Training in endocrine diagnostic techniques and hormone analysis of thermally challenged female Atlantic salmon broodstock

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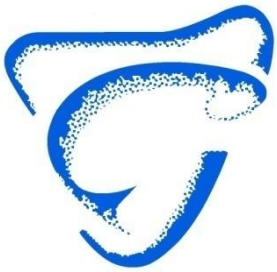

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Non-technical summary

The purpose of the research was to determine whether the endocrine profiles of plasma follicle stimulating hormone (FSH) and luteinizing hormone (LH) changed as a result of thermal exposure, broodstock age or hormonal treatment in farmed female Atlantic salmon. FSH promotes the production of testosterone, oestrogen and oocyte (egg) growth while LH controls oocyte maturation and ovulation. Results indicate that it is unlikely that circulating FSH is affected by stock age; however, the same can not be said for temperature. There is some evidence to suggest that fish reared at 22 °C (equivalent of a warm Tasmanian summer) have higher levels of circulating FSH compared to fish reared at 14 °C (ideal temperature) and this is probably due to abnormal oestrogen levels (and therefore feedback mechanisms) in fish reared at high temperature. Additionally, implantation with gonadotropin releasing hormone or oestrogen failed to stimulate the production of FSH or LH at 22 °C which demonstrates the complexity of the hormonal cascade controlling reproductive development. No obvious differences were observed in circulating levels of LH for though the preovulatory surge of LH was not captured by our sampling schedule.

An *in vitro* experiment was also performed in Seattle that investigated the affect of elevated temperature and hormonal treatment on the expression levels of genes involved in testosterone production in ovarian fragments isolated from coho salmon. For the first time, it has been shown that the relative expression of several genes involved in testosterone production was impaired by increased temperature *in vitro*. Interestingly, treatment with FSH appeared to temporarily stimulate the expression of some genes incubated at 14 °C but did not promote gene expression at all in 22 °C ovarian fragments. This could indicate that gene expression of FSH receptor (FSHr) is impaired, or the binding affinity of the receptor is reduced by thermal insult which would limit the effectiveness of hormonal therapy both *in vitro* and *in vivo*. From this trial, a suite of candidate genes that may contribute to low T levels and therefore reproductive performance in Atlantic salmon reared at 22 °C have been identified.

These results combined with our previous work (Pankhurst., unpublished) demonstrate that dysfunction at multiple levels in the endocrine cascade controlling reproductive development in thermally challenged female Atlantic salmon is likely. This work also shows that formulating management strategies that improve reproductive performance in Atlantic salmon reared at elevated temperature is no easy feat. However, as we are learning more about how reproductive physiology changes with various therapeutic, biological (i.e. age) and environmental conditions, we becoming better positioned to make informed choices that will benefit the aquaculture industry in Australia.

Acknowledgements

This research was supported by a CRC travel grant with in-kind contribution from Dr. Penny Swanson at the Northwest Fisheries Science Centre, Seattle, USA. Samples that were analysed in Seattle were obtained from a study involving the Salmon Enterprises of Tasmania (Dr. Harry King and Dr. Nicole Ruff), Griffith University (Prof. Ned Pankhurst and Dr. Patricia Pankhurst) and the University of the Sunshine Coast (Prof. Abigail Elizur). Extensive training and assistance was provided by Dr. Penny

Swanson, Dr. Adam Luckenbach and Dr. Yoji Yamamoto in Seattle. Thanks are also extended to Dr. Graham Mair from the seafood CRC for his assistance and encouragement when preparing the grant application.

Background

Salmon Enterprises of Tasmania (Saltas) is the leading producer of Atlantic salmon (*Salmo* salar, AS) smolts in Australia. Their production data shows 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, the majority of egg production comes from first time 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 negative reproductive effects seen here are purely due to broodstock age. However, the combined effect of rearing temperature and age on reproductive performance is not completely understood, although it is known that elevated temperature can negatively affect reproductive performance (King et al., 2007, King and Pankhurst, 2003). Currently, broodstock rearing temperatures may exceed 20 °C in Summer (King and Pankhurst, 2000) which is at the upper limit of thermal tolerance (22-23.5 °C) for the species (Pennell and Barton, 1996). For this reason it is unclear as to whether the differences in reproductive performance are due to age class, thermal challenge or a combination of both.

Reproductive development in fish is controlled by the brain-pituitary-gonad-liver (BPG-L) axis which is a complex endocrine cascade involving many genes, receptors, enzymes and hormones (Martinez-Chavez et al., 2008, van Aerle et al., 2008). Gonadotropin-releasing hormone (GnRH) is a key regulator of the BPG-L axis which stimulates the pituitary to produce two gonadotropins (GTHs); follicle stimulating hormone (FSH) and luteinizing hormone (LH). These hormones are responsible for stimulating oocyte growth and causing a shift in steroidogenesis that promotes oocyte maturation before spawning respectively (Nagahama et al., 1993). Specifically, as reproductive development progresses FSH levels increase which promotes steroidogenesis. This results in the production of testosterone (T) which is then converted to estradiol-17β (E_2) by the enzyme aromatase A (Cyp19a1a). E_2 is then responsible for triggering the hepatic synthesis of the egg yolk precursor vitellogenin (Vtg) and zona pellucida proteins (ZP) which are the building blocks of a healthy egg (Celius and Walther, 1998). Vtg is sequestered by the developing oocyte and becomes the source of nourishment for the developing embryo; while ZPs will ultimately form the 'egg shell' that surrounds and protects the embryo post fertilisation (Celius and Walther, 1998). At the end of vitellogenesis and zonagenesis (Vtg and ZP production respectively), an LH surge causes a shift in steroidogenesis (from production of T) and 17α, 20β-dihydroxy-4-pregnen-3-one (maturation-inducing hormone, MIH) is produced within the follicle (Nagahama and Yamashita, 2008). Once MIH is synthesised, the signal is transmitted into the oocyte resulting in the formation and activation of M-Phase promoting factor (MPF); which in turn, ensures that the final stages of oocyte maturation occur and ovulation can occur (Nagahama and Yamashita, 2008).

