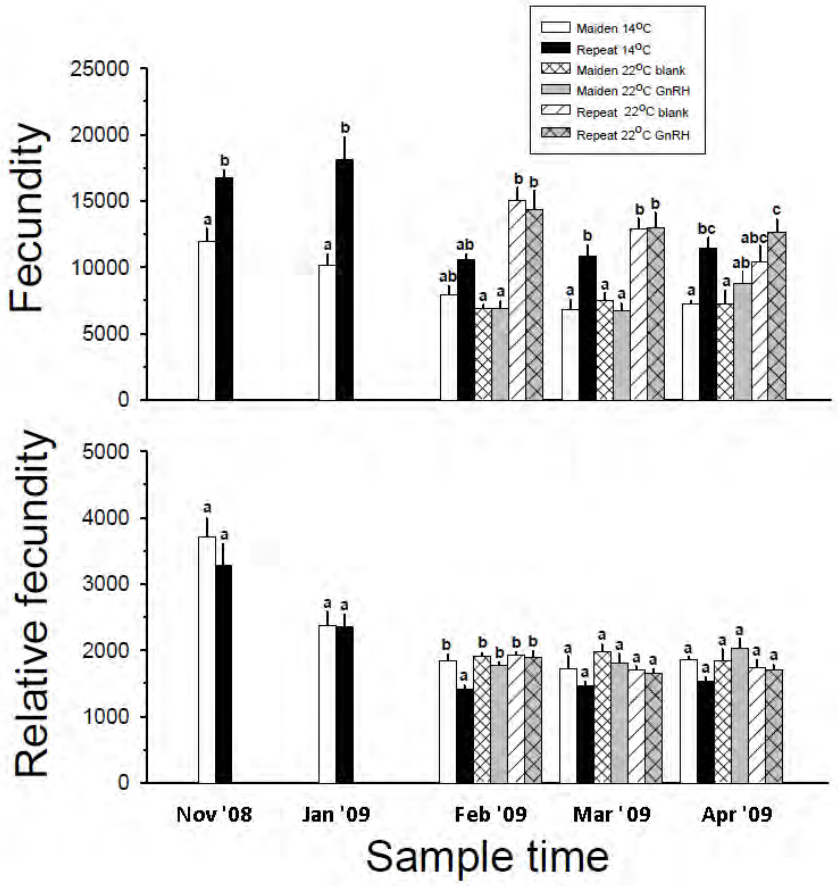

Figure 6.2 Mean absolute fecundity and relative fecundity of maiden and repeat spawners. Other details as for Fig. 6.1.

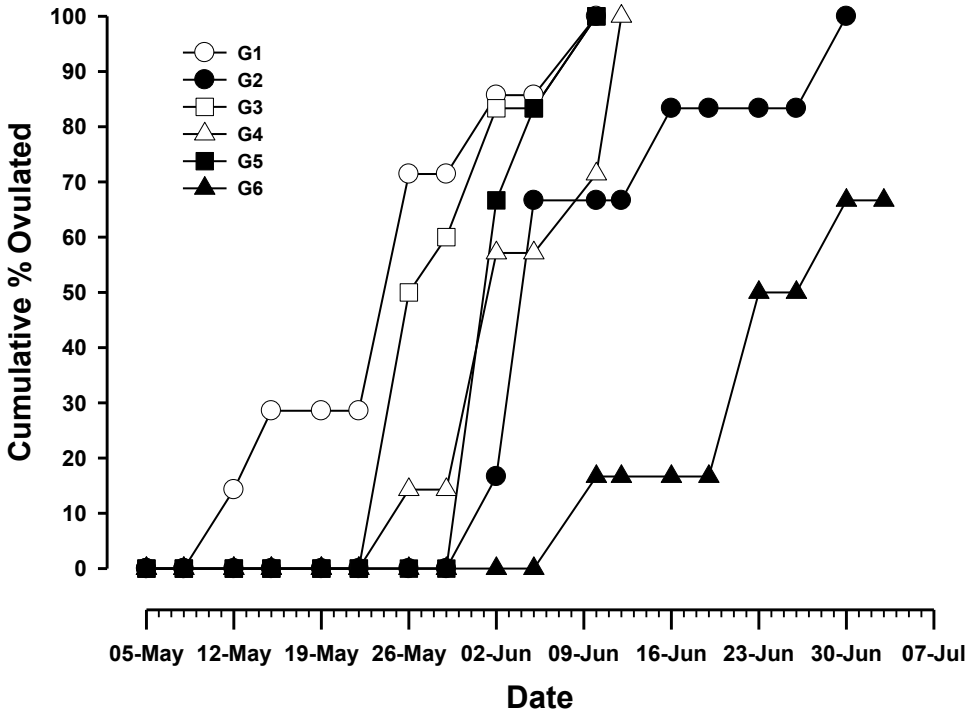

Figure 6.3 Cumulative ovulation of maiden and repeat spawners. G1 (Group 1) – maidens at 14 °C; G2 – repeats at 14 °C; G3- maidens at 22 °C, G4- maidens at 22 °C treated with Gnrh; G5 – repeats at 22 °C, and G6- repeats at 22 °C treated with Gnrh.

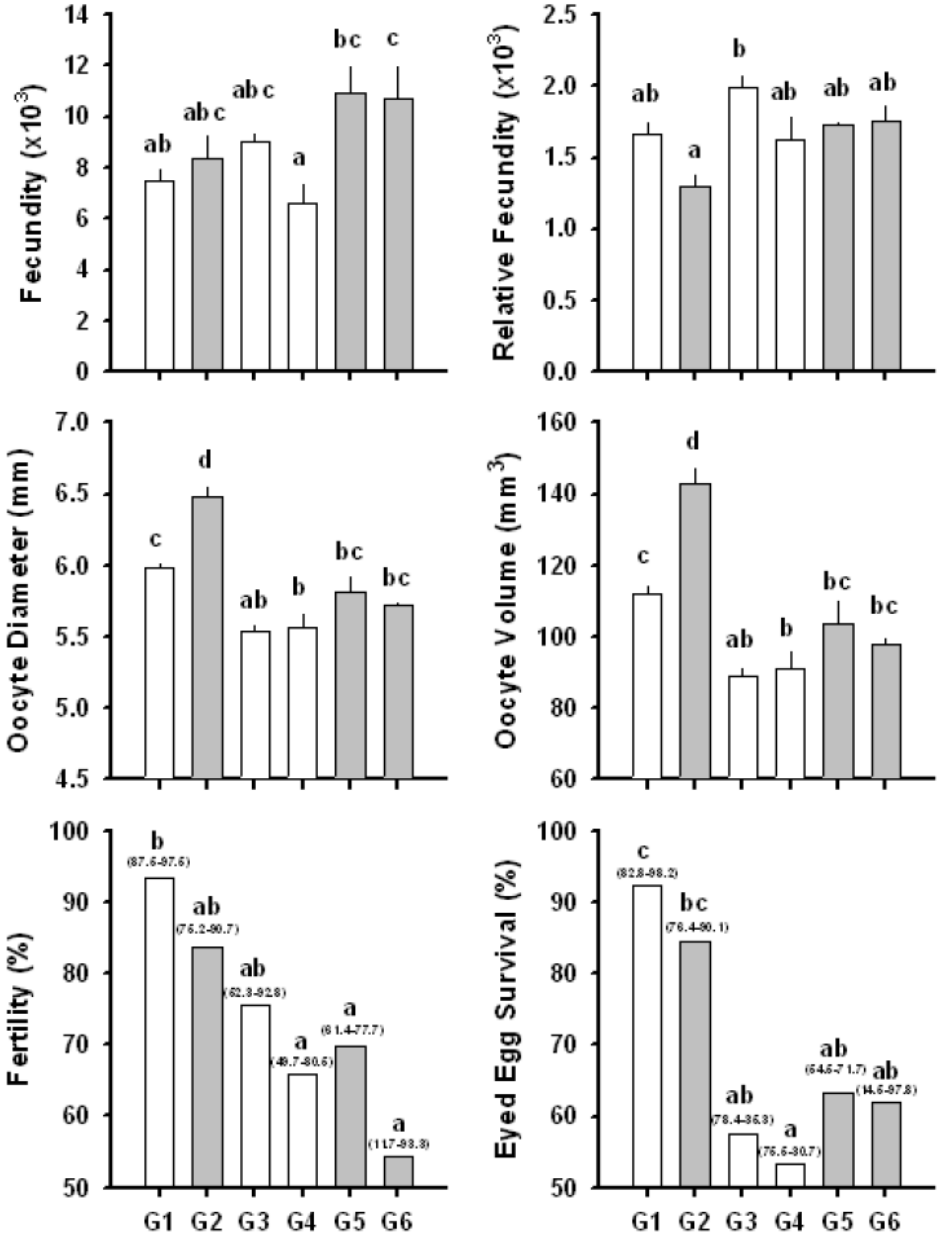

Figure 6.4 Fecundity, relative fecundity, egg size (at time of stripping), fertility and survival (to the eyed stage) for maiden and repeat spawners exposed to 14 or 22 °C with or without Gnrh implantation. G1 (Group 1) – maidens at 14 °C; G2 – repeats at 14 °C; G3- maidens at 22 °C, G4- maidens at 22 °C treated with Gnrh; G5 – repeats at 22 °C, and G6- repeats at 22 °C treated with Gnrh. For fertility and egg survival the numbers above each bar represent the 95% confidence interval. Different superscripts denote significant differences ($p \leq 0.05$).

6.4.3 Plasma hormones and Vtg

There was no difference in the level of plasma Fsh between groups of fish at Sample 1, 2 or 3 (Fig. 6). At Sample 4, plasma Fsh was elevated in treated and untreated maiden fish reared at 22 °C relative to the untreated group maintained at 14 °C, and Fsh levels were similar among both maiden groups at 22 °C. There was no significant difference among plasma Fsh levels in repeat fish at Sample time 4. There was no significant difference in plasma Fsh levels between any groups of fish at Sample 5. There were no differences in the mean (±SEM) plasma Lh levels between any groups of fish at Sample 4 (0.91 ± 0.08 $ng.ml^{-1}$) or 5 (0.85 ± 0.037 $ng.ml^{-1}$) (data not shown).

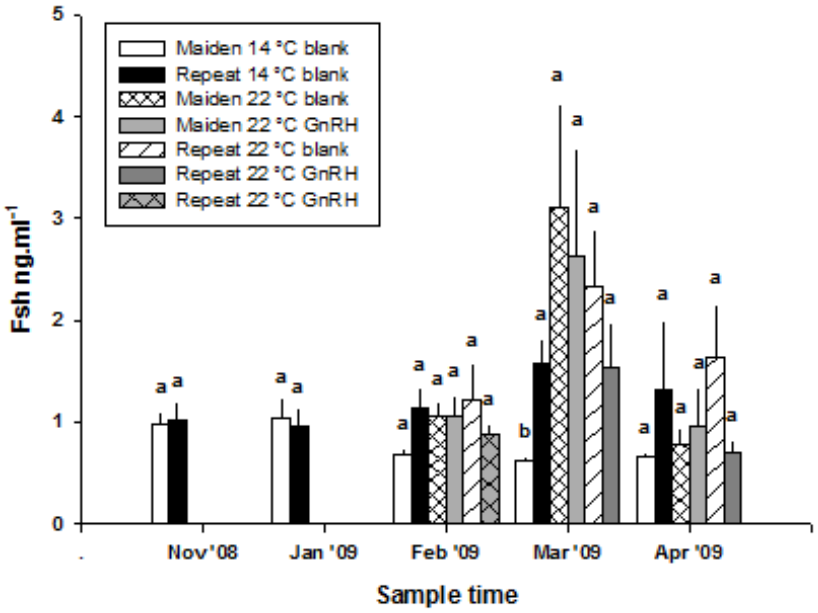

Figure 6.5 Mean plasma Fsh levels in maiden and repeat spawners exposed to 14 or 22 °C with or without Gnrh implantation. Fsh levels below the lower limit of assay detection are displayed as 0.6 $ng.ml^{-1}$. Other details as for Fig. 6.1.

Plasma E_2 levels were suppressed at Sample time 3 in both groups of maiden spawning, but not repeat spawning fish reared at 22 °C relative to their respective control at 14 °C (Fig. 6.6). At Sample 4, plasma E_2 levels in maiden spawning fish were suppressed in both groups at 22 °C and there was also suppression at 22 °C in repeat spawning fish treated with Gnrh. At Sample 5, all 22 °C groups were suppressed relative to maiden spawning fish held at 14 °C. T levels were not different between groups at Sample time 1 and 2, and repeat spawning fish treated with Gnrh had higher T levels than the corresponding control group at 22 °C at Sample 3 (Fig. 6.6). Plasma T levels were suppressed at 22 °C in maidens treated with Gnrh at Samples 4 and 5 relative to maidens at 14 °C, and were suppressed in both groups of repeat spawning fish held at 22 °C at Sample 5 relative to the control group at 14 °C. Mean (± SEM) plasma cortisol levels were elevated in maiden (15.7 ± 2.0 $ng.ml^{-1}$) relative to repeat (3.3 ± 1.2 $ng.ml^{-1}$) spawning fish at Sample 1, but there were no differences among groups at Sample 2, 3 or 4 (data not shown). At Sample 5, cortisol was elevated in repeat spawning fish at 14 °C (23.1 ± 6.4 $ng.ml^{-1}$) relative to all other groups (range 2.4 - 8.8 $ng.ml^{-1}$).

Plasma Vtg levels increased through reproductive development and were similar among groups until Sample 3 when levels were suppressed in maidens at 22 °C relative to 14 °C (Fig. 6.7). At Sample 3, suppression of plasma Vtg was also observed for repeat spawning fish treated with Gnrh, and to some extent the untreated 22 °C group relative to the corresponding control at 14 °C. There was some recovery in plasma Vtg levels at Sample 4, with only group 6 (repeat spawning fish treated with Gnrh) showing significant suppression at 22 °C. At Sample 5, there were no differences between fish at 14 and 22 °C for either age class; although, maiden spawning fish had higher levels of plasma Vtg than repeat spawning fish at 14 °C.

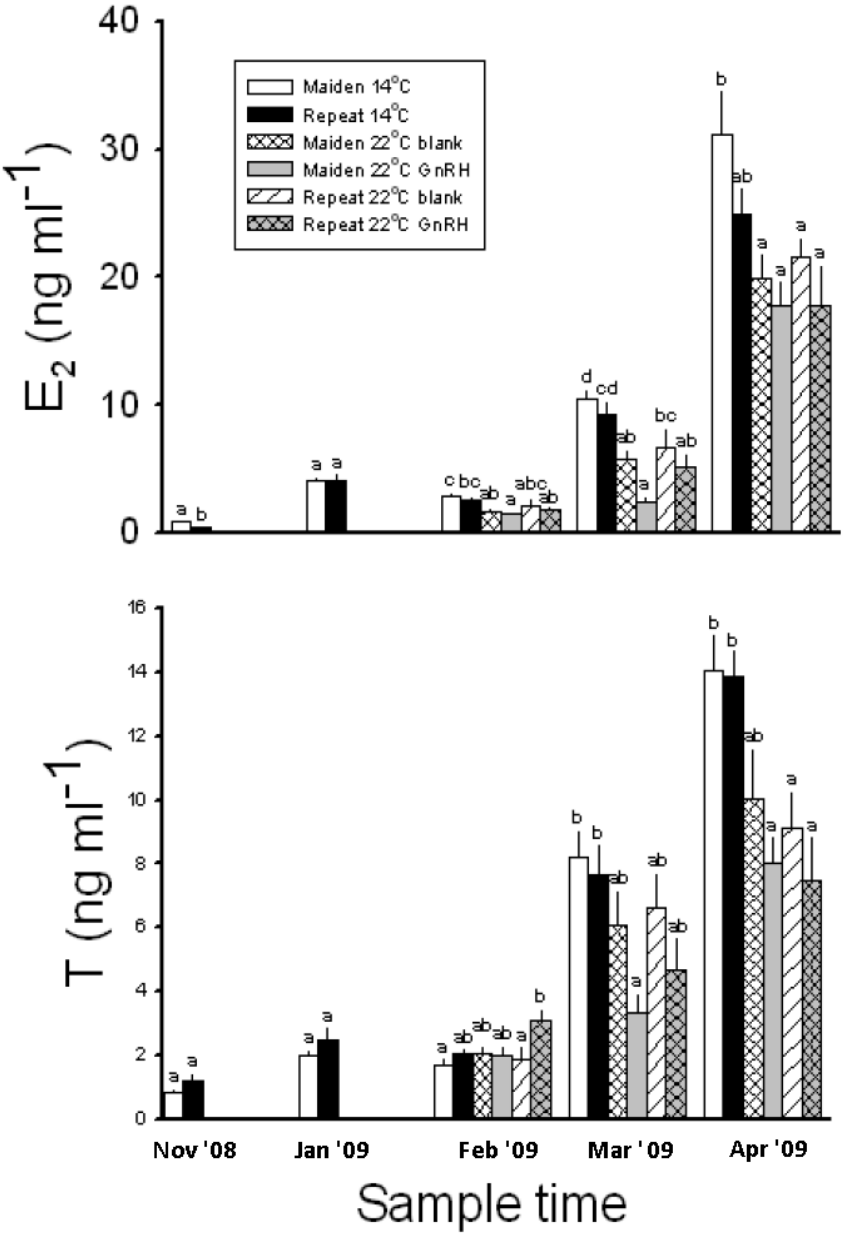

Figure 6.6 Mean plasma 17β-estradiol (E_2) and testosterone (T) levels of maiden and repeat spawners. Other details as for Fig. 6.1.

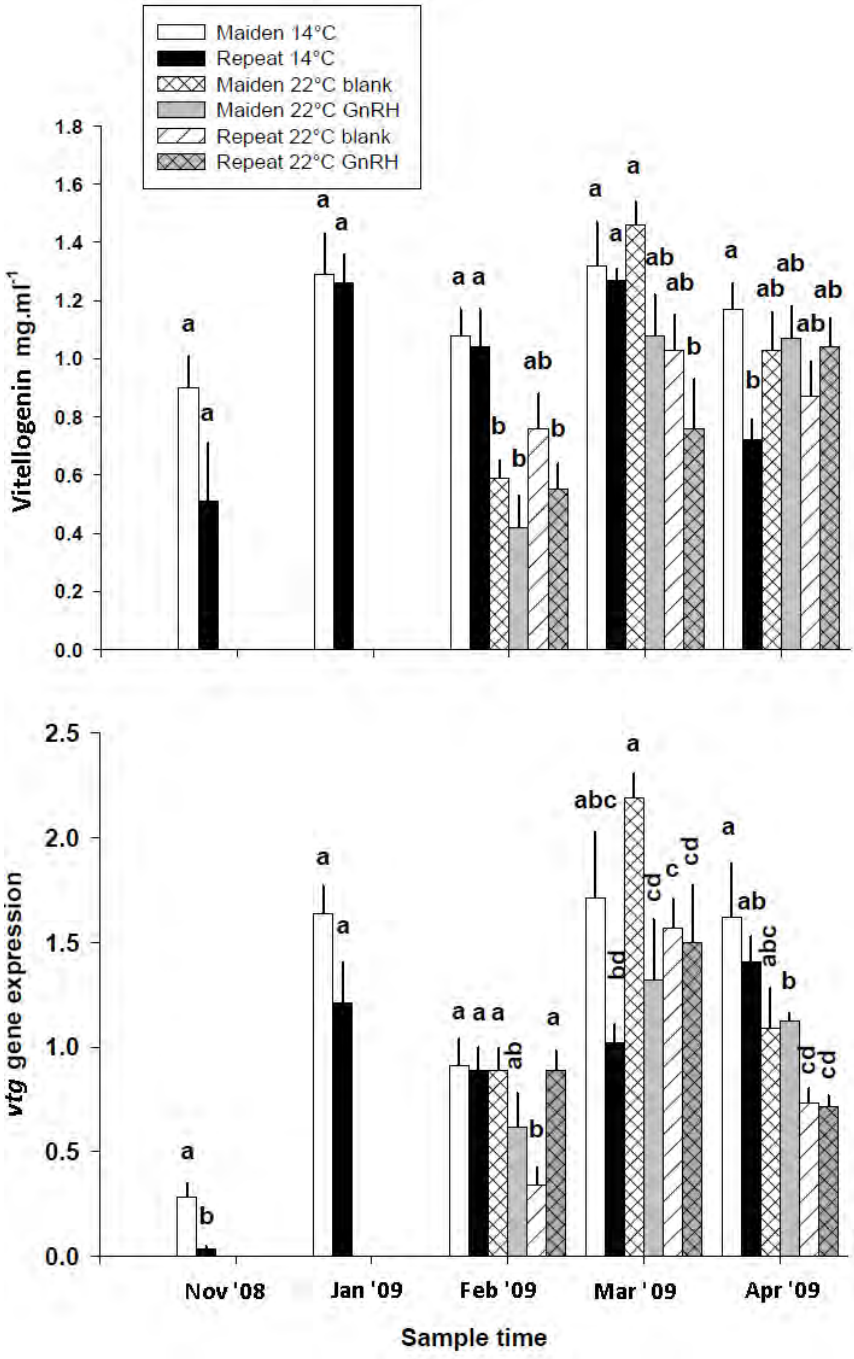

Figure 6.7 Mean plasma *vitellogenin* (Vtg) levels, and hepatic *vtg* gene expression levels of maiden and repeat spawners exposed to 14 or 22 °C with or without Gnrh implantation. Gene expression was normalised to *tata binding protein*. Other details as for Fig. 6.1.