A more complete understanding of the endocrine processes (mentioned above) that determine egg quality is a crucial aspect of formulating management strategies that enhance egg quality female Atlantic salmon. In response to this realisation, a large research effort was initiated in 2007 to investigate the affect of broodstock age and thermal challenge on the reproductive performance of Tasmanian female Atlantic salmon and trail novel management strategies targeted at improving reproductive performance.

Experiment 1: Maiden and repeat female Atlantic salmon were reared at either 14 or 22°C prior to spawning to determine the effects of temperature and stock age on egg quality. Results from this research show that E_2 production is impaired (at least in part due to suppression of CYP19a1a gene expression) in thermally challenged maiden and repeat spawning female Atlantic salmon. This had the 'knock on' effect of reducing egg quality in these fish because vitellogenesis and zonagenesis did not occur 'normally' in E_2 deprived fish. While these results improved our understanding of reproductive dysfunction in Atlantic salmon an important question was raised: are other processes involved in E_2 production (and therefore egg quality) affected in fish exposed to elevated temperature?

As mentioned above, oocyte development and maturation is controlled by gonadotropins which are secreted for several months prior to spawning. However, data concerning how elevated temperature affects circulating levels of FSH and LH in maiden and repeat female Atlantic salmon was unavailable. Thus, it was proposed that analyzing the circulating levels of gonadotropins (that control E_2 synthesis) would shed some light on whether upstream processes (at the level of the pituitary) contribute to the endocrine dysfunction observed in the ovary.

Experiment 2: In an effort to stimulate the endocrine system which controls egg quality, thermally challenged maiden and repeat spawning Atlantic salmon were implanted with slow release GnRH pellets and the effects on egg quality were determined. Treatment with GnRH was ineffective at recovering egg quality in fish reared at elevated temperature; this is probably due to the fact that there is thermal inhibition of multiple steps in the endocrine cascade. However, it is unclear as to whether GnRH implantation successfully triggered the production and release of FSH and LH yet the gonadotropins were ineffective at raising estrogen levels and later advancing ovulation, or if GnRH did not stimulate the creation of FSH and LH at all. By measuring FSH and LH levels in fish from this experiment, we will help to determine why GnRH therapy was unsuccessful in thermally challenged Atlantic salmon.

Experiment 3: Ideally, the Tasmanian salmon industry would like to use first time maiden spawning fish for egg production as there is a large cost and risk associated with production strategies which utilise repeat spawning fish. However, the industry's ability to do this is limited by the fact that maiden fish are more sensitive than repeats to the negative effects of elevated temperature in terms of egg quality (major finding from experiment 1). Results from experiment 2 showed that treatment with GnRH was ineffective at offsetting the negative effects of elevated temperature and is therefore not a suitable means of enhancing egg quality. Therefore, in an attempt to develop a commercial broodstock management strategy that improves reproductive performance, E_2 therapy was trialled in female Atlantic salmon. By measuring circulating levels of FSH and LH in fish from this experiment, we will discover whether estrogen therapy stimulates the endocrine system to the point

of recovery. We will also gain valuable information about the biological responses of salmon to temperature challenge and hormonal replacement therapy.

By measuring circulating levels of FSH and LH, we aim to build on, and add to, the information already collected in our studies on the physiology of maiden and repeat spawning female Atlantic salmon exposed to various temperatures. The additional information attained from this work will help us to develop better management strategies that enhance egg quality and therefore increase productivity, lower running costs and increase sustainability of the industry.

Need

The variable spawning success of Atlantic salmon broodstock has left the industry with the ongoing problem of lack of predictability of egg production, and a difficult management challenge. To produce the required number of smolts, the number of maiden spawning fish which produce lower quality eggs 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 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 and breeding with repeat spawners (Taranger and Hansen, 1993, Pankhurst and King, 2010). In fact, it is expected that in the future all broodstock holding facilities in Tasmanian AS hatcheries will be thermally regulated with the introduction of recirculation systems and temperature control (Battaglene et al., 2008). However, the initial capital investment required to set up a thermally controlled broodstock facility is significant, and this does not include ongoing cost of cooling large volumes of water on a yearly basis (King, H 2007, pers. comm., 6 February). In addition, 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 mean 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 an commercially acceptable level.

Objectives

Objectives as outlined in the CRC travel grant application

1) Training in endocrine diagnostic techniques

To learn how to analyse blood samples from Atlantic salmon for the gonadotropins: follicle stimulating hormone (FSH) and luteinizing hormone (LH) using either a radio immuno-assay (RIA) developed by Dr Swanson or an enzyme-linked immunosorbent assay (ELISA) that I will develop whilst there.

2) Hormone analysis of thermally challenged female Atlantic salmon broodstock

To analyse plasma samples for FSH and LH from the CRC and FRDC approved projects (2008/762, 2008/217 and 2009/085) with the working titles: "Effect of temperature on reproductive development of maiden and repeat spawning Atlantic salmon", "Developing methods for endocrine and thermal manipulation of salmon stocks to achieve desired fertility outcomes" and "Mitigation of climate change effects on salmon broodstock: effects of estrogen therapy" respectively.

Additional objectives formulated while in NWFSC, Seattle

1) To determine whether FSH, insulin-like growth factor 1 (Igf1) or a combination of FSH/Igf1 enhances/maintains steroidogenic processes in isolated coho salmon ovarian follicles incubated at elevated temperature.

As objectives one and two were completed in a timely fashion, an additional experiment was conducted to investigate the affect of hormone treatment on steroidogenesis in isolated ovarian follicles of female coho salmon incubated at elevated temperature. This experiment was designed to complement the FRDC/CRC funded project (2008/762) which found that steroidogenesis is impaired in thermally challenged female Atlantic salmon. Carrying out this trial gave me the opportunity to learn how to do *in vitro* studies on animal tissue. This is a powerful technique for the investigation of (in this case) ovarian response to a range of environmental and/or hormonal treatments.

Methods

Fish Husbandry, experimental treatments and procedures used to obtain the samples analysed in Seattle

<u>Experiment 1:</u>

Maiden (first spawning 2+ year old fish) and repeat (second spawning 3+ year old fish) adult females 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 at 16 °C from the end of August 2007 until January 2008. In mid-January 2008, fish were divided into the following treatment groups and transferred to temperature-controlled $4m^3$ Rathbun tanks under simulated ambient photoperiod. Fish were not fed from the time of transfer to the temperature controlled systems.

Treatment Groups

1. Maidens held at 14 ^{o}C

2. Repeats held at 14 ^{o}C
3. Maidens held at 22 ^{o}C
4. Repeats held at 22 ^{o}C

All fish were maintained at the nominated temperature (14 or 22^{o}C) from January until late March when all fish were exposed to a temperature ramp down to 8^{o}C (Fig. 1) to induce final oocyte maturation and ovulation (King and Pankhurst, 2000).

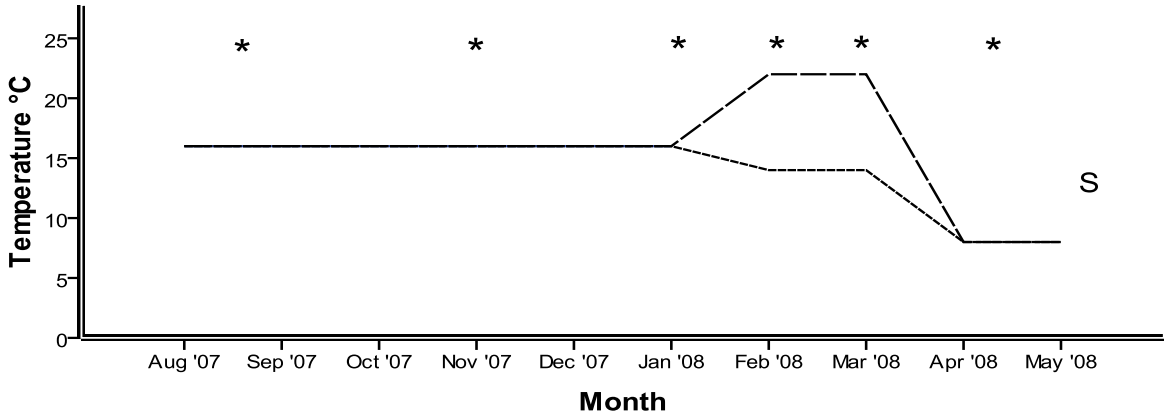

Figure 1. Thermal regime for maiden and repeat spawning Atlantic salmon in simulated 'cool or warm summer' treatments in experiment 1. The stars show sampling points while the S is the approximate start of the spawning period.

Experiment 2:

Fish husbandry and thermal regime for this experiment was identical to experiment 1 except sampling took place between November 2008 and April 2009 and the treatments/experimental groups were as follows.

Treatment Groups

1. Maidens held at 14^{o}C;
2. repeats held at 14^{o}C;
3. maidens held at 22^{o}C;
4. maidens held at 22^{o}C and treated with a GnRH pellet (see 'sampling protocol' for details);
5. repeats held at 22^{o}C and
6. repeats held at 22^{o}C and treated with a GnRH pellet.

Experiment 3:

Fish husbandry and thermal regime for this experiment was identical to experiment 1 except sampling took place between February and April 2010 and the experimental groups were as follows.