6.4.4 Hepatic gene expression

Hepatic levels of relative *vtg* gene expression increased markedly from Sample 1 to 2 (November and January Samples), and in February (Sample 3), *vtg* expression was suppressed in repeat spawning fish at 22 °C but this effect appeared to be offset by treatment with Gnrh (Fig. 6.7). In maidens, *vtg* gene expression was unaffected by maintenance at 22 °C or treatment with Gnrh at Sample 3. At Sample 4 (March), *vtg* expression was suppressed in Gnrh-treated maiden fish at 22 °C, compared to the 22 °C maiden control group, but not maidens at 14 °C. There were no differences among repeat spawning Gnrh-treated and control fish held at 22 °C. At Sample 5 (April), maiden spawning fish held at 22 °C and treated with Gnrh showed suppression relative to maidens at 14 °C, and both groups of repeats held at 22 °C showed suppressed expression relative to repeat spawning fish at 14 °C.

zpb gene expression was down-regulated in repeat relative to maiden spawning fish at Sample 1; although there was no difference in gene expression by Sample 2 between groups (Fig. 6.8). In February (Sample 3), *zpb* expression was significantly reduced in maiden spawning fish at 22 °C receiving Gnrh implantation relative to maidens at 14 °C, and both groups of repeat spawning fish at 22 °C showed suppression relative to repeat spawners at 14 °C. At Sample 4 (March), *zpb* expression was suppressed in both groups of maidens spawning fish held at 22 °C, and repeats spawning fish at 22 °C treated with Gnrh. At Sample 5 (April), there were no significant differences between treatments even though there was a general trend towards lower gene expression at elevated temperature. At Sample 1 and 2, the level of *zpc* gene expression was not different between maiden and repeat spawning fish (Fig. 6.8). At Sample 3, *zpc* expression was higher in repeat than maiden spawning fish at 14 °C, and gene expression was higher in maiden fish at 22 °C than at 14 °C. There was suppressed *zpc* expression in maidens held at 22 °C treated with Gnrh, relative to maidens at 14 °C at Sample 4. In April (Sample 5), the same effect was present. There were no significant

differences among fish held at 22 °C at Sample 5; however, there was a general trend towards lower gene expression levels at elevated temperature.

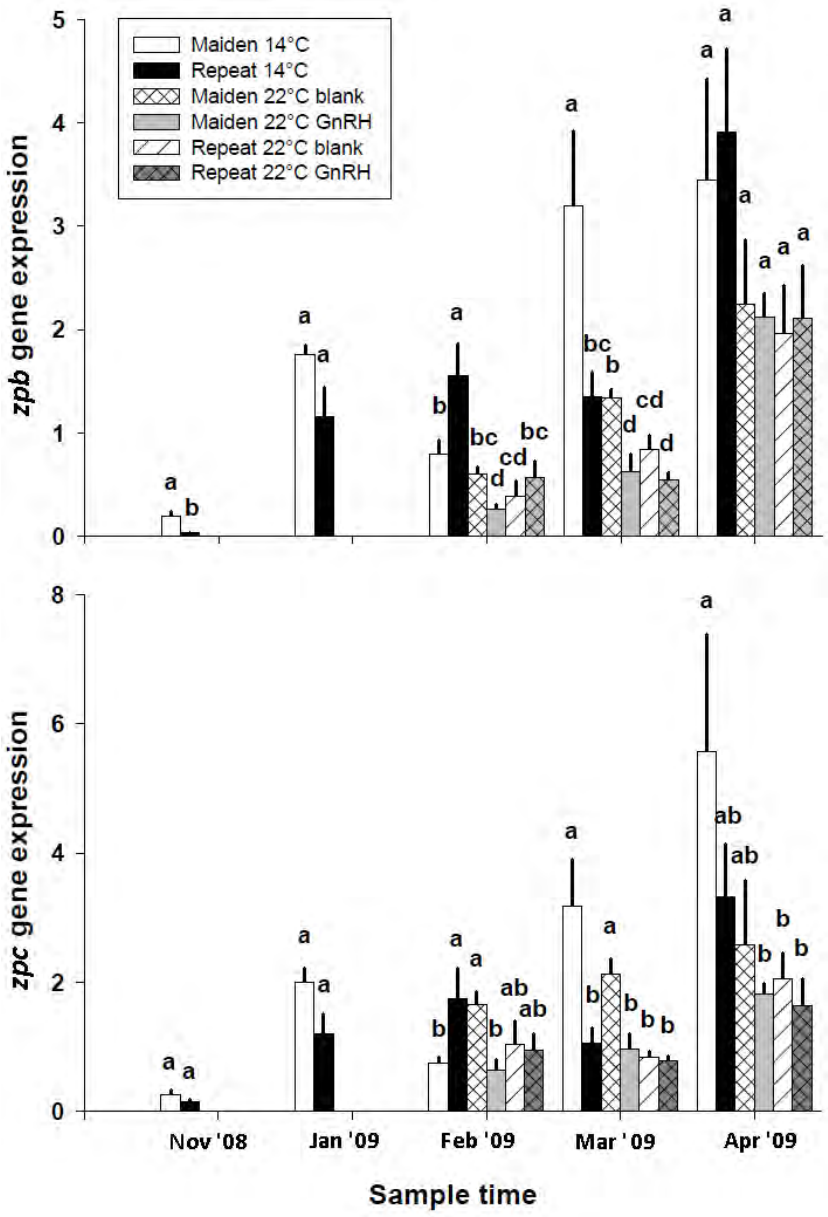

Figure 6.8 Mean hepatic *zona pellucida b* and *c* (*zpb* and *zpc* respectively) gene expression levels of maiden and repeat spawners exposed to 14 or 22 °C with or without Gnrh implantation. Gene expression was normalised to *tata binding protein*. Other details as for Fig. 6.1.

6.5 Discussion

Maintenance at 22 °C with or without treatment with Gnrh did not affect body weight relative to fish reared at 14 °C. CF was initially lower in repeat spawning fish at Sample 1 (November); however, CF was very similar between all groups from Sample 2 (January) onwards. The variation in CF during early reproductive development appears to be a consistent feature for this population of female Atlantic salmon. We recently reported the same age class effect, and suggested that the initial variation in CF is a reflection of the increased energy demand associated with recovery from the previous reproductive cycle for repeat spawning fish (Ch 4: Pankhurst et al., 2011). As a consequence, plasma levels of E_2, and hepatic gene expression levels of *vtg* and *zpb* were suppressed in repeat spawning fish relative to maidens spawning fish at Sample 1 in the present study, but repeat spawning fish had caught up in terms of plasma E_2 and hepatic gene expression by Sample 2 as CF increased. The onset of ovulation was delayed by 21 days in repeat spawning fish relative to maiden spawning fish reared at 14 °C, and this is consistent with the slower rate of development in repeat fish relative to maiden spawning fish reared at 14 °C during early vitellogenesis.

In maidens, treatment with Gnrh did not affect the timing of ovulation relative to the control group reared at 22 °C. In contrast, ovulation was delayed in repeat spawning fish treated with Gnrh relative to the control group at 22 °C; however, this was not associated with a further reduction in egg fertility and embryo survival. Delays in ovulation at high temperature in salmonids are thought to be caused by dopamine mediated inhibition of Lh secretion (Gillet et al., 1996), in combination with lower circulating levels of *maturation inducing hormone* 17,20β-dihydroxy-4-pregnen-3-one (17,20βp) prior to ovulation (King and Pankhurst, 2004b). In the present study, there was no difference in basal Lh plasma concentration as a result of exposure to 22 °C at least one month (depending on group) prior to spawning which is consistent with our previous work on Atlantic salmon maintained at elevated temperature (Ch 5: Anderson et al., 2012). Treatment with Gnrh also had no effect on plasma Lh levels; however, due to the timing of sampling relative to the time of ovulation, the sampling schedule was

unlikely to have detected the preovulatory rise in plasma Lh associated with FOM. Therefore, the cellular basis for delayed ovulation in Gnrh-treated fish remains unclear, but it is possibly due to a combination of reduced Lh release, reduced follicular responsiveness to Lh and reduced oocyte sensitivity to 17,20βp of the type described for Arctic charr (*Salvelinus alpinus*) (Gillet et al., 2011). Furthermore, it is unlikely that Gnrh implantation directly affected the events associated with FOM and ovulation, as the Ovaplant™ pellet has a sustained release profile of approximately 24 days total at 10 °C, and the second implantation occurred approximately 3 months before ovulation commenced in untreated repeat spawning fish at 22 °C.

Exposure to elevated temperature alone or in combination with prolonged Gnrh-treatment did not affect GSI, relative fecundity or fecundity for maiden or repeat spawning fish during oocyte development or at ovulation. Gnrh-treatment also did not affect follicle diameter during vitellogenesis, and at Sample 5 (April) all Gnrh-treated and untreated groups maintained at 22 °C displayed oocyte diameters that were smaller than those from fish maintained at 14 °C. Uptake of plasma Vtg into the developing oocyte results in an increase in oocyte size, and the products of vitellogenesis make up a significant proportion of the egg's volume in salmonids (reviewed in Brooks et al., 1997). Plasma Vtg levels were lower in Gnrh-treated maiden and repeat spawning fish maintained at 22 °C at Sample 3, and repeat spawning fish only at Sample 4, relative to the corresponding control group at 14 °C. Since treatment with Gnrh failed to stimulate the production of Vtg at 22 °C in maiden and repeat spawning fish, Vtg uptake by oocytes was presumably reduced and follicle diameter consequently smaller in Gnrh-treated fish compared to the respective control group at 14 °C. At the time of stripping, all maiden and repeat spawners reared at 22 °C with or without hormonal treatment had lower egg diameters and volumes compared to the corresponding control group at 14 °C, which is consistent with the smaller follicle diameters observed for these groups during late vitellogenesis. Egg fertility and survival was generally reduced in maiden and repeat spawning fish at 22 °C relative to the respective control at 14 °C, and consistent with our previous work, repeat spawning fish appeared to be slightly more resilient to

the effects of thermal challenge (Ch 4: Pankhurst et al., 2011). Gnrh therapy during vitellogenesis did not help to maintain egg quality in maiden or repeat spawning fish reared at 22 °C, with the lowest fertility and eyed egg survival rates recorded for Gnrh-treated fish.

In the present study, Gnrh-therapy during vitellogenesis failed to elevate plasma Fsh levels in maiden and repeat spawning fish maintained at 22 °C above those recorded for the corresponding control group at 22 °C. In salmonids, Gnrh-treatment is able to stimulate Fsh production and secretion in primary pituitary cell cultures (Dickey and Swanson, 2000), although pituitary responsiveness to Gnrh stimulation is dependent on the stage of reproductive development (Ando et al., 2004). Since treatment with Gnrh did not affect plasma Fsh levels at 22 °C in the present study, it is possible that Gnrh signal transduction may have been impaired as a result of thermal challenge. In red seabream (*Pagrus major*), maintenance at 24 °C caused a reduction in pituitary *gonadotropin-releasing hormone receptor* (*gnrhr*) gene expression levels relative to fish reared at 17 °C for 10 days (Okuzawa et al., 2003). The up-regulation of *gnrhr* in response to Gnrh-treatment has been shown *in vivo* in masu salmon (*Oncorhynchus masou*) (Jodo et al., 2005), and *in vitro* in hybrid tilapia (*Oreochromis aureus* x *Oreochromis niloticus*) (Levavi-Sivan et al., 2004). Therefore the expected stimulatory effect of Gnrh-treatment on *gnrhr* gene expression may have been offset by the inhibitory effects of elevated temperature in the present study.

In rainbow trout, treatment with a dopamine agonist inhibited the (Gnrh-induced) release of Fsh from the pituitary during late vitellogenesis (Vacher et al., 2000), and in Arctic charr, it is thought that dopamine is involved in the thermal inhibition of ovulation (Gillet and Breton, 2009). In the latter study, treatment with Gnrh in combination with a dopamine antagonist was more effective at inducing ovulation than treatment with Gnrh alone at high temperature (Gillet and Breton, 2009). Therefore, it is possible that Gnrh-treatment during vitellogenesis could be more effective at 22 °C if administered in combination with a dopamine antagonist. However, it should be noted that plasma Fsh levels were unchanged as a result of thermal challenge in untreated

repeat spawning fish, and in maiden spawning fish, plasma Fsh levels where higher at 22 °C relative to the control group at 14 °C. Sex steroids such as E_2 are known regulators of pituitary hormones (reviewed in Pankhurst, 2008); for example in coho salmon, E_2 has been shown to down-regulate pituitary *fshβ* gene expression during secondary oocyte growth (Dickey and Swanson, 1998). In the present study, plasma E_2 was significantly suppressed in maiden but not repeat spawning fish maintained at 22 °C at Samples 3 and 4. Therefore a reduction in negative feedback by E_2 on the pituitary may have resulted in an increase in plasma Fsh in maidens maintained at 22 °C as suggested previously for this stock of Atlantic salmon (Ch 5: Anderson et al., 2012).

There was no evidence to suggest that treatment with Gnrh at 22 °C compensated for the negative effects of elevated temperature on the production of T in the present study, other than at Sample 3 in repeat spawning fish when plasma T was higher in Gnrh-treated fish than in the corresponding control group at 22 °C. The stimulatory effect of Gnrh on plasma T for repeat spawning fish at that Sample did not correspond to an increase in plasma E_2 for the same or following months. In a previous study on Atlantic salmon, treatment with Gnrh increased plasma T levels relative to sham treated fish during vitellogenic development (Crim et al., 1986). This implies that any stimulatory effect of Gnrh-treatment achieved in the present study was not significant enough to compensate for impairment of endocrine function at 22 °C. Fsh is known to play a role in regulating the expression of steroidogenic enzymes in mammals and coho salmon (reviewed in Payne and Hales, 2004, Luckenbach et al., 2011), and can increase the gonadal production of T. Exposure to elevated temperature resulted in the down-regulation of ovarian *follicle stimulating hormone receptor* (*fshr*) in Japanese flounder (*Paralichthys olivaceus*) during sexual differentiation (Yamaguchi et al., 2007), and adult female pejerrey (*Odontesthes bonariensis*) during vitellogenesis (Soria et al., 2008). Additionally, we recently demonstrated that *cholesterol side chain cleavage protein* (*cyp11a1*) gene expression was impaired in adult female Atlantic salmon reared at 22 compared to 14 °C during vitellogenesis (Ch 5: Anderson et al., 2012). Since plasma Fsh was not suppressed in fish reared at 22 °C in the present study, it is possible

that the aforementioned mechanisms of thermal impairment may have operated here, which counteracted the action of Fsh and subsequently impaired the production of T.

In the present study, plasma E_2 levels were generally lower in maiden and repeat spawning fish treated with Gnrh at 22 °C compared to the corresponding control group at 14 °C. This is presumably due to the inability of Gnrh to stimulate the production of T at 22 °C. In addition, we recently demonstrated that *cyp19a1a* gene expression was significantly reduced in maiden and repeat spawning adult female Atlantic salmon maintained at 22 °C relative to 14 °C (Ch 5: Anderson et al., 2012). It is likely that impaired *cyp19a1a* gene expression contributed to the suppression of plasma E_2 levels in our previous study, and it is therefore likely that the same mechanism was operating here in combination with thermal impairment of steroidogenesis upstream of T. In pink salmon (*Oncorhynchus gorbuscha*), prolonged treatment with Gnrh effectively elevated plasma E_2 levels during reproductive development only when administrated in combination with T in fish reared at between 8.9-12.3 °C (Crossin et al., 2010). However, co-treatment of fish with Gnrh and T may not promote vitellogenic development at higher temperatures in Atlantic salmon if there is thermal inhibition at the level of gonadal *cyp19a1a*.

Plasma cortisol was elevated in maiden relative to repeat spawning fish at Sample 1, which is likely to be a reflection of the crowding method used to capture this group of fish early in the experiment (Ch 4: Pankhurst et al., 2011). For Samples 2, 3 and 4 plasma cortisol levels in all fish were lower than those typically observed in stressed Atlantic salmon (Thomas et al., 1999) which indicates that treatment with Gnrh and exposure to high temperature was not perceived as stressful, and the endocrine profiles obtained were a result of experimental manipulation and not stress. At Sample 5, plasma cortisol was elevated in repeat spawning fish reared at 14 °C relative to all other groups. However, the mean (± SEM) concentration of plasma cortisol in repeat spawning fish maintained at 14 °C (23.14 ± 6.4 $ng.ml^{-1}$) was similar to the level observed in unstressed Atlantic salmon and rainbow trout (~15 $ng.ml^{-1}$ for each study) (Thomas et al., 1999, Pankhurst and Van Der Kraak, 2000), and it is therefore unlikely that the

cortisol level in repeat spawning fish was high enough to significantly impact reproductive development in the present study.

Treatment with Gnrh at 22 °C had no effect on *vtg* gene expression in maiden or repeat spawning fish, except at Sample 4 where *vtg* gene expression was down-regulated in maiden spawning fish relative to the corresponding control group at 22 °C, and at Sample 3 where *vtg* gene expression was up-regulated in repeat spawning fish after Gnrh-treatment. This is the only case where treatment with Gnrh appeared to offset the negative effects of thermal inhibition on the endocrine system at the level of the liver. However, the stimulatory effect of Gnrh-treatment observed for repeat spawning fish at Sample 3 did not cause a subsequent increase in plasma Vtg. Treatment with Gnrh at 22 °C had no effect on plasma levels of Vtg in maiden and repeat spawning female Atlantic salmon; except at Sample 4 when plasma Vtg was significantly suppressed in Gnrh-treated repeat spawning fish at 22 °C relative to the control at 14 °C. This is not surprising since Vtg production is regulated by E_2 (reviewed in Lubzens et al., 2010), and Gnrh-treatment failed to elevate plasma E_2 or *vtg* gene expression levels at 22 °C.

Treatment with Gnrh did not help to maintain *zpb* gene expression levels at 22 °C in repeat spawning fish at any time during reproductive development. For maidens, treatment with Gnrh had an inhibitory effect on *zpb* gene expression at Samples 3 and 4, and no effect at Sample 5. Similar to what was observed for *zpb*, *zpc* gene expression was down-regulated in response to Gnrh-treatment in maiden but not repeat spawning fish at Samples 3 and 4 and there was no significant effect on any fish at Sample 5. This suggests that female Atlantic salmon respond differently to Gnrh-treatment on the basis of stock age in terms of Zp transcription, although the molecular basis for this phenomenon is unclear given the fact that plasma E_2 levels were similar between Gnrh-treated maiden and repeat spawning fish throughout the study.

The present study was undertaken to determine whether reductions in egg quality caused by thermally induced endocrine impairment could be compensated for by prolonged treatment with Gnrh during vitellogenic development. It is clear from the

present experiment that treatment with Gnrh alone is not an effective strategy for enhancing the endogenous production of plasma E_2 and maintaining egg quality at elevated temperature. While maiden and repeat spawning fish responded in a similar way to treatment with Gnrh for almost all of the parameters measured, we have provided evidence suggesting that Atlantic salmon broodstock respond differently to hormonal manipulation in terms of Zp gene expression although the molecular basis for this effect is unknown at the present time. If, as the present study suggests, multiple endocrine events upstream of E_2 production are compromised by exposure to elevated temperature, it is possible that treatment of Atlantic salmon with Gnrh alone may not be effective, although co-treatment with a dopamine antagonist may help to offset some thermal inhibition. Alternatively, the direct administration of E_2 may warrant investigation, as artificially elevating plasma E_2 levels may stimulate the down-stream synthesis of Vtg and Zps and maintain egg quality at high temperature in female Atlantic salmon broodstock.

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Chapter 7- Effect of elevated temperature on estrogenic induction of vitellogenesis and zonagenesis in juvenile Atlantic salmon

7.1 Abstract

Atlantic salmon in Tasmania are routinely reared towards the upper limit of thermal tolerance for successful reproduction. In eggs from thermally challenged female fish, fertility and embryo survival rates are often low, presumably due to endocrine dysfunction at the level of ovarian 17β-estradiol (E_2) production. The present study used juvenile Atlantic salmon to assess whether hepatic tissue still remained responsive to stimulation by E_2 at the elevated temperatures typically encountered during austral summer by Tasmanian salmon stocks. E_2 administration stimulated expression of *vitellogenin* (*vtg*) and *estrogen receptor alpha* (*erα*) genes at both 14 °C and 22 °C although induction of *vtg* gene expression occurred more rapidly at 22 °C. In conjunction with more rapid *vtg* gene expression at 22 °C, plasma Vtg levels increased at a faster rate and reached a plateau more quickly at 22 °C than at 14 °C. *Zona pellucida* (*zp*) *b* and *c* gene expression was significantly reduced in E_2-treated fish at 22 °C compared to 14 °C. These results show that the Vtg gene remains highly responsive to estrogen at high temperature unlike Zp b and c genes whose expression pattern displayed traits of thermal inhibition. This suggests that estrogen replacement therapy in adult female Atlantic salmon will offset some, but not all of the inhibitory effects of elevated temperature on reproductive function.