Treatment Groups

1. Maidens held at 14 ^{o}C
2. Maidens held at 22 ^{o}C
3. Maidens held at 22 ^{o}C and treated with multiple E_2 silastic pellet implants

Sampling timelines

Experiment 1:

Fish from both maiden and repeat groups that were maintained at 16 ^{o}C were sampled on the 31st August and 2nd November 2007, and 7th January 2008 to cover the initiation of vitellogenesis for each age class. After introduction to the controlled temperature regimes (14 and 22 ^{o}C) on the 14th February 2008, fish were sampled on the 28th of March and 25th of April (Fig. 1). Destructive (fatal) sampling was carried out on seven fish from each group at each sample time, and 7 fish from each treatment proceeded through to ovulation and stripping after April (non-destructive).

Experiment 2:

Fish from both maiden and repeat groups (16 ^{o}C) were sampled in late October 2008 to establish a previtellogenic hormonal baseline for each group, and on the 22nd January 2009 (16 ^{o}C). Subsequent samples were taken from all 6 groups over February 25-27, March 26-27, and April 15-16. Six to seven fish were sampled from each group at each sample time, and 7 fish from each treatment proceeded through to ovulation and stripping, after the final destructive sample in April. Fish from treatment groups 4 (maidens at 22 ^{o}C) and 6 (repeats at 22 ^{o}C) were implanted with an Ovaplant ™ (Syndel, Canada) cholesterol pellet containing 37µg of the D-Arg^{6},Pro^{9}NEt analogue of salmon GnRH on 22nd January, and again over February 26-27 to give repeat doses of approximately 7-12µg.kg^{-1}. The dose chosen was designed to stimulate or maintain pituitary secretion of follicle stimulating hormone (FSH), but remain below the threshold likely to stimulate a luteinizing hormone (LH) surge and possible premature stimulation of oocyte maturation and ovulation (King and Pankhurst, 2004a, King and Pankhurst, 2004b).

Experiment 3:

7 Maiden fish maintained at 16 ^{o}C were sampled on the 17th of February 2010 to establish a baseline for subsequent hormonal measurements. Fish from each of the 3 groups were then sampled on the 5th of March, 19th of March and 9th of April via destructive sampling and 7 fish from each treatment proceeded through to ovulation and stripping in April as per previous experiments. At the first 3 sampling points, fish from group 3 were implanted with silastic E_2 pellets at a dose of 10 mg kg^{-1}.

Sampling protocol (blood only)

Fish were netted from the holding tanks, terminally anaesthetised in Aqi-S ™ (Crop & Food, New Zealand, 50ppm lethal, 25ppm non-lethal), weighed, measured and then blood was sampled by caudal puncture using pre-heparinised syringes fitted with 22G needles. Plasma was separated out by centrifugation then stored at -20 ^{o}C for later measurement of plasma hormones.

FSH and LH measurement (Seattle)

FSH and LH measurements were done using a routinely used RIA developed by Swanson et al., (1989) for coho salmon with minor modifications. This was originally based on a similar assay developed by Suzuki et al., (1988). Briefly, standards, controls and samples were aliquotted into 12 x 75 mm test tubes (volumes were adjusted based on pre-screening) then mixed with an appropriate volume of phosphate buffered saline with 1 % bovine serum albumin (PBS-BSA, pH 7.4) to 100 µl. 100 µl of primary antibody (rabbit anti-coho LHβ or FSHβ) diluted in normal rabbit serum (NRS)-PBS was added to the appropriate test tubes, vortexed gentle and incubated overnight at 4 °C. The following day, 100 µl of label (^{125}I-FSH or ^{125}I-LH, prepared by iodogen method) containing ~9-10000 cpm was added to the appropriate tubes; tubes were then vortexed and incubated overnight at 4 °C. On day 3, 200 µl of secondary antibody (goat anti-rabbit IgG) was added to the appropriate tubes; all tubes were then vortexed and incubated overnight at 4 °C. On day 4, 500 µl 4 % polyethylene glycol was added as needed, tubes were vortexed then centrifuged at 3000 rpm for 30 min. The supernatant was aspirated carefully and tubes were read/counted for 3 min each.

Even though the GTH RIA has been validated for cross-species use in Atlantic salmon previously (Oppen-Berntsen et al., 1994, Antonopoulou et al., 1998), validity was checked using the parallel displacement curve method.

Methodology for additional *in vitro* trial (Seattle)

To determine whether FSH, insulin-like growth factor 1 (Igf1) or a combination of FSH/Igf1 enhances/maintains steroidogenic processes in isolated coho salmon ovarian follicles incubated at elevated temperature, an *in vitro* experiment was set up at NWFSC as follows.