7.2 Introduction

Worldwide, piscine aquaculture will face new challenges as a result of predicted global increases in water temperature. For example, in Tasmania, farmed Atlantic salmon (*Salmo salar*) already experience warm conditions which accelerate growth rate and shorten production time (Jungalwalla, 1991). Natural summer water temperatures of 18 °C are regularly reported for most farms and higher temperatures (up to and exceeding 22 °C) have been reported during summer months and are predicted to occur more frequently under conditions of global warming (Battaglene et al., 2008). In both southern and northern hemisphere stocks, it has been previously shown that elevated temperature can reduce reproductive performance in terms of egg fertility and embryo survival (reviewed in Pankhurst and King, 2010). In an attempt to minimise exposure of broodstock to elevated temperature, the salmon industry in Australia is moving towards re-circulating systems that allow complete thermal regulation of broodstock (Battaglene et al., 2008, Pankhurst and King, 2010). Unfortunately, this approach to thermal security requires significant capital investment and may not be a viable option for much of the aquaculture sector due to scale, site and other logistical restrictions (Pankhurst and King, 2010).

Thermal insult which leads to reduced egg quality is thought to occur as a result of temperature induced effects at a number of levels of the reproductive endocrine cascade (Pankhurst and King, 2010). These effects include suppression of plasma levels of 17β-estradiol (E_2) in fish reared at 16 °C relative to the more optimal temperatures of 6 and 11 °C (King and Pankhurst, 2000), and in fish reared at a more challenging 22 °C compared to 14 °C (King et al., 2003, King et al., 2007) during vitellogenesis**.** Furthermore, King et al. (2003) reported that testosterone (T) levels during vitellogenesis were generally higher in fish reared at 22 °C than in fish reared at 14 or 18 °C, suggesting that conversion of T to E_2 was also impaired, probably due to inhibition of *p450 aromatase a* (Cyp19a1a). An *in vitro* study by Watts et al. (2004) further supported this suggestion with increased T production by isolated ovarian follicles incubated in the presence of 17-hydroxyprogesterone and androstenedione at 14, 18

and 22 °C, but inhibition of conversion of T to E_2 at 22 °C. Additionally, an *in vitro* competitive binding study showed that hepatic estrogen receptors (Er) from Atlantic salmon reared at 22 °C during vitellogenesis had significantly reduced E_2 binding affinity compared to Er from fish maintained at 14 and 18 °C (Watts et al., 2005). This suggests that signal transduction and processes downstream from E_2 synthesis may also be involved in the inhibitory effects of elevated temperature. Measurable downstream effects do include reduction in both hepatic gene expression and plasma levels of *vitellogenin* (Vtg), and hepatic gene expression of *zona pellucida* proteins (Zp) (King et al., 2007, Ch 4: Pankhurst et al., 2011), both of which are essential for embryo development and survival. What is less clear is whether these effects are only the result of reduced estrogen tone, or whether other endocrine defects are associated with exposure to high temperature.

An alternative management strategy which could offset the effects of thermal insult might involve administration of exogenous hormones to stimulate the endocrine system and prevent the impairment on endocrine function (Pankhurst and King, 2010). In earlier studies on juvenile salmonids, treatment with E_2 has been shown to induce the expression of hepatically-expressed genes at a variety of temperatures. In juvenile Arctic char (*Salvelinus alpinus*) reared at 10 °C, *vtg*, *zona pellucida b* (*zpb*), *zona pellucida c* (*zpc*) and *er* mRNA levels had increased within 12 hours post injection with E_2 (Westerlund et al., 2001), and in brown trout (*Salmo trutta*), treatment with ethinylestradiol (EE_2) resulted in higher levels of *vtg* and *erα* mRNA in fish held at 19 °C compared to 12 °C (Körner et al., 2008). Similarly, in E_2-treated juvenile rainbow trout (*Oncorhynchus mykiss*) maintained at 15 °C, plasma Vtg was detectable 24 h after the initial injection and peaked at >70 $mg.ml^{-1}$ after 10 days, compared to ~9 $mg.ml^{-1}$ in fish held at 9 °C (Mackay and Lazier, 1993). The stimulatory effects of increasing temperature across normal ranges have also been demonstrated *in vitro* in primary hepatocytes from rainbow trout that were maintained at either 14 or 18 °C and exposed to E_2 (Pawlowski et al., 2000); *vtg* gene expression was significantly higher at 18 °C than at 14 °C. However, whether estrogen-dependent gene expression remains intact at the

higher temperatures where endocrine suppression occurs, when circulating E_2 is not a limiting factor, remains to be established. Successful demonstration of maintenance of estrogen sensitivity at high temperature would confirm the potential of endocrine manipulation to offset thermal effects. Therefore using juvenile Atlantic salmon as model animals, the present study investigated whether E_2 implantation could generate circulating E_2 levels that were a good approximation of those observed in developing adults. Accordingly, the E_2 responsiveness of juvenile fish was assessed in terms of hepatic expression of *vtg*, *erα*, *zpb* and *zpc*, and plasma levels of Vtg at 14 and 22 °C for 14 days.

7.3 Methods

7.3.1 Fish and sampling

Eighty-four juvenile female Atlantic salmon (mean weight 217.2 ± 4.68 g) were housed at the Salmon Enterprises of Tasmania Pty. Ltd. (Saltas), Wayatinah Hatchery, Tasmania, Australia during February (summer), in 4 separate 1000-L tanks (1 x 1 x 1 m, 21 fish per tank), with independent recirculating fresh water for each temperature treatment. At day 0, all fish were anesthetised with Aqui-S™ (25 ppm), weighed, implanted with a blank or E_2-containing pellet and placed in thermo-regulated tanks in 1 of 4 experimental groups (n = 21 per group, see below). Fourteen and 22 °C represent cool and warm Tasmanian summers respectively. At 3, 7 and 14 days post implantation, 7 fish were sacrificed from each group using a lethal dose of Aqui-S™ (50 ppm). Blood was taken from the caudal vasculature using heparinised syringes and centrifuged at 12000 ×g for 5 min to separate the plasma component which was then stored at -20 °C. Sections of liver were dissected, immersed in RNA Later™ (Ambion) overnight at 4 °C then stored at -80 °C until processed. Experiments were conducted under approval from the Animal Ethics Committee of the University of the Sunshine Coast (approval number AN/A/07/35).

Experimental groups:

1) Blank silastic pellet at 14 °C

2) Blank silastic pellet at 22 °C

3) E_2 pellet at 14 °C

4) E_2 pellet at 22 °C

7.3.2 E_2 implants

Slow release E_2 pellets were made according to Pankhurst et al. (1986) with some modifications. Briefly, E_2 (Sigma) was mixed with unpolymerised elastomer (Silastic 382 medical grade elastomer, Dow Corning Corporation) at 50 $mg.g^{-1}$ elastomer, and spread into 2 x 2 x 20 mm molds after the addition of 8 µl of accelerant (stannous octoate, Sigma) to give a final E_2 concentration of 2.2 $mg.cm^{-1}$ pellet. E_2 pellets were cut at various lengths to give a total dosage of 10 $mg.kg^{-1}$ body weight; blank or E_2 pellets were then ventrally implanted into the peritoneal cavity using a 15 G needle.

7.3.3 Plasma E_2 and Vtg measurement

Plasma levels of E_2 were determined by radioimmunoassay using the reagents and procedure described in Pankhurst and Carragher (1992). Further details for this assay can be found in Chapter 4 (Pankhurst et al., 2011).

Plasma Vtg levels were measured by enzyme linked immunosorbent assay using the reagents and protocol as described in Watts et al. (2003). Further details for this assay can be found in Chapter 4 (Pankhurst et al., 2011).

7.3.4 RNA isolation, cDNA synthesis and qPCRs

Total hepatic RNA was isolated and used to synthesis cDNA using the procedure outlined in Chapter 4 (Pankhurst et al., 2011). In addition, RNA quality was determined

using the method described in Chapter 5 (Anderson et al., 2012). qPCRs were conducted using the cycler, cycling conditions, reaction components and controls outlined in Chapter 4 (Pankhurst et al., 2011).

GSPs for target genes (*vtg*, *zpb* and *zpc*) previously optimized and validated for qPCR (Ch 4, Pankhurst et al., 2011) were used to amplify hepatic transcripts in the present chapter. GSPs described in Chapter 5 (Anderson et al., 2012) were used to amplify hepatic *erα* transcripts. GSPs developed in Chapter 4 (Pankhurst et al., 2011) were used to quantify the expression of candidate reference genes, namely *tata binding protein* (Tbp), *hypoxanthine phosphoribosyltransferase 1* (Hprt1) and *elongation factor 1 alpha* (Ef1α). The suitability of using *tbp*, *hprt1* and *ef1α* as reference genes for normalisation was assessed using the method outlined in Chapter 3 (Anderson and Elizur 2012). As a result of this analysis (Ch 3: Anderson and Elizur 2012), expression data from all 3 reference genes was used for accurate target gene normalisation using the Rest© software 2008 package, V2.0.7 (Pfaffl et al., 2002). This package was also used to calculate the level of target gene expression relative to a calibrator sample analysed in every run, and adjust the level of target gene expression to account for inter-assay variability which was negligible (the %CV was extremely low: < 0.85% for all qPCR assays).

7.3.5 Statistical analysis

One-way ANOVA coupled with Tukeys-b was used to test for statistically significant differences in the plasma E_2 and Vtg data ($p \leq 0.05$). Differences in relative gene expression levels were detected non-parametrically as in Chapter 4 (Pankhurst et al., 2011).

7.4 Results

7.4.1 Quantification of plasma E_2 and Vtg

Plasma E_2 levels were low (< 0.4 $ng.ml^{-1}$) throughout the experiment in fish receiving a blank silastic pellet (Fig. 7.0). E_2-treated fish had significantly higher plasma E_2 than their respective control fish at all Sample times. E_2 levels were also consistently higher at 14 than at 22 °C with a general trend of decreasing plasma E_2 concentration over time in E_2 treated fish. Plasma Vtg levels were also low but detectable in controls at 3 and 7 day Samples, and significantly elevated at those times in fish treated with E_2 (Fig. 7.0). At 3 days post-implant, Vtg levels were higher in E_2-implanted fish at 22 °C than at 14 °C; at 7 days there was no difference between treatments and at 14 days post-implant, plasma Vtg was lower at 22 than at 14 °C. At the 14 day Sample, there was also an elevation in plasma Vtg in the blank pellet group at 14 °C relative to the 22 °C blank group, but to a lower level than in either of the E_2-treated groups.

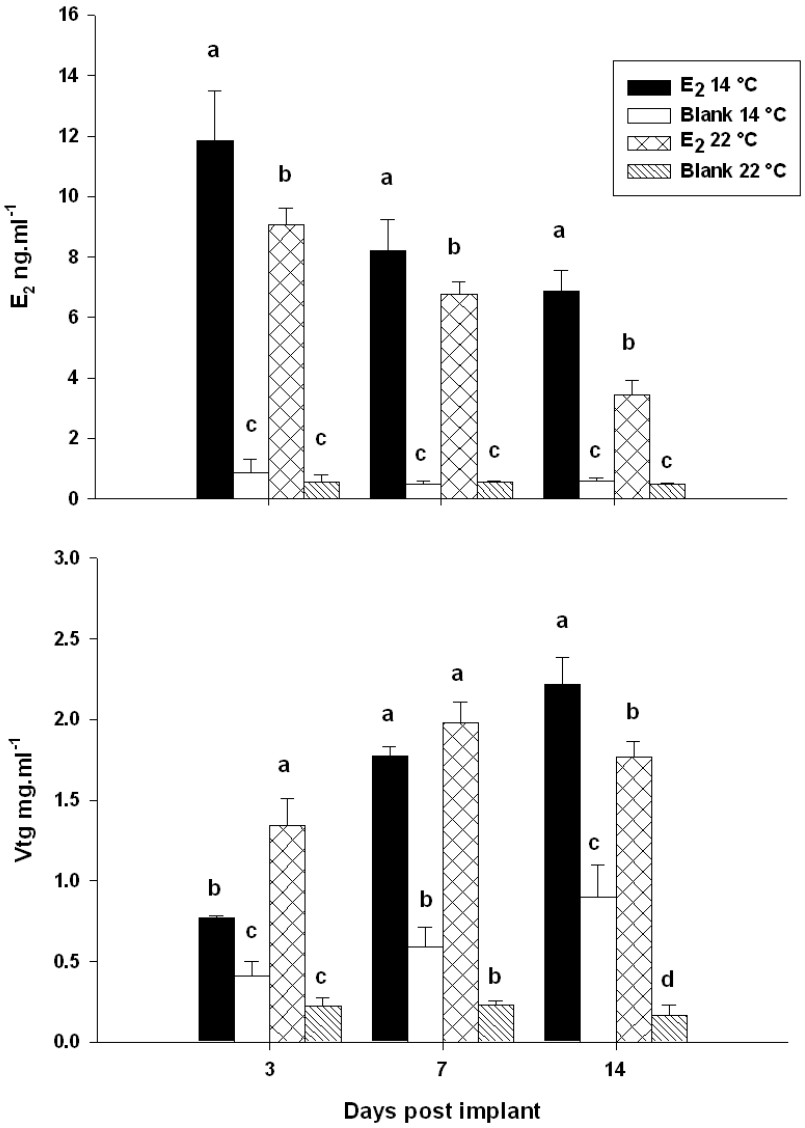

Figure 7.0 Plasma levels (mean + SE, n = 7) of E_2 and Vtg in juvenile Atlantic salmon given a blank implant at 14 °C or 22 °C, or an E_2 implant (10 mg.kg^{-1}) at 14 °C or 22°C. Fish were sampled at 3, 7 and 14 days post implantation. Different superscripts at each sampling time denote significantly different means ($p \leq 0.05$).

7.4.2 Hepatic gene expression

At day 3, *erα* gene expression in the E_2-treated groups at 14 and 22 °C were similar (Fig 7.1). Gene expression levels for E_2-treated groups were significantly higher than those observed for control groups at both temperatures on day 3. At day 7, *erα* gene expression was elevated in the 14 °C blank, 14 °C E_2 and 22 °C E_2 groups relative to the

22 °C blank. By day 14, the levels of *erα* gene expression in both E_2-treated groups were significantly elevated over the levels in their respective controls. *erα* gene expression was also significantly higher in the 14 °C control group than in the 22 °C control group on day 14.

At 3 days post-implant, *vtg* gene expression was significantly higher in 22 °C E_2-treated fish than any other group (Fig. 7.1). The mean gene expression level of E_2-treated fish at 14 °C was also higher than its corresponding control group. *vtg* gene expression in E_2-treated groups was elevated over controls for the remainder of the experiment. Gene expression was higher in E_2-treated fish at 22 °C than that of the E_2-treated group at 14 °C at day 7; however, this difference had disappeared by day 14. Gene expression was consistently low in control groups for the duration of the experiment.

At day 3, *zpb* and *zpc* gene expression levels were elevated in E_2-treated animals at both temperatures relative to their respective controls (Fig 7.2). *zpc* gene expression was also significantly higher in E_2-treated fish at 22 °C compared to E_2-treated fish at 14 °C. *zpb* and *zpc* gene expression increased over time in the E_2-treated 14 °C group. At 22 °C, *zpb* and *zpc* gene expression levels were consistently higher than those of the control; however, the level of expression for these genes did not steadily increase over time. Additionally, gene expression was lower at 22 °C than 14 °C for *zpb* at days 7 and 14, and for *zpc* at day 14.

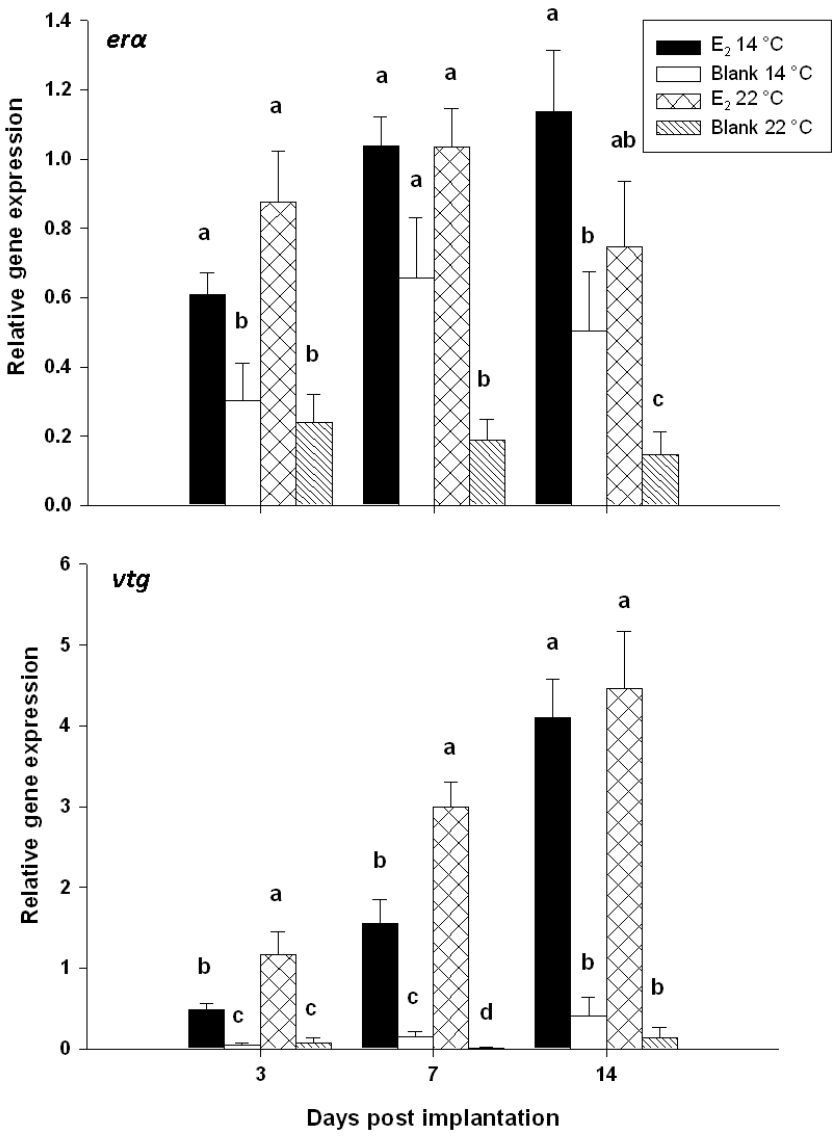

Figure 7.1 Relative gene expression levels (mean + SE, n = 7) of hepatic *erα* and *vtg* in juvenile Atlantic salmon given a blank implant at 14 °C or 22 °C, or an E_2 implant (10 mg.kg^{-1}) at 14 °C or 22°C. Fish were sampled at 3, 7 and 14 days post implantation. Gene expression levels were normalised to *tbp*, *hprt1* and *ef1α*. Different superscripts between groups at each sampling time denote significantly different means ($p \leq 0.05$).

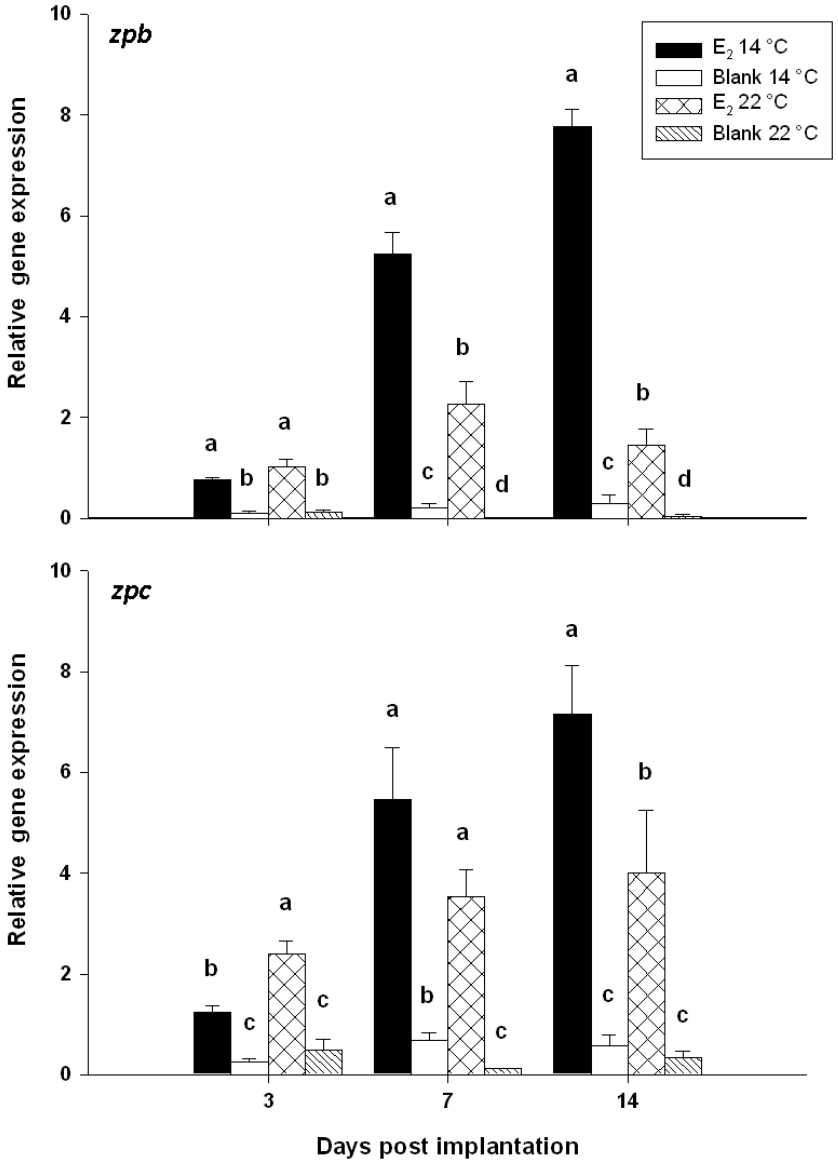

Figure 7.2 Relative gene expression levels (mean + SE, n = 7) of hepatic *zpb* and *zpc* in juvenile Atlantic salmon given a blank implant at 14 °C or 22 °C, or an E_2 implant (10 mg.kg^{-1}) at 14 °C or 22°C. Fish were sampled at 3, 7 and 14 days post implantation. Gene expression levels were normalised to *tbp*, *hprt1* and *ef1α*. Different superscripts between groups at each sampling time denote significantly different means ($p \leq 0.05$).