6 female coho salmon (2 + yo, 595 ± 75g body weight) were given a lethal dose of (tricaine methane sulphinate) MS222. All fish were then weighed, total body and fork length was measured, then their ovaries were removed and temporarily stored in chilled L-15 culture medium (Invitrogen, Carlsbad, CA) supplemented with 0.2 % BSA (henceforth referred to as L-15). To set up the cultures, the anterior and posterior end of each ovary was removed and discarded while the remaining sections of ovary were cut into 83.5 ± 7.5 g pieces while immersed in chilled L-15 culture medium. Ovarian fragments were spread into 24 well polystyrene culture plates so that each treatment would receive 1 fragment from each fish. At this time, the fragments were pre-incubated with 1 ml of fresh L-15 medium for 2 h with gentle orbital shaking. Then, time zero fragments were snap frozen in liquid N_2 to set a baseline. For all other fragments the medium was removed and replaced with fresh L-15 containing no hormone, highly purified FSH (100 ng/ml), Igf1 (100 nM) or a combination of FSH and Igf1 (100 ng/ml and 100 nM each respectfully). Trays containing each culture treatment were then incubated at either 14 or 22 °C with gentle orbital shaking and sampled at 3 and 24 h. At these sampling points, a sub sample of 10 follicles were taken from the appropriate wells for measurement of caspase activity (not included in this report) while the remaining fragments (61.7 ± 9.8 g) were snap frozen in N_2 for subsequent RNA isolation.

RNA isolation, DNase treatment and cDNA synthesis

Total RNA was extracted from homogenised ovarian fragments using Tri-Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's specifications. To remove potentially contaminating DNA, all RNA samples were treated with the DNA Free kit's "rigorous" protocol (Ambion/Applied Biosystems). RNA yield and quality were determined by NanoDrop (ND-1000 Spectrophotometer; NanoDrop Technologies, Rockland, DE) and gel electrophoresis. cDNA synthesis was performed using the Superscript II kit (Invitrogen, Carlsbad, CA) with random primers, RNase inhibitor from Promega (Madison, WI). Negative reverse-transcription controls were included for a sub-set of samples (n = 10).

Quantitative polymerase chain reactions

Quantitative polymerase chain reactions (qPCRs) for genes involved in ovarian steroidogenesis were performed according to a SYBR green method developed and validated by Luckenbach and Swanson

(unpublished data) except a 384 well plate format was utilised. The gene expression of 3-β-hydroxysteroid dehydrogenase (3β-HSD), cholesterol side-chain cleavage enzyme (CYP11a1), CYP17a (hydroxylase and lyase activity), CYP19a1a and steroidogenic acute regulatory protein (StAR) was determined along with the reference genes elongation factor 1 (Ef1α) and TATA binding protein (TBP).

Statistical analysis

Statistical comparisons (where applicable) were made using the Kruskal-Wallace test ($P \leq 0.05$) coupled with Bonferroni's correction for post-hoc analysis on SPSS Inc. version 17.0.

Results/Discussion

Experiments 1, 2 and 3

Preliminary results indicate that circulating levels of FSH were generally similar between maiden and repeat spawning fish from August '07 until February '08 (experiment 1) with the exception of repeat spawning fish in August '07 (Fig. 1). Up to and including February, the plasma concentration of FSH did not exceed 5 ng/ml (with the exception of 1 fish) which is similar to what was previously reported in a northern hemisphere stock of Atlantic salmon (Oppen-Berntsen et al., 1994). In March, there is some evidence to suggest that fish reared at 22 °C have higher levels of FSH than fish reared at 14 °C irrespective of age. This could be due to the fact that fish maintained at 14 °C have higher levels of circulating E_2 (Pankhurst et al., in press), and E_2 is known to exert negative feedback on FSH in rainbow trout, *Oncorhynchus mykiss* (Saligaut et al., 1998). FSH was detectable in only 2 fish maintained at 14 °C in April which was approximately 1 month prior to ovulation for these groups of fish. This is similar to what was observed in other Atlantic salmon by Oppen-Berntsen et al., (1994) where a general decline in FSH was detected close to ovulation. Therefore, the elevated FSH levels in 22 °C fish in April may be reflection of the delayed ovulation observed for these groups compared to the 14 °C groups (Pankhurst et al., in press).

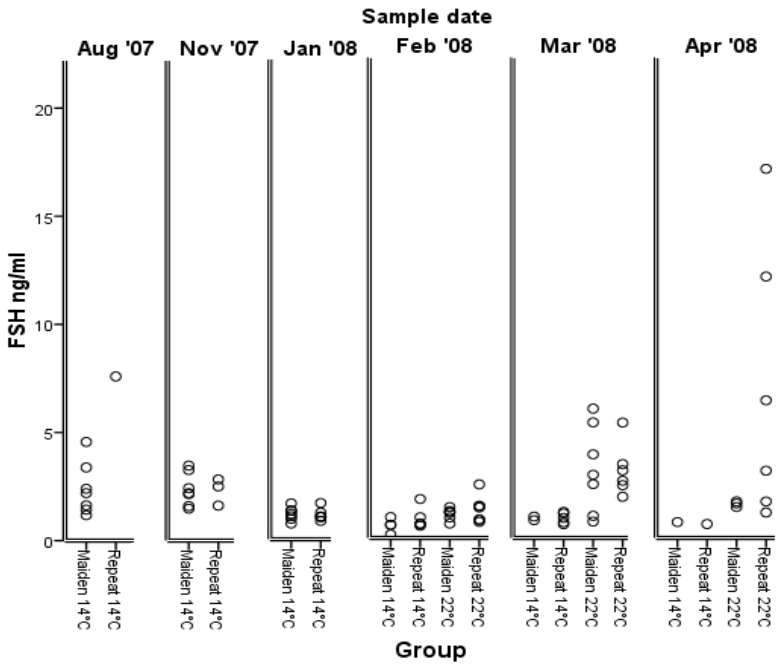

Figure 1. Plasma FSH concentrations for individual maiden and repeat spawning fish from experiment 1 'effect of temperature on reproductive development of maiden and repeat spawning Atlantic salmon' (2007-2008). Fish whose level of FSH was below the limit of detection (LOD) for the assay are not displayed. N.B statistical analysis has not been performed on this data as FSH levels were below the LOD for some fish; assigning these fish a value of '0' is not statistically or biologically correct.