7.5 Discussion

Atlantic salmon are routinely reared in Tasmania at temperatures towards the upper limit of thermal tolerance for successful reproduction. Egg fertility and embryo survival rates are often low at high temperatures, presumably due to endocrine dysfunction at the level of ovarian E_2 production. Assessment of the role of sustained E_2 levels in this process required a treatment protocol that produced elevations in plasma E_2 in implanted fish that were similar to those in normally maturing adults. By day 3 in the present study, circulating E_2 had risen to ~12 and 9 $ng.ml^{-1}$ in E_2-treated fish maintained at 14 °C and 22 °C respectively. For the same type of pellet, Pankhurst et al. (1986) observed a rapid fall in plasma E_2 level post-implantation that lasted for approximately 6 hours, after which a steadier decline in plasma E_2 was observed. Based on the plasma E_2 profiles obtained by Pankhurst et al. (1986), it is likely that the plasma concentrations of E_2 in juvenile fish prior to day 3 were initially higher than those observed in fish reared at both 14 and 22 °C on day 3. The concentration of plasma E_2 decreased over time for the duration of the present experiment similar to what was observed in other studies using the same type of silastic E_2 implant (Pankhurst et al., 1986). E_2 levels in fish maintained at 22 °C were consistently lower at each sampling point relative to fish from the 14 °C treatment which suggests that E_2 was utilised and/or cleared at a faster rate at 22 °C than at 14 °C. Similarly, Mackay and Lazier (1993) observed lower plasma E_2 levels in E_2-treated rainbow trout maintained at 15 °C compared to 9 °C, and also suggested that this difference resulted from increased metabolic rate and steroid clearance at the higher temperature. Adult Atlantic salmon reared at 14 °C typically have plasma E_2 concentrations of ~4 and 8.5 $ng.ml^{-1}$ during February and March respectively (which coincides with a period of advancing vitellogenesis), while levels in fish held at 22 °C remain low at <1 $ng.ml^{-1}$ and 2 $ng.ml^{-1}$ for the same months (Ch 4: Pankhurst et al., 2011). In the present study, the highest E_2 levels in juveniles held at 22 °C were measured 3 days after implantation (9 $ng.ml^{-1}$) and decreased to ~3.5 $ng.ml^{-1}$ over a 2 week period. After 2 weeks, the E_2 level observed in thermally challenged juvenile fish was still higher than that typically observed in untreated broodstock reared at 22 °C

during February and March (Ch 4: Pankhurst et al., 2011). This illustrates that E_2 implantation of thermally challenged broodstock at 10 $mg.kg^{-1}$ during February and March could elevate plasma E_2 levels to a level which is comparable to the physiological levels observed in broodstock reared under optimal conditions, or at the very least could keep E_2 levels above the 1-2 $ng.ml^{-1}$ baseline for a minimum of 2 weeks. This demonstrates the potential of E_2-implantation to at least partially restore circulating E_2 levels in thermally compromised adult broodstock.

In order to accurately quantify the relative abundance of hepatic mRNA transcripts for the various target genes of interest, a reference gene (or panel of genes) that does not respond to temperature or estrogen was required. As described in Chapter 3 (Anderson and Elizur 2012), it was determined that the stability of the candidate reference genes was *ef1α* > *hprt1* > *tbp* through independent statistical analysis. Despite its apparent lower stability, using *tbp* in addition to *ef1α* and *hprt1* significantly improved the stability value of candidate panel according to the GeNorm algorithm, and using 3 genes for normalisation reduced the level of noise in a target gene dataset (Ch 3: Anderson and Elizur 2012). For these reasons, all 3 reference genes were used for accurate target gene normalisation in the present chapter.

For juvenile fish reared at 14 °C, *vtg* gene expression was significantly elevated at 3 days post implantation and this increased further over the course of the experiment. This is broadly consistent with previous observations in juvenile salmon, where E_2-treatment induced *de novo* synthesis of *vtg* mRNA, and gene expression increased over time (Mackay and Lazier, 1993). In E_2-treated fish maintained at 22 °C, *vtg* gene expression was significantly higher than that in the corresponding group of fish reared at 14 °C at day 3, indicating that gene expression was induced more quickly at the higher temperature. On day 7, the difference in gene expression was maintained; however by day 14 expression levels were approximately equal. In ectotherms, an increase in temperature causes an increased rate of physiological processing (Sokolova and Lannig, 2008), which in this case appears to have resulted in the faster induction and increased synthesis of *vtg* mRNA for the first week of the experiment. Similarly, Mackay and

Lazier (1993) reported that juvenile rainbow trout held at 15 °C responded more quickly in terms of *vtg* mRNA synthesis to E_2 injection than fish maintained at 9 °C, and had *vtg* mRNA levels that were 3-fold higher at the end of the experiment (day 10). Since it has been shown that a rise in temperature can cause an increase in liver somatic index, total protein output (Korsgaard et al., 1986) and basal metabolic rate (Johnston and Dunn, 1987), we suggest that the faster induction and increased *vtg* gene expression at higher temperature in the present study can be attributed to more rapid utilisation and clearance of plasma E_2.

Plasma Vtg levels generally reflected *vtg* mRNA levels for the duration of the experiment. Circulating levels of Vtg in E_2-treated fish were higher in the 22 °C than the 14 °C group at day 3 which is consistent with the faster induction of *vtg* gene expression at the higher temperature. However, despite the initially more rapid accumulation of Vtg in 22 °C E_2-treated fish, plasma Vtg levels were approximately equal in E_2-treated fish at both temperatures by day 7, and by day 14, plasma Vtg levels were higher at 14 °C. The more rapid production of Vtg at the elevated temperature shows that the rate of short term response to E_2 in terms of Vtg production is modulated by temperature and the maximum response is reached more rapidly at higher temperature. This is consistent with observations of adult fathead minnows (*Pimephales promelas*) that were exposed to a mixture of estrogenic chemicals and maintained at either 20 or 30 °C for 2 weeks, where plasma Vtg was significantly higher after 24 h at 30 °C (Brian et al., 2008). Consistent with the present study, no difference in Vtg concentration could be detected between temperature treatments after a longer period of exposure (Brian et al., 2008). Brian et al. (2008) also concluded that temperature alters the short term response of the animal to estrogenic stimulation, but may not have an impact on the final magnitude of response. In contrast, other studies on salmonids have reported different temporal responses to exogenous E_2. Exposure of Atlantic salmon post-smolts and juvenile rainbow trout to elevated temperature resulted in greater total accumulation of plasma Vtg in response to E_2 over time (Korsgaard et al., 1986, Mackay and Lazier, 1993). The apparent difference in plasma Vtg profiles between those and

the present study is likely to be a function of dose and route of exposure (implantation in the present study versus injection), and the temperature ranges used in the respective studies. The study undertaken by Korsgaard et al. (1986) utilised temperatures of 3 and 10 °C while Mackay and Lazier (1993) maintained fish at 9 and 15 °C compared with the upper temperature of 22 °C in the present study. Collectively our *vtg* mRNA and protein data show that the mechanisms by which E_2 induces and sustains the production of Vtg are still functional in juvenile Atlantic salmon at the relatively high temperature tested in the present study.

The relative expression of *zpb* and *zpc* genes in the present study was up-regulated in a time-dependent fashion by E_2-treatment in fish reared at 14 °C, which is consistent with previous reports on salmonids. In juvenile Arctic charr and rainbow trout, E_2-treatment significantly up-regulated the gene transcription and translation of ZPs at temperatures of 10 and 16 °C respectively (Celius et al., 2000, Westerlund et al., 2001). In the present study, consistent with the broad effects of increased rate of metabolic processing, E_2-treatment caused a more rapid of induction of *zpc* at 22 °C relative to 14 °C. *Zpb* gene expression was also induced at day 3 at high temperature, but unlike *zpc* the level was not greater than that observed in the corresponding group at 14 °C. At day 7 and 14, gene expression of *zpb* was higher in the E_2-treated group at 22 °C than its corresponding control group, but was significantly lower than the level observed for E_2-treated fish reared at 14 °C. A similar trend was also observed for *zpc*, although there was no statistical difference in gene expression levels for E_2-treated groups at day 7. Furthermore, there was an apparent difference in the expression profiles as gene expression for both *zpc* and *zpb* at 22 °C did not increase over time as observed for E_2-treated fish at 14 °C. This suggests that unlike *vtg*, the responsiveness of juvenile Atlantic salmon to exogenous E_2 in terms of *zp* transcription is partially compromised at 22 °C, which may have implications for the usefulness of E_2 therapy in maintaining reproductive function in thermally challenged adult broodstock.

The promoter region of Vtg genes contain estrogen responsive elements (EREs) and enhancer sequences that appear to be relatively simple (Teo et al., 1998, Bouter et

al., 2010), and regulation of Vtg is under strict E_2 control (Lubzens et al., 2010). However, it has been suggested elsewhere that the regulatory mechanism of Zps may differ to that of Vtg (Berg et al., 2004). Due to the range of different reproductive strategies and sites of *zp* gene expression among fish species, it is quite difficult to make generalisations about the regulatory mechanisms of genes in the Zp family (reviewed in Babin et al., 2007). For example, in zebrafish (*Danio rerio*) where Zps are exclusively expressed in the ovary, *zp* expression cannot be induced by E_2, and the promoter region of these genes lack EREs (Mold et al., 2001, Liu et al., 2006). It is thought that in zebrafish, *zp* expression is at least party regulated by nuclear transcription factor Y through CCAAT boxes (Mold et al., 2009) and through E-boxes (Mold et al., 2001) that bind basic helix-loop-helix proteins such as factor in the germ cell α. The promoter region of medaka (*Oryzias latipes*, liver *zp* expression) contains multiple E-boxes (Kanamori et al., 2003) and perhaps other regulatory elements, and the promoter region of winter flounder (*Pseudopleuronectes americanus*, liver *zp* expression) contains imperfect EREs and CCAAT and TATAAA boxes (Lyons et al., 1993). While hepatically expressed Zps are inducible by E_2 (Berg et al., 2004, Knoebl et al., 2004), it has recently become apparent that many factors besides E_2 could potentially be involved in the regulation of Zps. Furthermore, no data are available on the promoter regions of Atlantic salmon Zp genes, or whether potential regulating factors of these genes are temperature sensitive. To our knowledge, this is the first report on the inhibitory effect of elevated temperature on the expression of Zp genes in an E_2-treated salmonid. This suggests that thermal sensitivity of the complex factors regulating *zp*, but not *vtg* gene expression, may have resulted in the differential effect of elevated temperature on *vtg* and *zp* gene expression observed in the present study. However, characterisation of the 5'-flanking promoter regions of the Zp genes from Atlantic salmon, and subsequent thermal exposure trials quantifying the expression of regulatory factors are required to clarify this possibility.

Exposure to E_2 at 14 °C significantly up-regulated *erα* gene expression by day 3 in the present study relative to the corresponding control group. Similarly, both *in vitro*

and *in vivo* studies have demonstrated that transcription of hepatic *er* can be induced by E_2 administration in salmonids (Flouriot et al., 1996, MacKay et al., 1996, Westerlund et al., 2001). Exposure of E_2-treated juvenile fish to 22 °C in the present study did not significantly alter the level of hepatic *erα* gene expression relative to fish reared at 14 °C for the duration of the experiment. In contrast to the present study, other studies have described a modulating effect of temperature on *er* transcription. Higher levels of *er* mRNA were detected in isolated hepatocytes from rainbow trout in response to E_2 at 18 °C compared to 14 °C (Pawlowski et al., 2000), and juvenile brown trout treated with EE_2 showed significantly enhanced *erα* gene expression at 19 °C compared to 12 °C (Körner et al., 2008). As increases in temperature are only stimulatory up to a certain point, the use of a higher temperature in the present study may account for the apparent lack of *erα* stimulation compared to previous studies. Even though *erα* expression was unaffected by high temperature treatment in the present study, other *in vitro* studies on Atlantic salmon and tilapia (*Oreochromis aureus*) have shown that exposure to high temperature can decrease hepatic Er binding affinity (Tan et al., 1999, Watts et al., 2005) and potentially affect E_2 signal transduction. The fact that E_2-treatment still significantly elevated *vtg* gene expression at 22 °C in the present study suggests that any negative effects on receptor affinity and therefore signal transduction were not sufficient to significantly decrease the transcription of other E_2-dependent genes. E_2-treatment resulted in high and sustained plasma E_2 levels so that any reductions in receptor affinity may have been offset by high ligand concentration and the effects of increased metabolic rate. It is still possible that signal transduction might be impaired at the lower ligand concentrations typically observed at elevated temperatures.

While it appears that temperature did not modify the expression of *erα* in juvenile Atlantic salmon, the effects of high temperature on other estrogen subtypes, such as estrogen receptor beta (Erβ), gamma (Erγ) or the novel isoform Erα2, are currently unknown. For male medaka, it has been suggested that each Er subtype specifically regulates a particular Vtg gene (Yamaguchi et al., 2009). For example, *vtgα* mRNA levels were regulated via Erα only; in contrast, *vtgb* mRNA levels were influenced

by both Erα and Erβ although their roles in initiation and enhancement of transcription seem to be different (Yamaguchi et al., 2009). Therefore, it is possible that Ers other than Erα are thermally sensitive which could have affected the expression of Zp or other Vtg genes not measured in the present chapter.

The expression of *erα* was significantly elevated in control fish in the present study at day 7 and 14, at 14 °C. This may indicate that there was waterborne estrogen in the 14 °C recirculating system which stimulated *erα* gene expression in the control group. When *erα* expression was elevated at day 7, the expression levels of both *zp* and *vtg* genes were also higher in the 14 °C than the 22 °C control group. This was also observed on day 14 for *zpb* gene expression and plasma Vtg levels, further supporting the possibility that unintended E_2 exposure may have occurred. Fish-to-fish transfer of steroid hormones has previously been demonstrated by Budworth and Senger (1993) where plasma T levels were found to be significantly elevated in the blood of saline-injected trout whose tank received water from a recirculating system that also held fish injected with T. The mechanism for fish-to-fish transfer appears to be loss and uptake across the gill epithelium (Vermeirssen and Scott, 1996, Scott et al., 2005). Therefore the unexpected gene expression detected in the 14 °C control group could be due to loss of E_2 via the gills in E_2-treated fish, subsequent contamination of the 14 °C recirculating system and uptake of E_2 by the control group. The level of *erα* expression was not different between the 14 °C control and E_2 treated group at day 7, while in the same fish, *vtg* and *zp* expression was elevated in the E_2 pellet group relative to controls. This difference could be due to differential sensitivity of these genes to E_2 stimulation. In rainbow trout a higher dose of E_2 was needed to stimulate the *in vitro* hepatic expression of *vtg* compared to *er* (Flouriot et al., 1996).

Thermal management of broodstock holding facilities is the most effective way to avoid endocrine dysfunction and ensure egg quality in Atlantic salmon during periods of high ambient water temperature (Pankhurst and King, 2010). However, for facilities where temperature control is not a desirable or viable option, E_2 therapy has been suggested as an alternative approach for maintaining endocrine function and

subsequent egg quality (King, H 2008, pers. comm., 15 August). In the present study we have demonstrated that juvenile Atlantic salmon remain responsive to E_2-treatment at 22 °C in terms of *vtg* and *erα* gene expression, and Vtg protein production. Our results indicate that the machinery by which E_2 exerts its action is still intact at 22 °C, and suggests that the compromised levels of *vtg* mRNA and protein observed in our previous study of adult animals (Ch 4: Pankhurst et al., 2011) occurred mainly as a result of reduced E_2 tone and not impairment of other downstream processes such as reduced E_2 signal transduction. We have also confirmed previous reports on the modulating effect of temperature on the speed of induction and duration of Vtg production which could have implications for the frequency of E_2-treatments in a commercial production setting. For the first time we have demonstrated that the hepatic zonagenic response to E_2-treatment in juvenile Atlantic salmon is significantly reduced at 22 °C. While the molecular basis for the observed dysfunction is unclear, we suggest that it may occur as the result of thermal impairment of (non-ERE) factors co-regulating the expression of both Zps but not Vtg. We conclude that the usefulness of E_2 therapy warrants investigation in thermally challenged adult broodstock as the liver remains responsive to E_2 at high temperature in terms of Vtg production, *erα* gene expression and to a lesser extent *zp* gene expression.

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Chapter 8- Effects of E_2-treatment on the reproductive physiology of thermally challenged maiden Atlantic salmon

8.1 Abstract

Rearing temperatures for Tasmanian Atlantic salmon can exceed 20 °C during the summer months which is near the upper-limit of thermal tolerance for this species. As a consequence, plasma 17β-estradiol (E_2) levels are typically low in salmon maintained at elevated temperature which is thought to impair down-stream vitellogenesis and zonagenesis, and reducing egg fertility and embryo survival. Therefore, the aim of the present study was to determine whether E_2-treatment could offset thermal impairment of endocrine function and maintain egg quality in maiden spawning female Atlantic salmon reared at 22 °C. Treatment with E_2 at 22 °C stimulated *vitellogenin* (*vtg*) gene expression and subsequent protein synthesis which promoted oocyte growth and increased egg size relative to untreated fish at 14 and 22 °C. However, E_2-treatment at 22 °C was not associated with an increase in egg fertility and embryo survival relative to untreated fish at 22 °C, despite the positive effects of E_2-treatment on vitellogenesis and oocyte growth. This could be due to the fact that treatment with E_2 did not offset the negative effects of maintenance at high temperature on the hepatic expression of *zona pellucida* (*zp*) genes during oocyte development. There was no evidence to suggest that the expression of *vtg* or *zps* was affected by a change in *estrogen receptor alpha* gene expression in the present study. Nevertheless, our results demonstrate that treatment with E_2 is not able to maintain *zp* gene expression or maintain egg quality in maiden Atlantic salmon at high temperature, even though *vtg* gene expression, protein synthesis and subsequent oocyte growth was promoted at 22 °C. The apparent lack of *zp* stimulation by E_2 implies that the mechanisms regulating the transcription of *zp*, but not *vtg* genes are impaired at elevated temperature in female Atlantic salmon broodstock, and highlights the remarkable complexity of thermally induced endocrine disruption in fish.

8.2 Introduction

Tasmanian Atlantic salmon (*Salmo salar*) are currently reared towards their upper-limit of thermal tolerance for successful reproduction (King and Pankhurst, 2003), and can sometimes experience temperatures up to and exceeding 20 °C during summer months (Battaglene et al., 2008). Under conditions of global warming, it is expected that the frequency of high-temperature days will increase for Tasmanian Atlantic salmon farms (Battaglene et al., 2008), and this is likely to impair reproductive endocrine function and result in a loss of egg quality and productivity (King et al., 2007, Ch 4: Pankhurst et al., 2011). There is growing evidence which suggests that the effects of thermal exposure on reproduction are mediated through the brain-pituitary-gonad axis, and endocrine function can be impaired at multiple levels (reviewed in Pankhurst and King, 2010). For example, in red seabream (*Pagrus major*), lower pituitary *gonadotropin-releasing hormone* (*Gnrh*) *receptor* (*gnrhr*) gene expression levels were observed in fish maintained at 24 °C relative to 17 °C (Okuzawa et al., 2003). Thermal impairment was also observed at the level of the ovary in female pejerrey (*Odontesthes bonariensis*) when exposure to 23 or 27 °C suppressed *follicle stimulating hormone* (*fsh*) *receptor* (*fshr*) mRNA and plasma 17β-estradiol (E_2) levels relative to a group at 19 °C (Soria et al., 2008). A similar suppressive effect is well documented for female Tasmanian Atlantic salmon (King et al., 2003, King et al., 2007, Ch 4: Pankhurst et al., 2011) where thermal impairment of *p450 aromatase a* (*cyp19a1a*) gene expression appears to contribute to the suppression of plasma E_2 (Ch 5: Anderson et al., 2012). Thermal sensitivity of *cyp19a1a* gene expression has also been demonstrated in juvenile fish during the process of sexual differentiation (Lim et al., 2003, van Nes and Andersen, 2006). It is therefore likely that impaired conversion of testosterone (T) to E_2 is in part responsible for lower-than-normal plasma E_2 levels measured at high temperature as suggested previously for Atlantic salmon (Watts et al., 2004, Ch 5: Anderson et al., 2012).

In Atlantic salmon maintained at high temperature, plasma levels of *vitellogenin* (Vtg) and hepatic gene expression levels of *vtg* and *zona pellucida* (*zp*) proteins were

generally depressed throughout reproductive development presumably as a result of E_2 suppression (King et al., 2003, Ch 4: Pankhurst et al., 2011). Endocrine impairment was most severe in Atlantic salmon reared at 22 °C relative to 14 and 18 °C, and as a consequence egg fertility was significantly reduced (King et al., 2003). Embryo survival was also lower in Atlantic salmon and rainbow trout (*Oncorhynchus mykiss*) maintained at elevated temperature just prior to ovulation or during vitellogenesis respectively (Taranger and Hansen, 1993, Pankhurst et al., 1996). This indicates that the salmon industry must adapt to ensure the production of high quality smolts as periods of high temperature become more frequent as a result of climate change (Pankhurst and King, 2010).

Currently, the most effective way to provide thermal protection for broodstock in Tasmania is to control water temperature at on-shore facilities (Battaglene et al., 2008). However, this approach requires significant capital investment and on-going costs, may not be practical for some farms, and will only be successful if the system never fails (Pankhurst and King, 2010). Our recent research has supported industry observations which suggested that 3+ year old (second-time spawning) 'repeat' spawning broodstock were slightly more resistant to the effects of thermal challenge in terms of egg quality than 2+ year old (first-time spawning) 'maiden' spawners (Ch 4: Pankhurst et al., 2011). Therefore, the use of repeat spawning fish over maidens may offer some level of thermal protection, but is still not a desirable option due to the considerable cost and risk associated with intensively maintaining such large fish for an additional year. A third management option includes administration of one or more exogenous hormones that will compensate for the negative effects of thermal challenge on endocrine function and subsequently maintain egg quality (Pankhurst and King, 2010). Gnrh analogue (Gnrha) injection in combination with a temperature reduction prior to ovulation was able to fully restore egg fertility in Atlantic salmon reared at 16 °C during late vitellogenesis (King and Pankhurst, 2004). However this treatment regime has never been tested at temperatures as high as 22 °C, and it is now known that even relatively short exposures to elevated temperature earlier in vitellogenesis can have

lasting negative effects on egg quality (King et al., 2007). In light of this, it has been suggested that hormonal therapy during the earlier stages of vitellogenesis may be an effective way to stimulate the endocrine system, and offset the effects of E_2 suppression at high temperature. At optimal temperatures, treatment with Gnrha during vitellogenesis caused an early elevation in plasma T and E_2, and promoted oocyte development in female Atlantic salmon (Crim et al., 1986). In contrast prolonged Gnrha therapy at 22 °C during vitellogenesis was unable to offset the negative effects of temperature on endocrine function and did not promote egg quality in maiden and repeat spawning Atlantic salmon (Ch 6). It is thought that in this case the action of Gnrha was blocked at multiple levels of the endocrine cascade, with the 'lowest' significant blockage probably occurring in the ovary at the level of *cyp19a1a* (Ch 6, Ch 5: Anderson et al., 2012). For this reason, administration of E_2 may by-pass *cyp19a1a* impairment in the ovary, and directly stimulate the downstream synthesis of Vtg and Zps at high temperature.

E_2 (or 17α-ethinylestradiol, EE_2) treatment has been shown to induce *de novo* synthesis of Vtg at elevated temperatures in juvenile salmonids (Westerlund et al., 2001, Körner et al., 2008, Ch 7). The effect of E_2-treatment on Zp synthesis at high temperature is less clear, although we have previously shown that in juvenile Atlantic salmon E_2-implantation can stimulate *zona pellucida b* (*zpb*) and *c* (*zpc*) gene expression to some extent at 22 °C (Ch 7). Given the industry's preference to use maiden fish for egg production, the aim of the present study was to determine whether E_2-therapy in maiden broodstock could overcome the effects of reduced endogenous E_2, and maintain egg quality at 22 °C. Accordingly, the effects of E_2-treatment on oocyte development and endocrine function were determined including plasma Fsh, *luteinizing hormone* (Lh) and Vtg levels, and hepatic gene expression levels of *vtg*, *zpb*, *zpc* and *estrogen receptor alpha* (*erα*) during vitellogenesis. Thereafter, the effects of hormonal and thermal manipulation on the timing of ovulation and subsequent egg fertility and embryo survival were assessed.

8.3 Methods

8.3.1 Fish husbandry and maintenance

Maiden (first spawning, 2+ year old) adult females (mean ± SE body weight of 4.7 ± 0.05 kg) from the Salmon Enterprises of Tasmania (Saltas) spawning stock were held in 200 m^3 circular tanks (2 x 2 x 1 m) at ambient photoperiod and temperature under standard conditions of husbandry, at the Saltas Wayatinah hatchery until February 2010. On the 17th of February, fish were transferred to temperature-controlled 4 m^3 Rathbun tanks (14 fish per tank) under simulated ambient photoperiod according to 3 treatment groups (see below). Fourteen and 22 °C represent cool and warm Tasmanian summers respectively. Fish were not fed from the time of transfer to the temperature controlled systems in January consistent with hatchery practice for management of this experimental stock of fish. All fish were maintained at the nominated temperature (14 or 22 °C) until late March when all fish were exposed to a gradual temperature ramp down to 8 °C to induce final oocyte maturation and ovulation approximately 1 week before the last sample point as described by King and Pankhurst (2000).

Treatment groups:
(1) 14 °C untreated
(2) 22 °C untreated
(3) 22 °C E_2 implant

8.3.2 Sampling protocol

Maiden fish maintained at ambient temperature were sampled (n = 7) on February 17th (Sample 1) to establish a vitellogenic base line for the rest of the experiment (Fig. 8.0). Then on March 5th (Sample 2), March 19th (Sample 3) and on April 9th (Sample 4), 7 fish from each group were sampled (fish were randomly taken from each replicate tank)

leaving 7 fish from each treatment to mature through to ovulation and stripping in May-June. Fish in group 3 were given E_2 pellets at Samples 1 and 2. For implantation, fish were anaesthetised in Aqui-S™ and a small ventral incision in the abdominal wall was made with a scalpel, the pellet was then inserted using forceps and the wound sealed with medical adhesive (Stoma Powder). Due to the logistical constraints surrounding handling of large fish, control groups 1 and 2 were not sham-implanted.

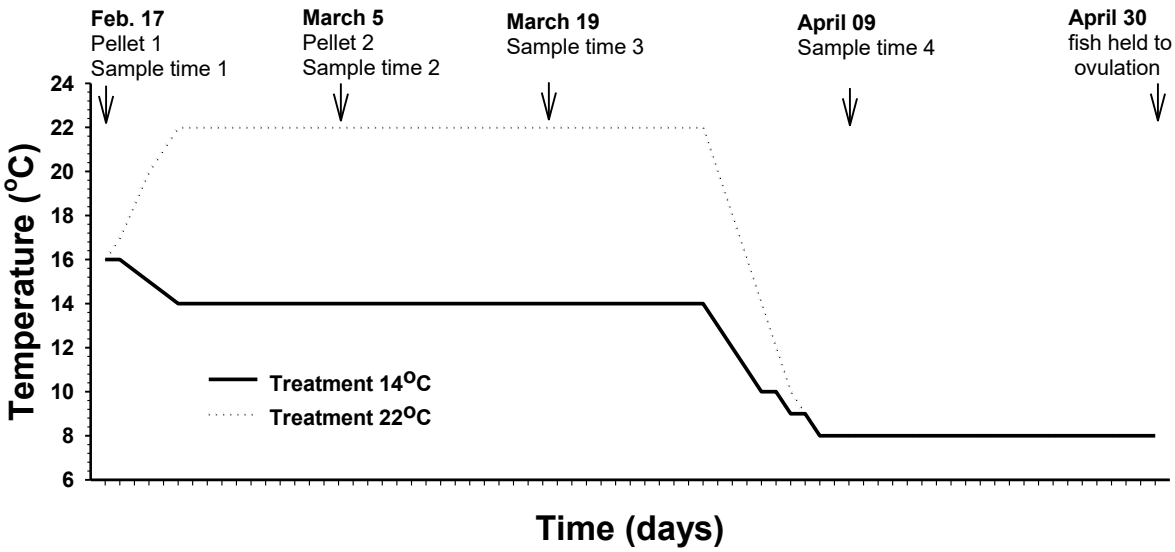

Figure 8.0 Thermal treatment regimes, hormone pellet implant dates and sampling times for maiden Atlantic salmon held at 14 °C and 22 °C in late summer and autumn.

For each sampling point, fish were randomly taken from both replicate tanks and handled humanely as outlined in Chapter 4 (Pankhurst et al., 2011). Furthermore blood was collected and centrifuged at 12000 ×g for 5 min, tissues were harvested and stored, and morphometric data was collected as described as in Chapter 4 (Pankhurst et al., 2011). For fish that were left to ovulate, ova were stripped and fertilised as described in King et al. (2003) for measurement of egg size, fertility and survival to the eyed stage at 250 degree-days of incubation. All animal experiments were conducted in accordance with Australian law under ethical approval issued by the Griffith University and University of the Sunshine Coast Animal Ethics Committees (ENV/01/10/AEC and AN/A/10/50 respectively).

8.3.3 E2 implants

Pellets containing E_2 were manufactured by combining 35 mg of crystalline E_2 with 500 µl of unpolymerised silastic elastomer (Dow Corning), adding accelerant and then spinning down the silastic mix in 1.5 ml Eppendorf tubes to provide solid cured pellets. Each fish (approximate weight 3.5 kg) received one pellet at each treatment to give a nominal dose of 10 $mg.kg^{-1}$.

8.3.4 Plasma hormone and vitellogenin measurement

Plasma Fsh (all sampling points) and Lh (March and April samples only) measurements were performed using an RIA developed for coho salmon by Swanson et al. (1989) with some modifications according to Chapter 5 (Anderson et al., 2012).

Plasma levels of E_2, T and cortisol were determined by radioimmunoassay using the reagents and procedure for E_2 and T described in Pankhurst and Carragher (1992), and for cortisol as in Pankhurst et al. (2008). Further details for these assays are available in Chapter 4 (Pankhurst et al., 2011).

Plasma Vtg levels were measured by enzyme linked immunosorbent assay using the reagents and protocol as described in Watts et al. (2003). Further details for this assay are available in Chapter 4 (Pankhurst et al., 2011).

8.3.5 RNA extraction, cDNA synthesis and qPCRs

Total hepatic RNA was isolated, handled and used to synthesis cDNA using the procedure outlined in Chapter 4 (Pankhurst et al., 2011). In addition, RNA quality was determined using the method described in Chapter 5 (Anderson et al., 2012). qPCRs were conducted using the cycler, cycling conditions, reaction components and controls outlined in Chapter 4 (Pankhurst et al., 2011).

Gene specific primers (GSPs) for target genes previously optimised and validated for qPCR (Ch 4, Pankhurst et al., 2011) were used to amplify the hepatic target (*vtg*, *zpc*, *zpb*) and reference gene (*tata binding protein*, *tbp*) transcripts. GSPs described in Chapter 5 (Anderson et al., 2012) were used to amplify hepatic *erα* transcripts. The suitability of using *tbp* as a reference gene for normalisation was assessed using the method outlined in Chapter 3 (Anderson and Elizur 2012).

Consistent with our previous work (Ch 3: Anderson and Elizur 2012), *tbp* was deemed to be stably expressed (data not shown) and was used for accurate target gene normalisation using the Rest© software 2008 package, V2.0.7 (Pfaffl et al., '02). This package was also used to calculate the level of target gene expression relative to a calibrator sample analysed in every run, and adjust the level of target gene expression to account for inter-assay variability which was negligible (the %CV was extremely low: < 0.85% for all qPCR assays).

8.3.6 Statistical analysis

Morphometric, plasma protein and gene expression data were analysed using the same procedure outlined in Chapter 4 (Pankhurst et al., 2011) for each data type.

8.4 Results

8.4.1 Morphometric data

There was no significant difference in the mean length or weight of fish in any treatment group (data not shown). Similarly, there was no difference in CF among groups at any time, but there was a significant increase in gonad mass in E_2-treated fish held at 22 °C at Sample time 3 (late March, data not shown). Relative gonad mass (expressed as GSI) was highest in E_2-treated fish at Sample 3, and higher in the control group at 14 °C and E_2-treated fish at 22 °C, than the control group at 22 °C at Sample 4 (April, Fig. 8.1).

Follicle diameter followed the same pattern of significance as GSI for the duration of the experimental period (Fig. 8.1).

There were no significant differences in absolute or relative fecundity between groups at any time (Fig. 8.2). However, fish from all groups showed the presence of atretic follicles in the ovary during vitellogenesis (Fig. 8.2), including quite high levels in fish held at 14 °C at Sample times 1, 2 and 3. At Sample 3, there were higher levels of atresia in E_2-treated fish held at 22 °C, than in the other 2 groups. At Sample 4, there were high levels of atresia in both groups of fish maintained at 22 °C. However, due to high levels of variation for both groups of fish at 22 °C, only the level of atresia for E_2-treated group at 22 °C was significantly different from fish maintained at 14 °C whose level of atresia appeared to decline from Sample 3 to 4. Histological characteristics of healthy, and atretic follicles are shown in Figs. 8.3 and 8.4, respectively.

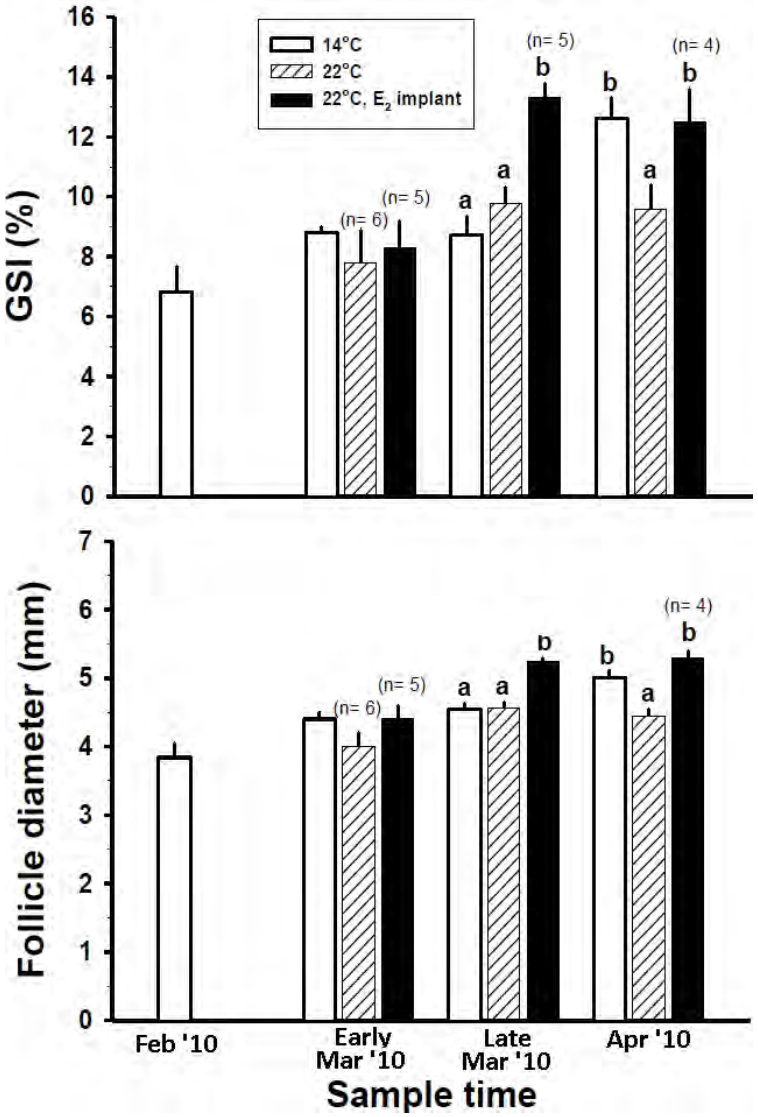

Figure 8.1 Gonadosomatic index (GSI) and follicle diameters among maiden Atlantic salmon without hormone pellet implants held at 14 °C, or 22 °C, and fish with E_2 pellet implants held at 22 °C. Values are means + SE (n = 7 unless stated otherwise). Different alphabetical superscripts at each sample time denote significant differences ($P<0.05$).

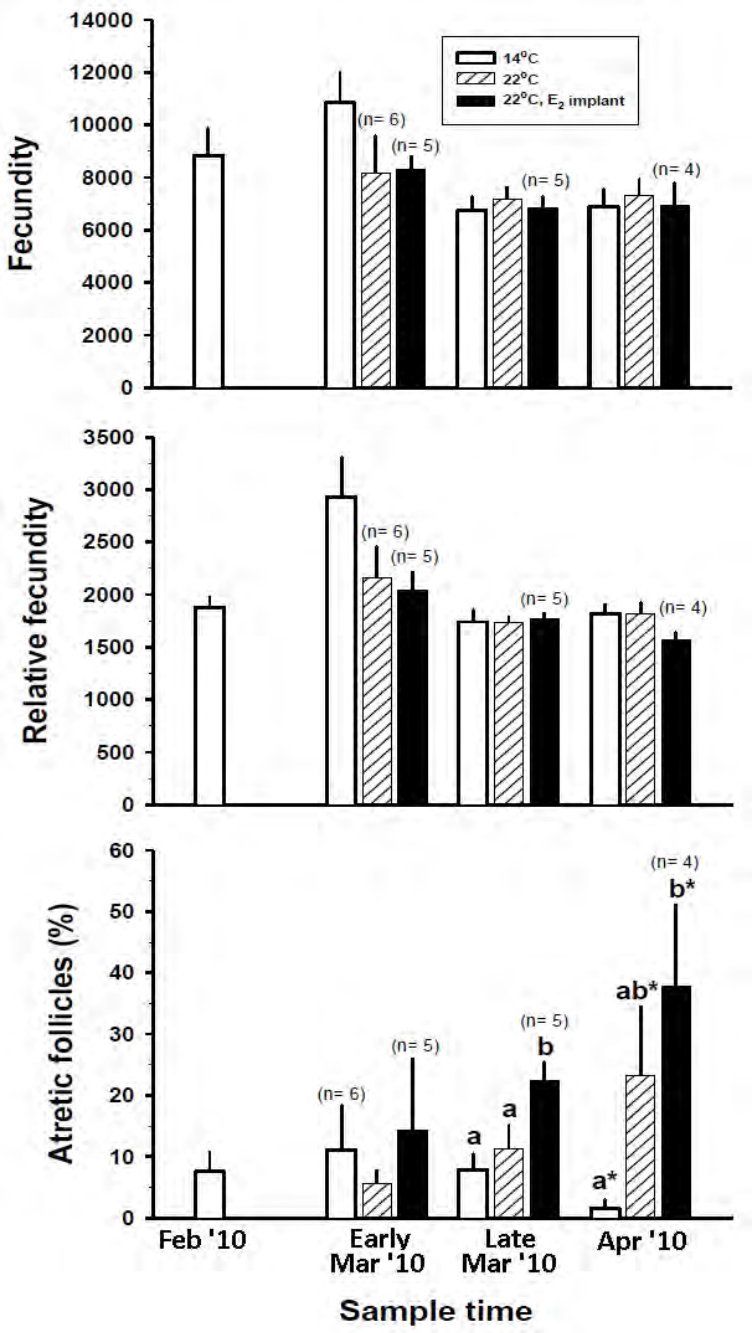

Figure 8.2 Absolute and relative fecundity and the proportions of atretic follicles in the ovaries of maiden Atlantic salmon without hormone pellet implants held at 14 °C, or 22 °C, and fish with E_2 pellet implants held at 22 °C. Values are means + SE (n = 7 unless stated otherwise). Different alphabetical superscripts at each sample time denote significant differences ($P < 0.05$) except for % atresia at Sample time 4 (*P<0.057; F = 3.486).