Plasma concentrations of FSH in fish from experiment 2 are broadly consistent with results from the previous year. The preliminary results indicate that circulating levels of FSH are similar between all groups tested from November '08 up to and including February '09. Then in March '09, again, there is some evidence that control (blank silastic pellet) fish reared at 22 °C had higher levels of FSH than fish reared at 14 °C (Fig. 2). Interestingly, GnRH implantation at elevated temperature failed to stimulate the production of FSH at the protein level with no obvious differences existing between GnRH implanted fish and their blank pellet counterparts. However, this is not unheard of in the literature; injection with GnRH alone or in combination with pimozide failed to promote FSH production in rainbow trout at a variety of reproductive stages (Breton et al., 1998) while injection with biodegradable GnRH microspheres failed to stimulate or advance oocyte growth in pink salmon (*Oncorhynchus gorbuscha*), presumably because FSH production was not enhanced (Crossin et al., 2010).

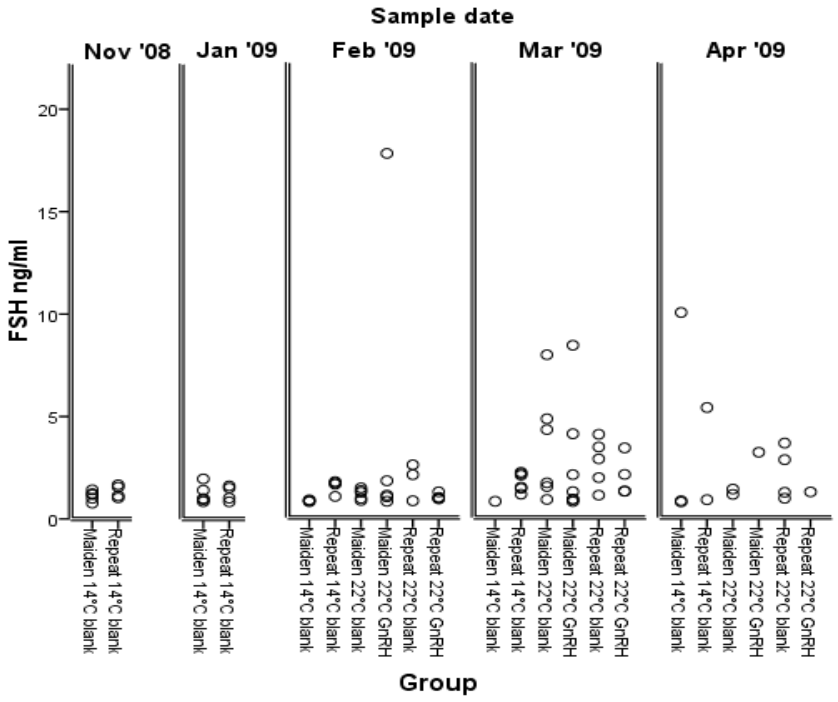

Figure 2. Plasma FSH concentrations for individual maiden and repeat spawning fish from experiment 2 'developing methods for endocrine and thermal manipulation of salmon stocks to achieve desired fertility outcomes' (2008-2009). Fish whose level of FSH was below the LOD for the assay are not displayed. N.B statistical analysis has not been performed on this data as FSH levels were below the LOD for some fish; assigning these fish a value of '0' is not statistically or biologically correct.

Preliminary results from experiment 3 demonstrate that administration of E_2 did not stimulate the release of FSH into the blood of thermally challenged maiden spawning Atlantic salmon (Fig. 3). In fact, E_2 treatment may have inhibited FSH production in March '10 as only 1 fish had a detectable level of plasma FSH. Furthermore, hormonal treatment did not improve reproductive performance of broodstock in terms of egg fertility and survival (Pankhurst et al., unpublished data) although at this point the reason for this is unclear (data analysis is not complete). However, it has been previously demonstrated that hormonal treatment with E_2 can dramatically affect the physiological response of the animal. For example, in Nile tilapia (*Oreochromis niloticus*) a 250 µg/kg dose of E_2 significantly impaired the release of FSH into the blood while a dose of 500 µg/kg increased FSH release though not by a statistically significant amount (Levavi-Sivan et al., 2006).

These results collectively show that FSH levels are mostly unaffected by broodstock age though there is some evidence of a temperature effect. FSH levels were generally elevated as a result of thermal challenge which is likely due to a lack of negative feedback in E_2 deficient 22 °C fish. For this reason, inappropriate E_2 and FSH levels in compromised fish may be the most significant drivers behind the delayed ovulation and reduced reproductive performance observed in our warm water groups (Pankhurst et al., unpublished data). Furthermore, hormonal therapy did not stimulate the

release of FSH into the blood of fish reared at 22 °C which demonstrates the complexity of the hormonal cascade controlling reproductive development.