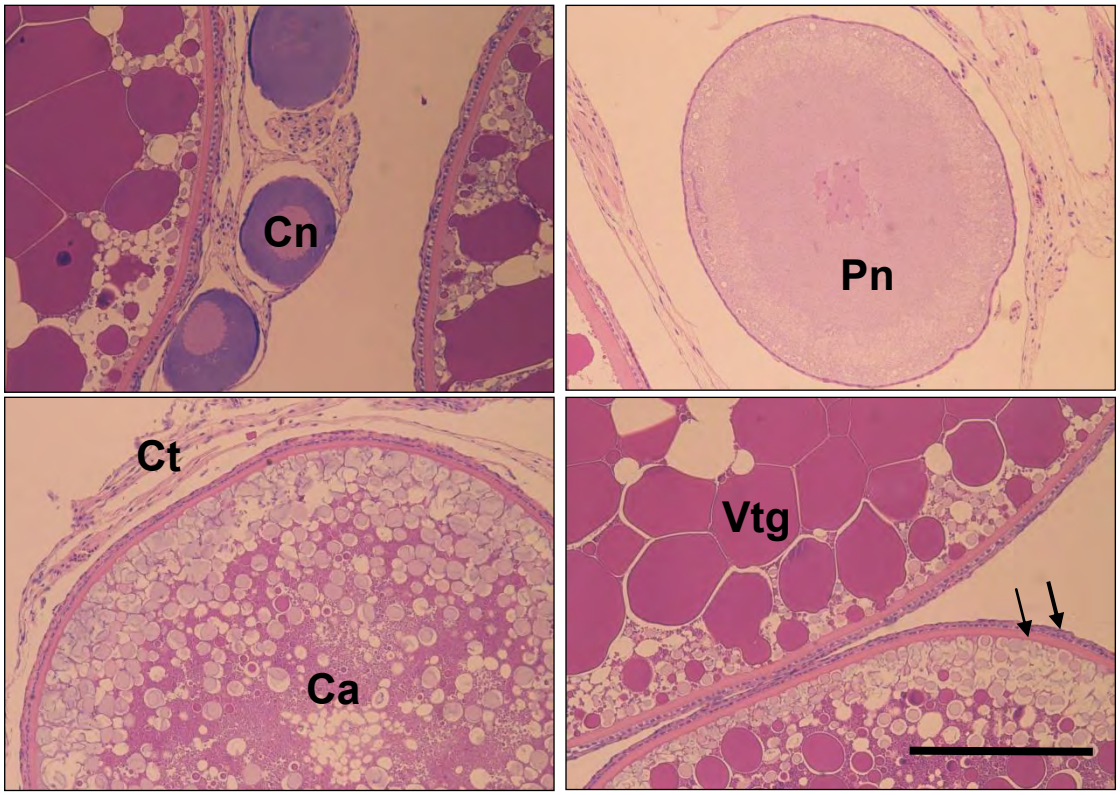

Figure 8.3 Micrographs of histological sections through a range of developmental stages of Atlantic salmon ovarian follicles showing healthy follicle structure: Cn – Chromatin nucleolus stage; Ca – Cortical alveolus stage; Pn - Perinucleolus stage; Vtg – Vitellogenic stage. Tissues were embedded in paraffin wax, sectioned at 5 µm, stained with hematoxylin and counterstained with eosin. Scale bar is 20 µm for all micrographs.

8.4.2 Ovulation, egg fertility and embryo survival

Fish held at 14 °C began ovulating in early June and had completed ovulation three weeks later (Fig. 8.5). The initiation of ovulation among untreated fish held at 22 °C was delayed by one week compared to fish maintained at 14 °C, and only 70 % of those fish had ovulated by the 22nd June. After the 22nd of June, no additional ovulation checks were performed as it was unlikely that the remaining fish would have ovulated, and in a commercial sense it was impractical to perform checks for an extended period of time. The pattern was similar in E_2-treated fish held at 22 °C, with the additional effect that only 50 % of the E_2-treated fish ovulated by the end of June (Fig. 8.5). For fish that did ovulate, there were no differences in absolute or relative fecundity between groups (Fig.

8.6). Egg diameters and volumes were larger in E_2-treated fish held at 22 °C than in both other groups, and eggs of untreated fish held at 14 °C were in turn larger than those from the control group at 22 °C (Fig. 8.6). In contrast, the reverse pattern was found for both egg fertility and survival to the eyed stage (Fig. 8.6). Fertility and survival was highest from untreated fish maintained at 14 °C, reduced in untreated fish at 22 °C, and further reduced in E_2-treated fish held at 22 °C.

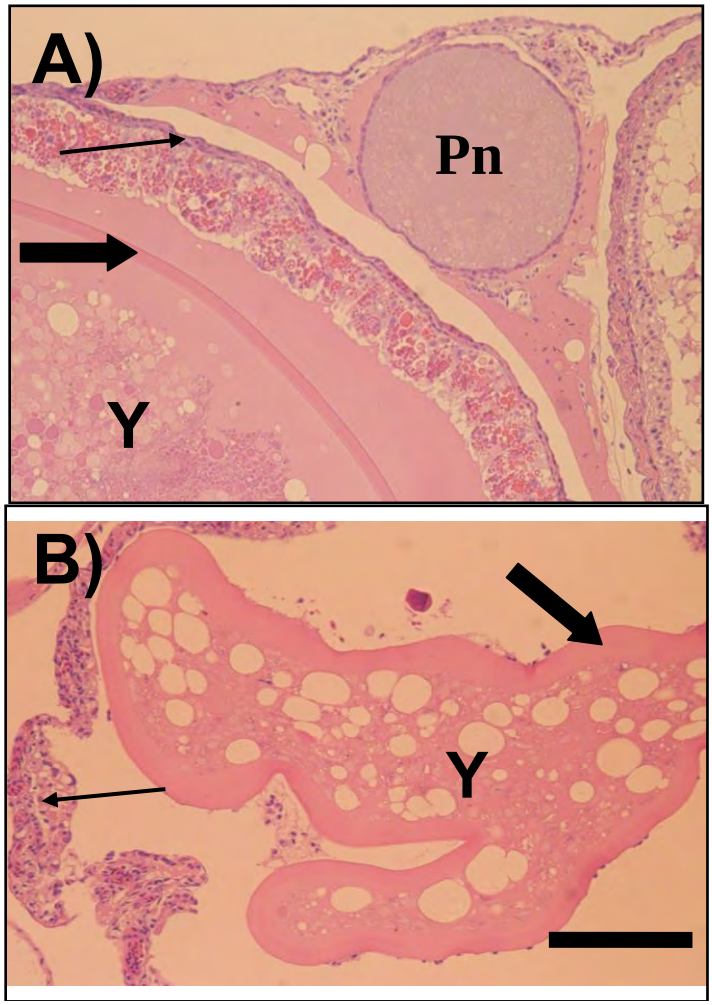

Figure 8.4 Photomicrographs of histological sections of atretic follicles. A) Perinucleolus (Pn) stage follicle lying adjacent to a vitellogenic follicle in which the onset of atresia was recent. B) Follicle showing advanced atresia in which most of the follicle contents have been resorbed. Thick arrows show remnants of the zona pellucida, and thin arrows indicate the thecal tissue. Y = yolk material. Scale bar is 10 µm for both micrographs.

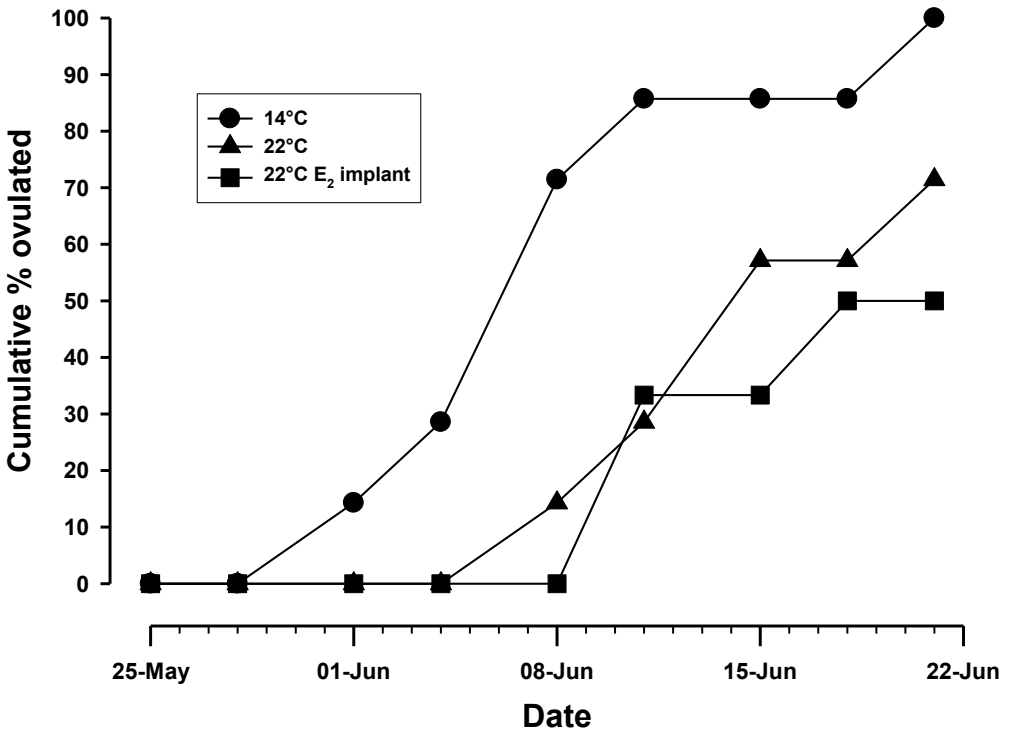

Figure 8.5 Cumulative ovulation in maiden Atlantic salmon spawners without hormone pellet implants held at 14 °C and 22 °C, and fish with E_2 pellet implants held at 22 °C during autumn.

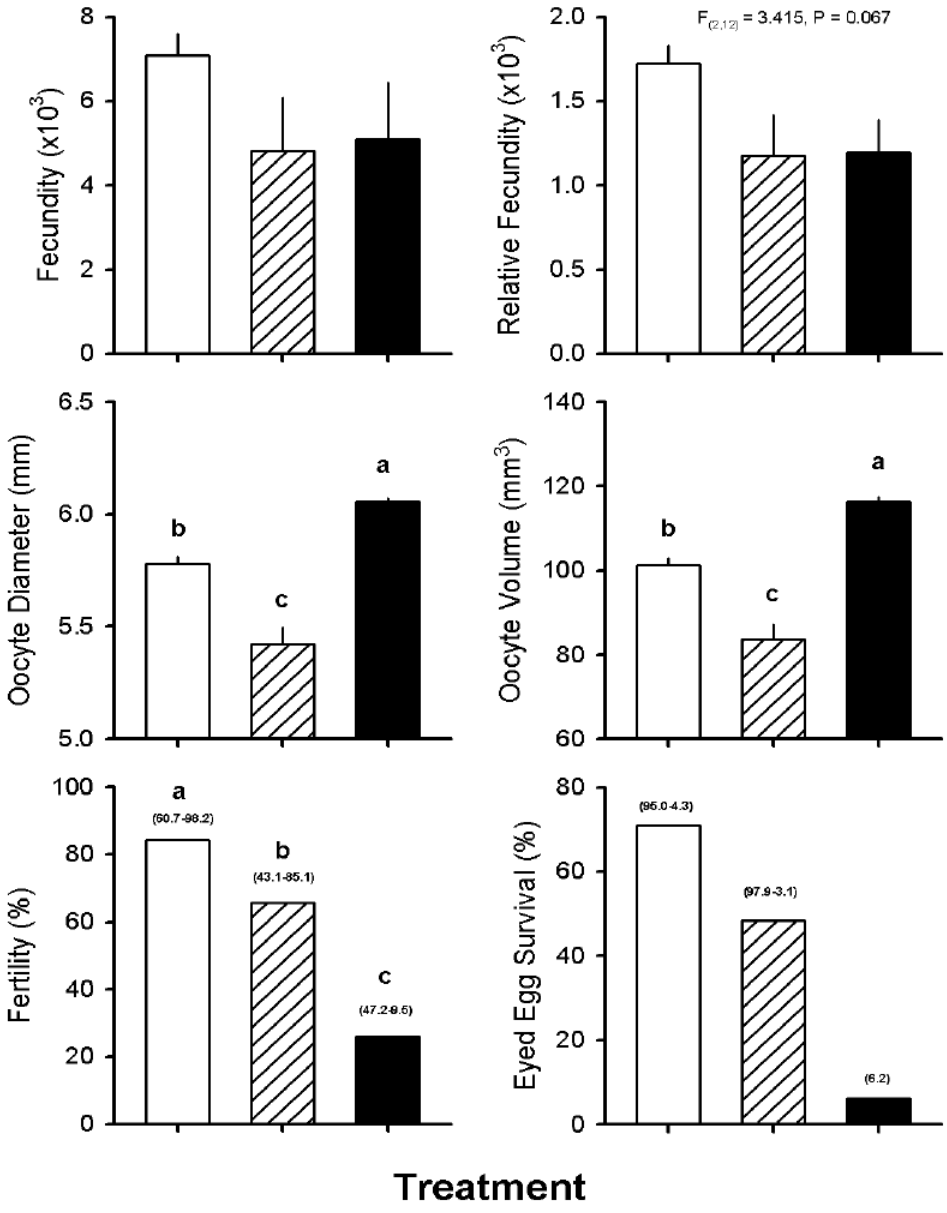

Figure 8.6 Post-ovulatory fecundity, fertility, oocyte diameter and volume, and survival to eyed egg stage among maiden Atlantic salmon without hormone pellet implants held at 14 °C (open bars) or 22 °C (cross-hatched bars), and fish with E_2 pellet implants held at 22 °C (black bars). Values are mean + SE (or 95 % confidence limits for % data) (n = 7, 5 and 3 for the three groups respectively; n = 1 for eyed egg survival in 22 °C + pellet group). For fertility and egg survival the numbers above each bar represent the 95% confidence interval. Different alphabetical superscripts among sample times denote significant differences ($P<0.05$) unless stated otherwise.

8.4.3 Plasma hormones and Vtg

Levels of plasma Fsh were low and very similar among all groups of fish (means ranged between 0.73 and 1.03 $ng.ml^{-1}$) at Sample points 1, 2 and 3 (data not shown). At Sample point 4, there were no statistically significant differences in the levels of plasma Fsh, although plasma Fsh was slightly higher in the 22 °C (1.61 ± 0.82 $ng.ml^{-1}$) than in the 14 °C group (0.80 ± 0.12 $ng.ml^{-1}$), and the E_2-treated group at 22 °C had an intermediate plasma Fsh level (1.06 ± 0.11 $ng.ml^{-1}$). Levels of plasma Lh ranged between (means) 0.55 and 0.81 $ng.ml^{-1}$ at Sample points 3 and 4, and no significant differences were detected between groups (data not shown).

Plasma T levels were depressed in maiden fish reared at 22 °C relative to 14 °C at Samples 2 and 4; while T levels in E_2-treated fish at 22 °C were lower than all other groups at Samples 2, 3 and 4 (Fig. 8.7). Plasma E_2 levels were suppressed in untreated fish held at 22 °C relative to 14 °C at Samples 2 and 3 (Fig. 8.7). E_2 levels were at an intermediate values in the E_2-treated group at Sample 2, but were the highest of any group at Sample 3. Plasma E_2 levels of all groups of fish were similar at Sample point 4. Plasma F levels were low (< 10 $ng.m1^{-1}$) in all fish at Samples 1 and 2 (Fig. 8.7). At Sample 3, plasma F was elevated in E_2-treated fish at 22 °C above those in untreated fish maintained at 14 and 22 °C. At Sample 4, plasma F levels were similar among treatment groups, but were generally higher than levels observed at other sample points. Plasma Vtg levels were relatively stable across sample times among fish held at 14 °C (Fig. 8.7). There was no significant suppression of plasma Vtg as a result of thermal challenge at any sample time; although levels were slightly lower in fish maintained at 22 °C relative to 14 °C at Samples 2 and 3. In contrast, plasma Vtg levels were elevated in E_2-treated fish held at 22 °C relative to untreated fish at 22 °C at Sample points 2, 3 and 4, and untreated fish at 14 °C at Samples 3 and 4.

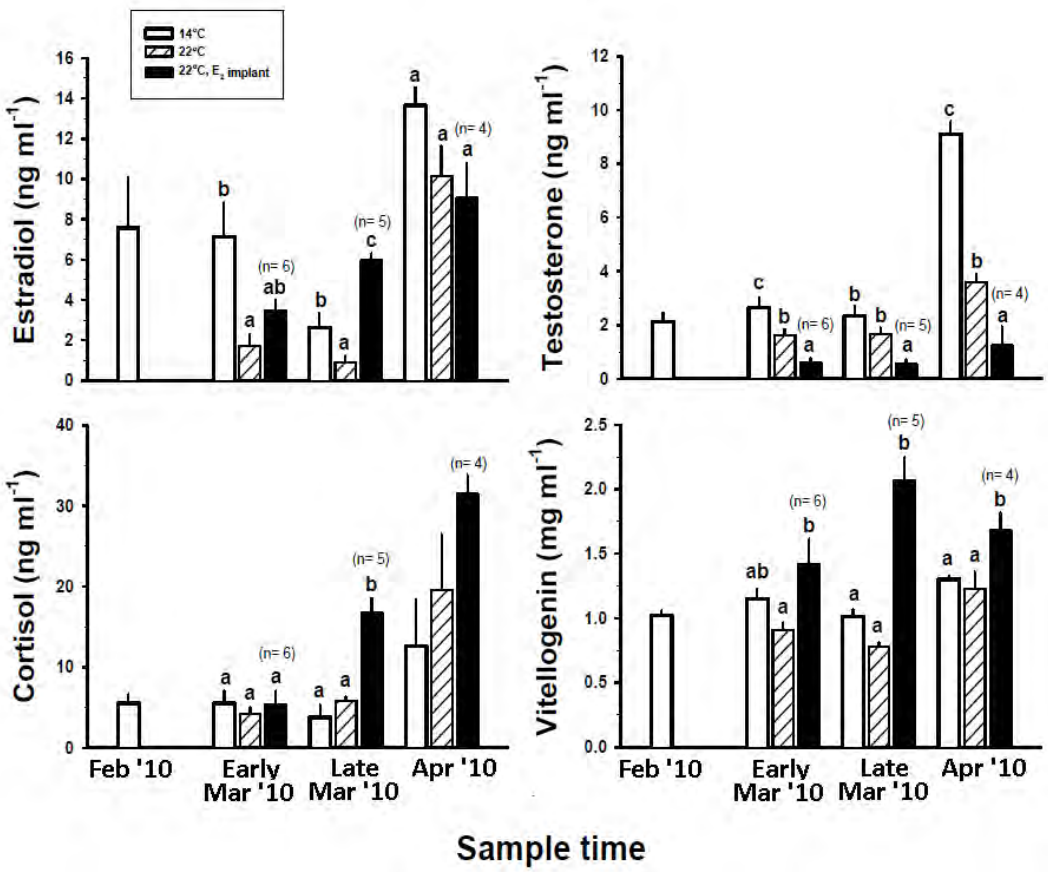

Figure 8.7 Plasma levels of 17β-estradiol, testosterone, cortisol and *vitellogenin* among maiden Atlantic salmon spawners without hormone pellet implants held at 14 °C or 22 °C, and fish with E_2 pellet implants held at 22 °C. Values are means + SE (n = 7 unless stated otherwise). Different alphabetical superscripts among sample times denote significant differences ($P<0.05$).

8.4.4 Hepatic gene expression

vtg gene expression was similar among all groups at Sample 2, and gene expression was higher in E_2-treated fish at 22 °C than all other groups at Sample 3 (Fig. 8.8). At Sample 4, there was no significant difference between the *vtg* gene expression levels of any group. *Zpc* gene expression levels were similar among groups of fish at Sample 2; however, gene expression was higher in the treated and untreated groups at 22 °C compared to the control at 14 °C at Sample 3 (Fig. 8.8). At Sample 4, thermal suppression of *zpc* gene expression was observed in the untreated and E_2-treated groups at 22 °C relative to the untreated group at 14 °C. Similarly to *zpc*, *zpb* gene

expression levels were not different between groups at Samples 2 and 3; but thermal suppression was present in both groups maintained at 22 °C at Sample 4 (Fig. 8.8). There were no significant differences in *erα* gene expression levels at any sample time, except for Sample 3, where expression was higher in the 22 °C control group relative to all other groups (Fig. 8.8).

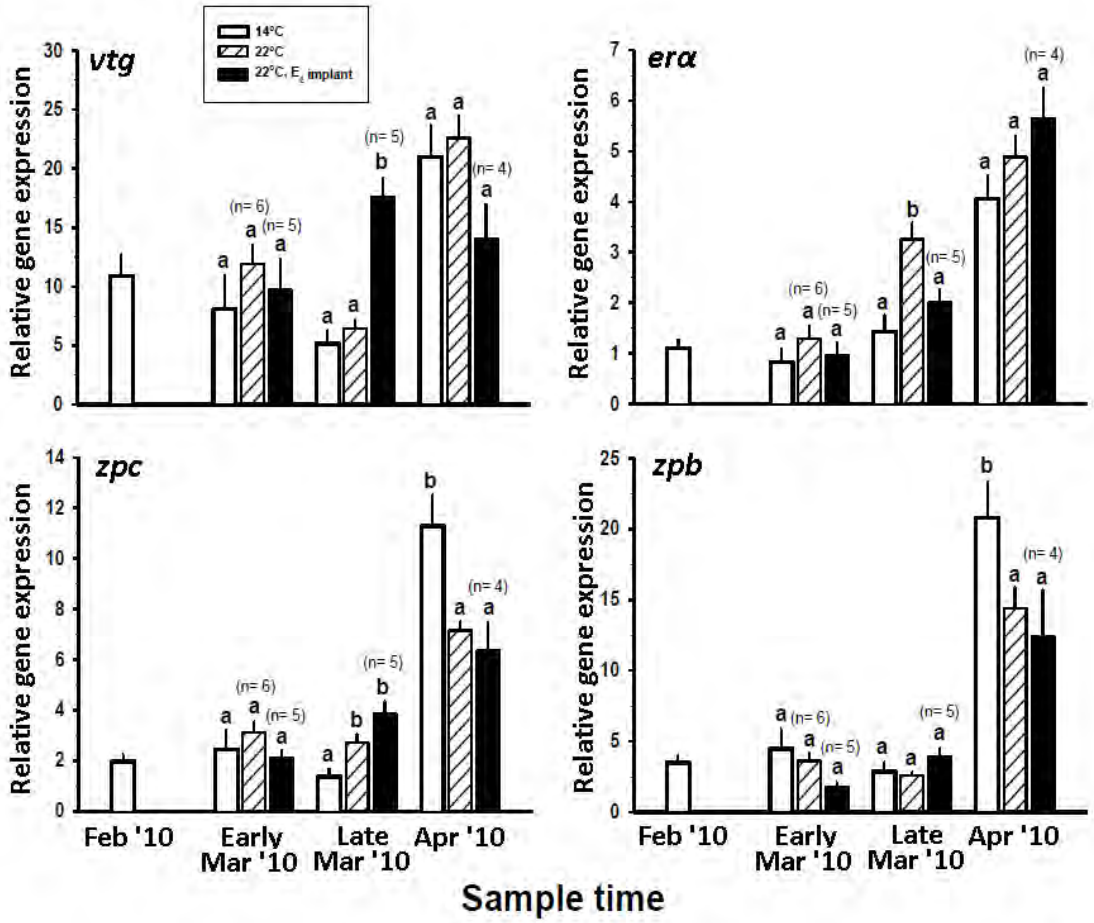

Figure 8.8 Relative gene expression of *vitellogenin*, *estrogen receptor alpha*, *zona pellucida b* and *zona pellucida c* among maiden Atlantic salmon without hormone pellet implants held at 14 °C (open bars) or 22 °C (cross-hatched bars), and fish with E_2 pellet implants held at 22 °C (black bars). Values are means + SE (n = 7 unless stated otherwise). Different alphabetical superscripts among sample times denote significant differences ($P<0.05$).

8.5 Discussion

The present study was performed to determine whether E_2-therapy during vitellogenesis could offset the negative effects of thermal challenge on oocyte development, endocrine function and subsequent egg quality in maiden spawning Atlantic salmon. Temperature and/or hormonal treatment had no effect on fish length, weight or condition factor indicating that the experimental outcomes were not biased by these parameters. In contrast, follicle diameter and subsequently GSI were higher in E_2-treated fish at 22 °C than in fish from untreated groups maintained at 14 and 22 °C at Sample 3 (late March), and untreated fish maintained at 22 °C at Sample 4 (April). It is established that ovarian growth occurs primarily as a result of Vtg uptake into the oocyte (Tyler et al., 1988). In the present study, E_2-treated fish at 22 °C displayed higher levels of plasma Vtg than the control group at 22 °C at Samples 2, 3 and 4 (see following discussion), and this was reflected by an increase in the rate of oocyte growth in E_2-treated relative to untreated fish at elevated temperature. These results demonstrate that E_2-treatment helped to maintain oocyte growth at 22 °C, and imply that the mechanisms governing Vtg uptake into developing oocytes are not impaired at high temperature in salmonids. This is in agreement with the findings of Tyler et al. (1987) who demonstrated that *in vitro* maintenance of rainbow trout follicles at 25 °C did not inhibit the uptake of Vtg from the culture medium relative to follicles held at 0, 5, 10, 15 and 20 °C (Tyler et al., 1987).

There was no significant difference in absolute or relative fecundity between the groups during reproductive development. However, histological analysis revealed the presence of atretic follicles in all fish (including those maintained at 14 °C) at all sample times. The presence of atresia in maiden fish reared at 14 °C was unexpected as our previous work has shown that atresia is either absent or affects very few follicles in maiden fish maintained at 14 °C (Ch 4: Pankhurst et al., 2011). Inspection of temperature records for the period prior to experimental set up showed that the maiden broodstock had experienced temperatures of between 18-22 °C during early

February 2010, whereas ambient pre-set up temperatures from our previous broodstock experiments did not exceed 17 °C. This occurred due to higher-than-normal ambient temperatures during summer in combination with a delay of experimental set-up due to an approaching bushfire. Therefore it appears that the maiden fish used in this study had some degree of unanticipated thermal exposure during February, a month that corresponds to a period of vitellogenesis that is particularly sensitive to high temperature (King et al., 2007). It appears that fish maintained at 14 °C recovered somewhat from the pre-set up thermal exposure as evidenced by a steady decrease in the rate of ovarian atresia from Sample 2 onwards. On the contrary, there was no evidence of recovery in fish reared at 22 °C, and the number of atretic follicles was generally higher in fish that received an E_2 implant relative to the control group at 22 °C. The presence of atretic follicles in both groups of fish maintained at 22 °C throughout development is broadly consistent with the general effects of elevated temperature on gonadal morphology in fish (Linares-Casenave et al., 2002, Soria et al., 2008, Ch 4: Pankhurst et al., 2011). In the present study, E_2-treated fish displayed an increased rate of atresia even though oocyte growth was promoted at 22 °C. In fathead minnow (*Pimephales promelas*) waterborne exposure to E_2 (27.3 $ng.l^{-1}$) resulted in a high incidence of ovarian atresia (Miles-Richardson et al., 1999). However, plasma E_2 levels in E_2-treated fish at 22 °C did not appear to exceed 12 $ng.ml^{-1}$ which is below the concentration of plasma E_2 observed in maiden fish (up to 20 $ng.ml^{-1}$) reared at 14 °C from our previous experiments that did not display significant ovarian atresia (Ch 4: Pankhurst et al., 2011). On the other hand, plasma cortisol levels were significantly higher in E_2-treated fish Sample 3 and higher to some extent at Sample 4 relative to untreated fish at 14 and 22 °C. This implies that the second hormonal implantation was perceived as stressful. In many species, stress and the subsequent production of cortisol has been linked to an increased incidence of ovarian atresia during reproductive development (Clearwater and Pankhurst, 1997, Cleary et al., 2000). Therefore it is possible that atresia occurred as a part of a stress response to E_2-implantation and not inappropriate levels of plasma E_2 per se.

Timing of ovulation was similar between the E_2-treated and control group at 22 °C, and the initiation of ovulation for these groups was delayed by 1 week relative to the control group at 14 °C. There is evidence suggesting that in salmonids, delays in ovulation at high temperature occur as a result of dopamine mediated inhibition of Lh secretion (Gillet et al., 1996) and suppression of plasma maturation-inducing hormone (King and Pankhurst, 2004). Unfortunately our sampling schedule did not capture the pre- rise in plasma Lh that typically occurs prior to ovulation, and basal plasma levels of Lh did not differ among groups up until at least 50 days prior to the onset of ovulation. This, combined with the fact that the timing of ovulation was similar between treated and untreated groups at 22 °C, suggests that implantation with E_2 did not significantly affect the events associated with FOM and ovulation in those fish that ovulated successfully. However, only 50 and 70 % of E_2-treated and untreated fish respectively ovulated at 22 °C compared to 100 % in the 14 °C control group. This may indicate that thermal exposure in combination with stress related to E_2-implantation inhibited ovulation in some fish, although any such inhibitory effects of temperature and cortisol were not reflected by the Lh data.

For fish that had ovulated by the end of June, absolute and relative fecundity did not change as a result of the experimental conditions. The largest and smallest eggs were produced by E_2-treated and untreated fish respectively at 22 °C, and fish from the 14 °C group produced eggs of an intermediate size. The production of large eggs in E_2-treated fish is consistent with the higher concentration of plasma Vtg and presumably Vtg uptake observed for this group at Samples 3 and 4 relative to all other groups. These results indicate that treatment with E_2 is able to offset the negative impacts of high temperature on egg size typically observed in Atlantic salmon. However, the stimulatory effect of E_2-treatment on oocyte growth did not translate into an increase in egg fertility or embryo survival. In fact, egg fertility and embryo survival from E_2-treated fish at 22 °C were markedly reduced relative to untreated fish maintained at 14 and 22 °C. While treatment with natural synthetic estrogens has been linked to reductions in egg quality at some concentrations (Lahnsteiner et al., 2006, Peters et al., 2007), it

appears that in the present study, plasma E_2 levels in E_2-treated fish at 22 °C were generally similar to those observed in the untreated group at 14 °C that displayed a higher level of egg fertility and survival. In addition, previous research has demonstrated that plasma E_2 levels are stable for between 7-14 days after implantation of a single silastic E_2 pellet in other species (Pankhurst et al., 1986) and juvenile Atlantic salmon (Ch 7). Therefore it is unlikely that the E_2 profiles in the present study were so inappropriate that egg quality was dramatically reduced as a result of E_2-implantation. On the other hand, plasma cortisol levels were elevated in the E_2-treated group after receiving a second hormonal implant which is an indicator of stress as previously mentioned. Stress is able to reduce reproductive performance in many species (reviewed in Schreck, 2010), and it is possible that egg quality was lower in E_2-treated fish as a result of the implantation process. However, this is hard to assess in the absence of data from fish receiving a blank implant, or a hormonal treatment via less invasive means. Plasma cortisol levels were not elevated after the first round of hormonal treatment, which suggests that use of a single implant with a slower rate of hormone release warrants investigation.

Plasma T was lower in untreated fish reared at 22 °C relative to 14 °C at Samples 2 and 4, and plasma T was further suppressed in E_2 treated fish relative to the 22 °C control groups at Samples 2, 3 and 4. Thermal inhibition of gonadal Fshr and *cholesterol side chain cleavage protein* (*cyp11a1*) gene expression is thought to contribute to impairment of T production in pejerrey and Atlantic salmon respectively (Soria et al., 2008, Ch 5: Anderson et al., 2012). In addition, E_2-treatment is able to regulate Cyp19a1a through a positive or negative feedback in the ovary depending on the species and stage of reproductive development (reviewed in Guiguen et al., 2010). Therefore, if a positive feedback loop was functioning at high temperature, plasma T may have been further depressed in E_2-treated fish due increased Cyp19a1a mediated conversion of T to E_2. However, this is unlikely as the gene expression of *cyp19a1a* is typically down-regulated in response to elevated temperature in both adult and juvenile fish (Lim et al., 2003, van Nes and Andersen, 2006, Ch 5: Anderson et al., 2012). On the other hand,

exogenous treatment with natural or synthetic estrogen has been shown to down-regulate the gonadal gene expression of various steroidogenic enzymes in several species (Kishida and Callard, 2001, Nakamura et al., 2009, Hogan et al., 2010). Therefore, it is possible that the additive effects of thermal and E_2-treatment induced impairment of steroidogenesis underpinned the significant depression of T levels in E_2 treated fish at 22 °C relative to all other groups.

For fish reared at 14 °C in the present study, plasma E_2 levels were generally lower than those observed in our previous work on Atlantic salmon reared at the same temperature (Ch 4: Pankhurst et al., 2011, Ch 6) which may be a lasting effect of thermal exposure prior to experimental set-up. E_2-treatment at 22 °C successfully elevated plasma E_2 to a level that was similar to, and then higher than the 14 °C control group at Samples 2 and 3 respectively, even though plasma levels of T were depressed in the E_2 treated group relative to all other groups. This demonstrates that E_2 implantation can effectively recover plasma E_2 levels in thermally challenged female Atlantic salmon, which is broadly consistent with previous studies on salmonids where treatment with exogenous E_2 raised plasma E_2 levels at high temperature (Mackay and Lazier, 1993, Ch 7).

In the present study, there was no evidence to suggest that E_2-treatment affected hepatic *erα* gene expression at any point during reproductive development. This is contradictory to many studies on salmonids where E_2-treatment resulted in an increase in the levels of hepatic *erα* mRNA at normal and elevated temperatures (MacKay et al., 1996, Westerlund et al., 2001, Arukwe et al., 2002, Ch 7). The apparent discrepancy between the present and aforementioned studies may have occurred as a result of cortisol mediated inhibition of *erα* gene expression. For example, in vitellogenic rainbow trout maintained at 12-15 °C, treatment with cortisol down-regulated the hepatic gene expression of *er* (Lethimonier et al., 2000). Therefore, the expected stimulatory effect of E_2-treatment on hepatic *erα* may have been offset by cortisol in the present study. Nevertheless, *erα* gene expression in fish maintained at 22 °C was not suppressed relative to fish reared at 14 °C, consistent with previous studies

(Pawlowski et al., 2000, Ch 7), and E_2-treated fish displayed *erα* gene expression levels that were similar to those observed in untreated fish at 14 and 22 °C for the duration of the experiment (except for the 22 °C untreated group at Sample 3). Therefore, even though both E_2-treatment and plasma cortisol are able to modulate *erα* gene expression in many species, it appears that there was no net change in *erα* gene expression in response to either of these variables in the present study.

Treatment with E_2 at 22 °C significantly increased *vtg* gene expression at Sample 3 relative to all other groups which is broadly consistent with the role of E_2 in Vtg regulation (Lim et al., 1991, Berg et al., 2004). Vtg up-regulation in response to E_2 demonstrates that hepatic responsiveness is maintained in adult broodstock at 22 °C, even though previous research in tilapia (*Oreochromis aureus*) and Atlantic salmon has shown that hepatic Er binding affinity is reduced at elevated temperature (Tan et al., 1999, Watts et al., 2005). This corroborates the findings from our previous work on E_2-treated juvenile Atlantic salmon where *vtg* transcription in fish held at 22 °C was not impaired relative to fish reared at 14 °C (Ch 7). At Sample 2, plasma Vtg was elevated in the E_2-treated group relative to the control at 22 °C prior to an increase in *vtg* mRNA at Sample 3. This may indicate that treatment with E_2 augmented the stability of *vtg* mRNA as observed in rainbow trout (Flouriot et al., 1996) which could make the mRNA available for translation for a longer period of time. In addition, E_2 exposure in combination with high temperature could have increased the rate of *vtg* mRNA translation relative to untreated groups, as observed previously in rainbow trout (Mackay and Lazier, 1993). For the remaining 2 Sample points, plasma Vtg was significantly higher in the E_2-treated group held at 22 °C than in all other groups, which is consistent with an increase of *vtg* mRNA and subsequent accumulation of Vtg in blood. It is promising that E_2-treatment elevated plasma Vtg levels given that fact that cortisol was also elevated in this group, and cortisol is known to have a deleterious effect on plasma Vtg levels in salmonids (Berg et al., 2004). In addition, a recent review by Guiguen et al. (2010) highlights the fact that even though E_2-treatment may have a suppressive effect on steroidogenesis in some species, exogenous E_2 is still able to

independently induce female-specific pathways during sexual differentiation. For example, in genetically male rainbow trout treated with EE_2 at 10 °C, levels of gonadal *cyp19a1a* gene expression were similar to those observed in untreated genetic males, and lower than those found in untreated genetic females (Vizziano-Cantonnet et al., 2008). Despite this, EE_2-treated males were feminised, and *cyp19a1a* gene expression was restored in the feminised gonads after EE_2-treatment had ceased (Vizziano-Cantonnet et al., 2008). Consistent with previous studies on the relationship between steroidogenesis and E_2-treatment during sexual differentiation, we have also demonstrated that E_2-treatment is a useful strategy for promoting female specific processes at 22 °C in adult fish, even though it is likely that gonadal steroidogenesis was thermally inhibited.

At temperatures within normal ranges, treatment with E_2 strongly induces Zp synthesis in salmonids such as rainbow trout and Arctic charr (*Salvelinus alpinus*) (Arukwe et al., 2002, Berg et al., 2004). However, in the present study, there was no evidence suggesting that E_2-treatment at 22 °C altered the hepatic gene expression of either *zpb* or *zpc* at any time during oocyte development. In a recent study on juvenile salmon, we recently demonstrated that E_2-implantation could induce *de novo* gene expression of *zpb* and *zpc* at 14 and 22 °C, however the magnitude of the response was greater at 14 °C for both genes (Ch 7). Therefore, there is mounting evidence to suggest that Vtg and Zps respond differentially to E_2-treatment at elevated temperature, although the cellular basis for this is currently unclear. Previous studies have shown that Er binding affinity is reduced at high temperature (Tan et al., 1999, Watts et al., 2005), and a reduction in E_2 signal transduction may explain the lack of zonagenic response to E_2-treatment at 22 °C. However, in our study this is unlikely to be the explanation, as Er-dependent Vtg synthesis was stimulated by E_2-treatment at 22 °C, implying that this pathway is still functional to a large extent at high temperature. It has been suggested previously that Vtg and Zps may be regulated by different mechanisms (Berg et al., 2004) and the promoter regions of hepatically expressed Zp genes appear to be more complex than those found in Vtg genes (Lyons et al., 1993, Teo et al., 1998,

Kanamori et al., 2003, Bouter et al., 2010). Therefore, the apparent lack of *zp* stimulation by E_2 implies that factors regulating the transcription of Zp but not Vtg genes are impaired at elevated temperature in both juvenile and adult Atlantic salmon, although the exact mechanism by with this occurs is yet to be explored.

Loss of Zp integrity in fertilised eggs has been linked to reduced egg viability in farmed Atlantic and chinook salmon (*Oncorhynchus tshawytscha*) (Barnes et al., 2003, King et al., 2003). In Atlantic salmon, the thermal sensitivity of hepatic *zp* gene expression during oocyte development has been previously demonstrated, and it is thought that Zp damage is present prior to spawning as a consequence (King et al., 2003, Ch 4: Pankhurst et al., 2011). Therefore, the reduction of *zp* gene expression in both groups of fish maintained at 22 °C relative to 14 °C at Sample 4 is consistent with our previous work, and could have contributed to the lower egg fertility and embryo survival observed in both groups maintained at 22 °C. However, at this time it is not clear whether only Zp transcription is depressed, or whether down-stream *zp* mRNA translation and subsequent protein assembly around the oocyte are also influenced by temperature.

The present study trialled E_2-therapy as a novel means of elevating plasma E_2, maintaining downstream E_2-dependent endocrine function and promoting high egg quality in maiden Atlantic salmon reared at elevated temperature. Treatment with E_2 at 22 °C stimulated *vtg* gene expression and subsequent protein synthesis which indeed promoted oocyte growth and increased egg size relative to untreated fish at 14 and 22 °C, but did not improve egg fertility and embryo survival. This could be due to the fact that treatment with E_2 did not offset the negative effects of maintenance at high temperature on the hepatic expression of Zp genes during oocyte development. There was no evidence to suggest that thermal impairment of Zp genes occurs as a result of lowered hepatic *erα* gene expression and thus, the cellular basis for the differential response of *vtg* and *zp* genes to E_2-treatment is currently unclear. In addition, the apparent stress caused by the double implant protocol in the present study seems to have resulted in a further reduction in egg quality in E_2-treated fish maintained at 22 °C,

and therefore may not be an appropriate means of hormonal delivery. Findings from the present study highlight that thermal impairment of endocrine function is complex, and our ability to promote egg quality at high temperature is reliant not only on the hormone chosen but the delivery method also. The development of alternate hormonal therapies (for example Gnrha and E_2) and delivery protocols may still warrant investigation, especially given the fact that large-scale water cooling is not a viable option for much of the production sector.

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Chapter 9- General discussion

In wild salmon, small variations in temperature can advance or delay the timing of ovulation which helps to ensure that alevins hatch at a time when conditions are optimal (Bromage et al., 2001). The upper-limit of thermal tolerance for Atlantic salmon has been reported as 22-23.5 °C (Pennell and Barton, 1996) although Tasmanian broodstock can encounter temperatures above 20 °C during summer (King and Pankhurst, 2000, Battaglene et al., 2008). As a consequence, even slight increases in temperature have a marked effect on physiology and are associated with impairment of reproductive development and inhibition of ovulation (King and Pankhurst, 2004, Watts et al., 2004). Due to the significant cost of setting up and maintaining broodstock rearing facilities that can cool large volumes of water, thermal control within hatcheries is not an economically viable option for much of the production sector. This has created a need for novel broodstock management strategies that maintain egg quality in the face of thermal challenge that occurs as a result of seasonal fluctuation or future climate change, where temperatures are expected to rise further (Battaglene et al., 2008).

The salmon industry's ability to adapt to such changes is hindered by a lack of understanding of the effects of thermal challenge on the endocrine system which ultimately determines egg quality. This matter is further complicated by the fact that the Tasmanian salmon industry uses maiden spawning fish for approximately 75 % of egg production due to their smaller size and the lower cost associated with their husbandry relative to repeat spawning fish. However, maidens appear to be more susceptible than repeat spawning fish to the effects of elevated temperature in terms of egg fertility and embryo survival which means that it is more difficult to predict egg quality when maidens are relied upon for production. Understanding the molecular basis for reduced egg fertilisation and embryo survival rates in both maiden and repeat spawning fish will enable the development of targeted management strategies that maintain normal endocrine function, promote high egg quality, and ensure industry

confidence in years of high temperature. Predictability of smolt production at current and predicted summer temperatures has become an industry priority, and thus has formed the underlying basis for the questions addressed in this thesis.