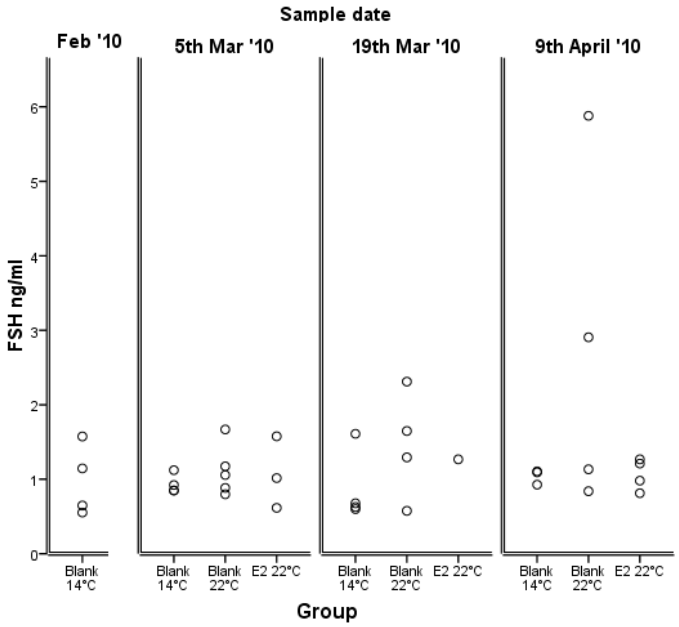

Figure 3. Plasma FSH concentrations for individual maiden spawning fish from experiment 3 'mitigation of climate change effects on salmon broodstock: effects of estrogen therapy' (2010). Fish whose level of FSH was below the LOD for the assay are not displayed. N.B statistical analysis has not been performed on this data as FSH levels were below the LOD for some fish; assigning these fish a value of '0' is not statistically or biologically correct.

Studies on reproductive development in Atlantic salmon (Oppen-Berntsen et al., 1994) and rainbow trout (Breton et al., 1998) have demonstrated that circulating LH levels remain low for most of the reproductive season and peak prior to ovulation. For this reason LH was analysed from March onwards which is approximately 2-3 months from ovulation. During March and April the level of LH was comparable between all groups of fish studied regardless of treatment and was 0.88 ± 0.33 ng/ml (Figs. 4, 5 and 6) which is consistent with concentrations reported previously for Atlantic salmon (Oppen-Berntsen et al., 1994). In rainbow trout, LH levels begin to increase approximately 12 days before ovulation with a minor peak occurring 8 days, and a major peak occurring 4 days before ovulation (Breton et al., 1998). The exact timing of the LH surge (or surges) in Atlantic salmon is less clear though Oppen-Berntsen et al., (1994) have shown that when blood samples are taken from all fish at the same time, then date of ovulation differs between fish, the LH surge for some fish can be missed all together. For this reason, it is apparent that the sampling schedule employed in this study was not optimal for detecting preovulatory differences in circulating LH. These results suggest that LH levels increase less than 1 month prior to ovulation in Atlantic salmon as in other species such as rainbow trout.

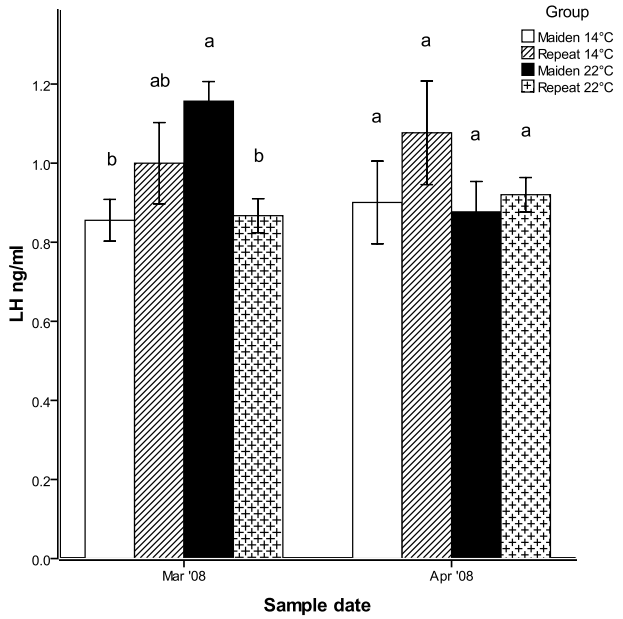

Figure 4. Plasma LH concentrations for individual maiden and repeat spawning fish from experiment 1 'effect of temperature on reproductive development of maiden and repeat spawning Atlantic salmon' (2007-2008).