As a first step, the molecular tools required to quantify the relative abundance of transcripts encoding genes that are relevant for reproductive function had to be developed for Atlantic salmon. Previous research has demonstrated that E_2 synthesis and subsequently Vtg production are impaired at high temperature (King et al., 2007), and it has been previously suggested that thermal inhibition of Cyp19a1a in combination with reduced Er binding affinity are at least partly responsible (Watts et al., 2004, Watts et al., 2005). For this reason ovarian enzymes that are involved in the stepwise production of E_2 (Cyp19a1a and Cyp11a1), E_2 signal transduction (Erα) and oocyte growth (Vtg) were chosen for qPCR analysis. In addition, exposure to elevated temperature resulted in a loss of zona pellucida integrity and a decrease in egg quality in Tasmanian Atlantic salmon which raised questions concerning the hepatic synthesis of Zp proteins at high temperature (King et al., 2003). Therefore, potential changes in the expression patterns of *zpb* and *zpc* genes at elevated temperature were of great interest, and were assessed in the present study. All of the mRNA sequences required to design gene specific qPCR primes were freely available from GenBank except for Zpc. Therefore a partial Zpc cDNA was amplified using degenerate primers, cloned, sequenced and submitted to GenBank. qPCR assays were subsequently developed, validated and made publically available for the aforementioned genes and the candidate reference genes Hprt1, Ef1α, Tbp and β-tubulin. The expression stabilities of candidate reference genes were then analysed in all relevant tissues on an experiment-to-experiment basis using freely available stability assessment programs and independent statistical analysis (Ch 3: Anderson and Elizur 2012). Results from GeNorm, NormFinder and BestKeeper were quite ambiguous, however the Kruskal-Wallis test combined with Bonferroni's correction, and assessment of candidate gene expression variation suggested that Tbp was the most, and β-tubulin was the least stably expressed gene in liver and ovary tissue from all experiments involving adult broodstock (Chs 3-6 and 8).

In contrast Ef1α and Tbp were the most and least stably expressed genes respectively in the E_2-treatment trial with juvenile salmon (Ch 3: Anderson and Elizur 2012, Ch 7). As part of the validation process, the consequence of using an unstable reference gene for target gene expression normalisation was demonstrated. The use of β-tubulin resulted in a loss of the ability to detect treatment-dependent changes in hepatic *vtg* gene expression at two Sample points in the first large-scale broodstock experiment. This result, in combination with previous reports (Dheda et al., 2005, Filby and Tyler, 2007) shows that without stringent reference gene validation significant experimental error may be introduced as a result of normalisation. In addition, the present research has highlighted the need to determine candidate reference gene stability on an experiment-to-experiment basis, and independently confirm the results obtained from freely available stability assessment algorithms. In accordance with increasing concern about, and subsequent research into the physiological effects of climate change, we have also provided a reference point for those wishing to use relative qPCR to study the effects of thermal challenge and/or hormonal treatment on expression of target genes in Atlantic salmon and other teleost species.

According to industry production data, eggs originating from maiden Atlantic salmon are typically of lower quality than those from repeat spawning salmon. Similar observations have been made in striped bass and Atlantic halibut, where there is evidence suggesting that fish are more likely to produce biochemically inferior eggs during their first reproductive cycle relative to subsequent cycles (Evans et al., 1996, Holland et al., 2000). If full reproductive capacity is not attained during pubertal development in maiden spawning fish, it is said that the first cycle of reproductive development is a 'dummy run'. We were interested to know not only if maiden fish produce eggs of lower quality under commercial conditions, but whether egg quality is reduced due to a general lack of reproductive competence as observed in other species exhibiting the dummy run phenomenon. As such, the aim of the first large-scale experiment was to compare the molecular mechanisms that underpin reproductive development and determine egg quality in maiden and repeat spawning fish under

optimal and challenging thermal regimes (Ch 4: Pankhurst et al., 2011, Ch 5: Anderson et al., 2012).

The present work confirmed industry observations that maiden fish are more susceptible to the effects of elevated temperature in terms of egg fertility and embryonic survival. However, it is unlikely that the basis for the differential response to temperature occurred due to a lack of reproductive competence during pubertal development in maidens. In striped bass, pubertal development was associated with a 34-fold increase in pituitary *fshβ* subunit mRNA from basal levels compared to a 218-fold increase in four year olds (Hassin et al., 1999). Similarly in rainbow trout plasma levels of Fsh were significantly higher in repeat spawning fish than in maidens (Prat et al., 1996). In contrast, our results showed that plasma Fsh levels were actually higher in maiden spawning fish during early vitellogenic development (before fish were divided into thermal manipulation groups) due to the initially slower rate of development displayed by repeat spawning fish as they recover from the energetic demands of the previous reproductive cycle (Ch 5: Anderson et al., 2012). Repeat spawning fish had caught up by mid-vitellogenesis as their CF increased, and for the remainder of the vitellogenic growth phase plasma Fsh levels were similar between maiden and repeat spawning fish maintained at 14 °C. Furthermore, the levels of plasma hormones and Vtg, and hepatic gene expression levels of *vtg*, *zpb* and *zpc* in maiden spawning fish were similar to or higher than those in repeat spawning fish throughout reproductive development (Ch 4: Pankhurst et al., 2011). Therefore it appears unlikely that maiden Atlantic salmon reared at 14 °C display the dummy run phenomenon that has been observed in other species during pubertal development. Another unique finding from the present research was that plasma Fsh levels increased in maiden and repeat spawning salmon maintained at 22 °C relative to 14 °C. E_2 is a known regulator of Fsh and is able to exert negative feedback on the pituitary during reproductive development (Dickey and Swanson, 1998, Saligaut et al., 1998). Since plasma E_2 was suppressed in response to high temperature in both maiden and repeat spawning salmon, it is possible that plasma Fsh levels were elevated due to a lack of negative feedback on Fsh

production by E_2. However, the higher levels of plasma Fsh did not cause a subsequent increase in E_2, suggesting that thermal suppression occurred in the gonad, perhaps at the level of *fshr* gene expression as observed in adult female pejerrey exposed to high temperature (Soria et al., 2008).

In both maiden and repeat spawning fish, exposure to elevated temperature suppressed plasma E_2 and T, and concurrently gene expression levels of *vtg*, *zpb* and *zpc* were reduced (Ch 4: Pankhurst et al., 2011). This was reflected by a reduction in egg quality in both groups of fish at 22 °C. Plasma levels of Vtg were generally similar between maiden and repeat spawning fish after introduction to the 14 and 22 °C treatments; however, there was one notable difference. Plasma Vtg was lower in maiden than repeat spawning fish in February, which is recognised as a period of increased thermal sensitivity. Previous studies on Atlantic salmon have shown that temperature exposure during February alone can have a lasting negative impact on egg quality (King et al., 2007). Therefore the increased susceptibility of maiden fish in February may have been enough to lower egg quality more severely, especially in combination with the reduced ability of maiden fish to sequester factors that promote egg quality due to their relatively smaller oocyte size during the same month. In the present study, there was no evidence to suggest that hepatic *erα* gene expression levels were modified as a result of exposure to high temperature which is in line with previous observations for thermally challenged rainbow trout and Atlantic halibut (Pawlowski et al., 2000, van Nes and Andersen, 2006). However, a study by Watts et al. (2005) demonstrated that maintenance at high temperature reduced the binding affinity of hepatic Ers. Therefore, diminished Er binding affinity could have potentiated the effects of lower plasma E_2 levels.

Prior to the present research, it had been proposed that thermal inhibition at the level of gonadal Cyp19a1a played an important role in impairing the synthesis of E_2 and subsequently plasma Vtg in fish that produce eggs of inferior quality (Watts et al., 2004). By establishing that gene expression levels of *cyp19a1a* are suppressed in maiden and repeat spawning fish maintained at 22 °C relative to 14 °C during vitellogenesis, we have

provided the first direct evidence supporting the link between elevated temperature and impairment of *cyp19a1a* in adult Atlantic salmon (Ch 5: Anderson et al., 2012). The present research has also raised questions about the integrity of factors that regulate the expression of *cyp19a1a* at high temperature such as Foxl2, a transcription factor that is thermally sensitive in Japanese flounder during sexual differentiation (Yamaguchi et al., 2007). In addition, *cyp11a1* gene expression was suppressed at 22 °C relative to 14 °C, indicating that like E_2, the molecular basis for reduced plasma T lies at least partly at the level of gonadal steroidogenesis.

With an increased understanding of endocrine impairment at high temperature in maiden and repeat spawning female Atlantic salmon, the next step was to develop and test alternate management techniques with the aim of maintaining endocrine function and subsequent egg quality in the face of thermal challenge. Treatment with Gnrh at the end of vitellogenic development is an effective way of triggering the release of endogenous Lh which induces FOM and synchronises ovulation in many species (reviewed in Zohar and Mylonas, 2001). In addition to its primary role in stimulating the events associated with FOM, Gnrh implantation during vitellogenesis caused a premature rise in plasma T and E_2 that promoted egg development in female Atlantic salmon (Crim et al., 1986). The increase in T and E_2 was most likely caused by a Gnrha-induced increase in pituitary gene expression of *fshβ* and subsequent release of Fsh as observed in Gnrha-treated pituitary cultures from coho salmon (Dickey and Swanson, 2000). Little information is available on the effects of prolonged Gnrha treatment on the endocrine system and oocyte development during vitellogenesis, especially at elevated temperatures. It was also unclear as to whether maiden and repeat spawning broodstock would respond in the same way to hormonal therapy under thermal challenge given their differential response to elevated temperature in terms of egg quality. However, in light of the demonstrated capacity of Gnrh to promote E_2 production and egg development during vitellogenesis in previous studies, it was hypothesised that treatment with Gnrha at 22 °C might maintain endocrine function in

female Atlantic salmon broodstock and compensate for the suppressive effects of high temperature (Ch 6).

Our results show that prolonged treatment with Gnrha during vitellogenesis did not compensate for the negative effects of thermal challenge on the timing of ovulation, egg size, egg fertility or embryo survival. The apparent lack of effectiveness was reflected by the endocrine data, where plasma Fsh levels were unchanged by treatment with Gnrha at 22 °C. This implies that if Gnrha treatment had a stimulatory effect, it may have been counteracted by elevated temperature, perhaps through suppression of *gnrhr* gene expression, as observed for red seabream maintained at 24 °C relative to 17 °C (Okuzawa et al., 2003). It should be noted that prior to the time that the Gnrha trial took place (late 2008 to mid 2009), plasma Fsh levels had not been analysed, i.e. for the first large scale experiment (Ch 5: Anderson et al., 2012) due a lack of access to Fsh specific antisera and protein standards. Plasma Fsh levels were subsequently determined for samples from all adult broodstock experiments in June 2010 after antisera and standards were made available by Dr. Penny Swanson at the Northwest Fisheries Science Centre. Therefore at the time of experimental set-up for the Gnrha trial, it was not known that plasma Fsh levels are not typically suppressed as a result of thermal insult. However as mentioned, the Gnrha experiment highlights that the action of Gnrha may have been partially blocked at the level of the pituitary which has not been demonstrated previously for Atlantic salmon. Furthermore, circulating Fsh was unable to stimulate the production of T and E_2 in the ovary which implies that gonadal steroidogenesis is impaired at high temperature, corroborating the findings from the first large-scale experiment. As plasma E_2 levels were unchanged in Gnrha-treated fish, it is not surprising that the transcription of *vtg*, *zpb* and *zpc* was not enhanced in Gnrha-treated fish relative to the controls at 22 °C. This is quite different to what was observed for Atlantic salmon reared at ambient temperature where Gnrha treatment approximately two months prior to the commencement of ovulation accelerated vitellogenesis and was not detrimental to egg quality (Crim et al., 1986), and demonstrates that thermally induced endocrine suppression at multiple levels is

complex and therefore difficult to overcome using hormonal therapy. However, in rainbow trout, treatment with a dopamine agonist inhibited the (Gnrh-induced) release of Fsh from the pituitary during late vitellogenesis (Vacher et al., 2000); and in Arctic charr, treatment with Gnrha in combination with a dopamine antagonist was more effective at inducing ovulation than treatment with Gnrh alone at high temperature (Gillet and Breton, 2009). Therefore, it is possible that Gnrha-treatment would be more effective at 22 °C if administered in combination with a dopamine antagonist. Alternatively, Gnrha treatment in combination with T promoted E_2 production in pink salmon reared at temperatures between 8.0-12.3 °C (Crossin et al., 2010). However, at high temperature, treatment with Gnrha in combination with T may not be successful due to upstream impairment of *cyp19a1a*. Therefore, we proposed that direct administration of E_2 may by-pass the aforementioned thermal impacts, help to restore plasma E_2 levels, stimulate downstream synthesis of Vtg and Zps, and maintain egg quality at temperatures relevant to the Tasmanian Atlantic salmon industry.

In earlier studies on juvenile salmonids, treatment with E_2 (or EE_2) was shown to induce the expression of hepatically-expressed genes such as *vtg*, *zpb*, *zpc* and *er* at a variety of temperatures (Westerlund et al., 2001, Körner et al., 2008, Mackay and Lazier, 1993). Even though the above studies examined hepatic responsiveness to E_2 at 'high' versus 'low' temperatures, the temperatures utilised were well within thermal tolerance ranges, and are considerably lower than temperatures experienced by Tasmanian Atlantic salmon broodstock. Therefore, before committing to a full scale E_2 therapy trial with adult broodstock, hepatic responsiveness to E_2 in terms of *vtg*, *zpb*, *zpc* and *erα* regulation was assessed at 14 and 22 °C in female juvenile Atlantic salmon (Ch 7).

Treatment with E_2 successfully raised plasma E_2 levels, and induced *de novo* hepatic gene expression of *vtg*, *zpb*, *zpc* and *erα* in juvenile Atlantic salmon maintained at 14 and 22 °C. The rate of induction for all genes (except *zpb*) was higher at 22 °C relative to 14 °C; however, plasma E_2 levels declined more rapidly in the group maintained at 22 °C. This presumably occurred due to a rise in basal metabolic rate and an associated increase in E_2 metabolism, as suggested for E_2-treated rainbow trout at

elevated temperature (Mackay and Lazier, 1993). Consistent with a rapid increase in *vtg* gene expression, plasma Vtg also increased at a faster rate, but reached a plateau more quickly at 22 °C relative to 14 °C. This indicates that the machinery by which E_2 exerts its action is still functional in juvenile fish at 22 °C, and suggests that suppression of Vtg production in adult fish occurs as a result of lower plasma E_2 levels, and not reduced E_2 signal transduction at 22 °C.

With respect to Vtg, the total magnitude of response at the transcriptional and protein level was similar between fish maintained at 14 and 22 °C, even with faster induction at 22 °C. This is consistent with a previous study on fathead minnow, whereby temperature modulated the short-term rate of response of the animals to estrogenic stimulation, but did not affect the overall concentration of plasma Vtg (Brian et al., 2008). On the contrary, E_2 stimulated the transcription of *zpb* and *zpc*, but expression levels of these genes at 22 °C were consistently lower than those observed at 14 °C (except for *zpc* at day 3). This suggests that unlike Vtg, the responsiveness of juvenile Atlantic salmon to exogenous E_2 in terms of *zp* transcription is partially compromised at 22 °C, and the molecular basis for this is currently unclear given the fact that E_2 signal transduction appears to be intact. The answer to this uncertainty may lie in differences between the promoter regions of Vtg and Zp genes. The promoter region of Vtg genes contains EREs and enhancer sequences that appear to be relatively simple (Teo et al., 1998, Bouter et al., 2010), and the regulation of Vtg is under strict control of E_2. The promoter regions of Zps appear to be more complex and may contain multiple E-boxes, imperfect EREs and CCAAT and TATAAA boxes, which are common in species exhibiting hepatic expression of Zps (Lyons et al., 1993, Kanamori et al., 2003). While the synthesis of Vtg and Zp proteins can both be triggered by E_2 in salmonids, it has been suggested that some aspects of their regulation may be quite different (Berg et al., 2004). This raises the question as to whether transcription factors specific to Zp genes are thermally sensitive, and suggests that characterisation of the 5' flanking promoter regions in Atlantic salmon Zp genes would be useful when trying to understand the molecular basis for the differential response of juvenile salmon to elevated temperature.

Given the fact that E_2-treatment was able to stimulate vitellogenesis and zonagenesis (to some extent) in juvenile Atlantic salmon maintained at 22 °C, a similar E_2-implantation protocol was trialled in adult female Atlantic salmon. Due to the industry's preference for using maiden fish, the last large-scale experiment examined the effects of E_2-treatment on egg development, endocrine function and egg quality in maiden spawners only at 22 °C (Ch 8).

In the present study, treatment with E_2 at 22 °C elevated plasma E_2 levels, stimulated *vtg* gene expression and subsequent protein synthesis which is broadly consistent with our previous results. Interestingly, plasma Vtg levels were elevated one month prior to a detectable increase in hepatic *vtg* mRNA levels in E_2-treated fish. This could indicate that E_2-treatment augmented the stability of *vtg* mRNA and/or E_2 exposure in combination with high temperature increased the rate of *vtg* mRNA translation relative to other groups as observed previously in rainbow trout (Mackay and Lazier, 1993, Flouriot et al., 1996). In contrast, E_2-treated and untreated fish had similar *zpb* and *zpc* gene expression levels at 22 °C, and gene expression for these groups was suppressed relative to the control group at 14 °C during late vitellogenesis. Consistent with our previous research, this implies that factors regulating the transcription of Zp but not Vtg genes is impaired at elevated temperature in adult and juvenile Atlantic salmon, although the effects appear to be more severe in adults, and the exact mechanism by with this occurs is yet to be explored.

E_2-treated fish displayed larger follicle diameters than untreated fish at 14 and 22 °C at one and two Sample points respectively during development, and this was carried through to ovulation whereby E_2-treated fish produced the largest eggs. Vtg is known to play a primary role in oocyte growth (Tyler et al., 1988); therefore, it seems that the higher plasma Vtg levels in the E_2-treated group were associated with an increased rate of Vtg uptake and oocyte growth relative to all untreated fish. These results also imply that the mechanisms governing Vtg uptake in to developing oocytes are not impaired at high temperature in salmonids as suggest by Tyler et al. (1987).

Egg fertility was highest in the control group maintained at 14 °C, followed by the control group at 22 °C, then the E_2-treated group at 22 °C, despite the stimulatory effect that E_2-treatment had on Vtg synthesis and oocyte growth. The reduction in egg quality in both groups of fish maintained at 22 °C relative to 14 °C may have occurred due to thermal inhibition of *zpb* and *zpc* gene expression. However, at this time it is not clear whether only Zp transcription is depressed, or whether down-stream *zp* mRNA translation and subsequent protein assembly at the oocyte are also impaired at elevated temperature. Furthermore, treatment with natural or synthetic estrogens has been linked to reductions in egg quality at some concentrations (Lahnsteiner et al., 2006, Peters et al., 2007). However, it appears that plasma E_2 levels in E_2-treated fish at 22 °C were generally similar to those observed in the control group at 14 °C that displayed a high level of egg fertility and embryo survival. In addition, previous research has demonstrated that plasma E_2 levels are stable for between 7-14 days after implantation of a single silastic E_2 pellet in juvenile Atlantic salmon (Ch 7) and other species (Pankhurst et al., 1986). Therefore it is unlikely that the E_2 profiles in the present study were so inappropriate that egg quality was further reduced as a result E_2-implantation. On the other hand, plasma cortisol levels were elevated in the E_2-treated group after receiving a second hormonal implant which indicates that the second implantation was perceived as somewhat stressful. Stress is able to reduce reproductive performance in many species (reviewed in Schreck, 2010) and it is possible that egg quality was lower in E_2-treated fish relative to the control group at 22 °C as a result of the implantation process. However, this is hard to asses in the absence of data from fish receiving hormonal treatment via less invasive means. Plasma cortisol levels were not elevated after the first round of hormonal treatment, this suggests that use of a single implant warrants investigation, but would need to be implemented in such a way that E_2 levels are maintained for a longer period of time.

9.1 Conclusion

In conclusion, we have confirmed industry observations that female maiden Atlantic salmon are more susceptible to the effects of elevated temperature in terms of egg fertility and embryo survival. We have also provided direct evidence that thermal impairment of endocrine function occurs at multiple levels of the reproductive axis. Treatment with Gnrha was not able to offset the impacts of elevated temperature; however treatment may be more effective if given in combination with a dopamine antagonist. In contrast, E_2-therapy was effective in maintaining vitellogenesis and oocyte growth at 22 °C, though stimulation of Vtg alone was not sufficient to maintain egg quality. The lower egg quality at higher temperature is apparently due to the impairment of zonagenesis. Our results suggest that it is difficult to overcome the impacts of higher temperature on the reproductive axis using a single hormonal treatment. Hormonal therapies in the future should simultaneously stimulate vitellogenesis and zonagenesis in order to maintain egg quality, and it is worth investigating whether a higher dose of E_2 could stimulate zonagenesis. It is evident that the method of hormonal delivery is of considerable importance and implantation techniques also warrant further investigation. Understanding the regulatory mechanisms governing the expression of Zp genes is justified, which could aid in the development of new management strategies that are essential for maintaining egg quality at high temperature without the added cost of intensive temperature control.

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