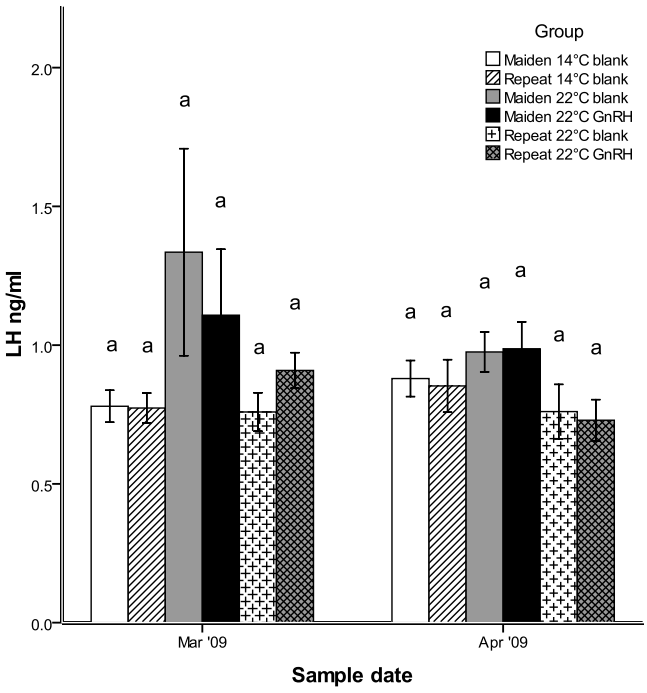

Figure 5. Plasma LH concentrations for individual maiden and repeat spawning fish from experiment 2 'developing methods for endocrine and thermal manipulation of salmon stocks to achieve desired fertility outcomes' (2008-2009).

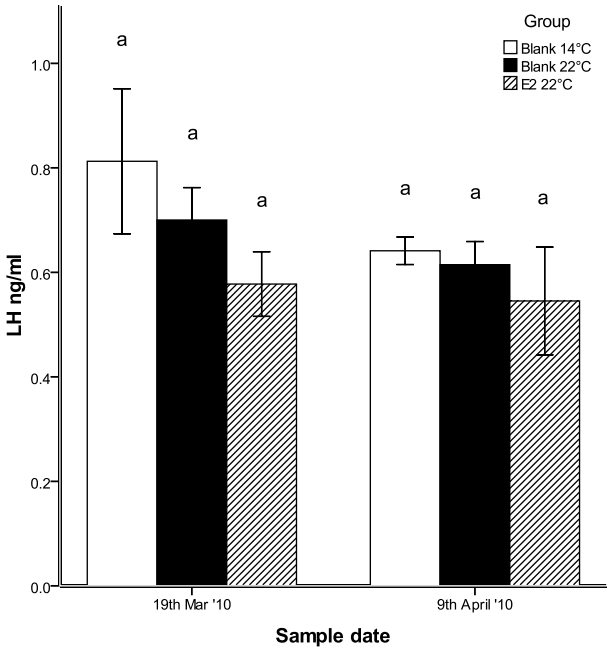

Figure 6. Plasma LH concentrations for individual maiden spawning fish from experiment 3 'mitigation of climate change effects on salmon broodstock: effects of estrogen therapy' (2010).

Investigating the affect of temperature and hormonal treatment on ovarian steroidogenesis in coho salmon: an *in vitro* trial

Recent studies in Atlantic salmon have demonstrated that E_2 synthesis is impaired in fish reared at 22 °C compared to lower temperatures (King et al., 2003, King et al., 2007). At least part of the observed endocrine dysfunction can be attributed to compromised CYP19a1a gene expression at elevated temperature (Anderson et al., unpublished data) as seen in other species. For example, exposure to 20 and 25 °C caused a significant reduction in CYP19a1a gene expression in the gonad of juvenile red seabream (*Pagrus major*) compared to the 15 °C treatment (Lim et al., 2003) and in flounder (*Paralichthys olivaceus*), thermal challenge suppressed CYP19a1a gene expression and induced male sex differentiation in a genetically female population (Kitano et al., 1999). Studies like these show that across species, ovarian steroidogenesis is a process can be negatively affected by higher than 'normal' temperatures. Therefore, an *in vitro* study was designed to determine whether other enzymes involved in steroidogenesis are thermally sensitive, and whether thermal sensitivity can be reduced through hormonal treatment.

The following graphs display un-normalised relative gene expression results and as such, are preliminary. Once a method of normalisation had been decided upon, all gene expression levels will be recalculated. Normalisation is a 'tidying up' process that accounts for differences in reverse transcription efficiency and sample loading volume, so it is expected that any changes to the data will be minor and the final outcome will not change. Due to the preliminary nature of the data, statistical analysis is yet to be performed; however, major trends in the data will be discussed here.

At 3 h, there is some evidence that elevated temperature generally down regulated CYP19a1a gene expression regardless of hormonal treatment, and this effect is more dramatic at 24 h (Fig. 7). Relative expression of CYP19a1a may have been upregulated as a result of FSH treatment at 14 (but not 22 °C) at 3 h but the same effect of FSH treatment was not seen at 24 h. A combination of FSH and Igf1 enhanced gene expression at 24 h but only at low temperature. The general impairment of CYP19a1a gene expression at elevated temperature agrees with our unpublished (in preparation) data concerning the *in vivo* affect of thermal challenge on the reproductive physiology of female Atlantic salmon (Anderson et al.), and *in vivo* studies on flounder (Kitano et al., 1999) and red seabream (Lim et al., 2003) where thermal inhibition of CYP19a1a was observed with increased temperature. These results support the growing body of evidence that suggests thermally challenged fish are unable to convert T to E_2 due to endocrine dysfunction at the CYP19a1a level, and this may affect downstream physiological processes that dictate reproductive performance.

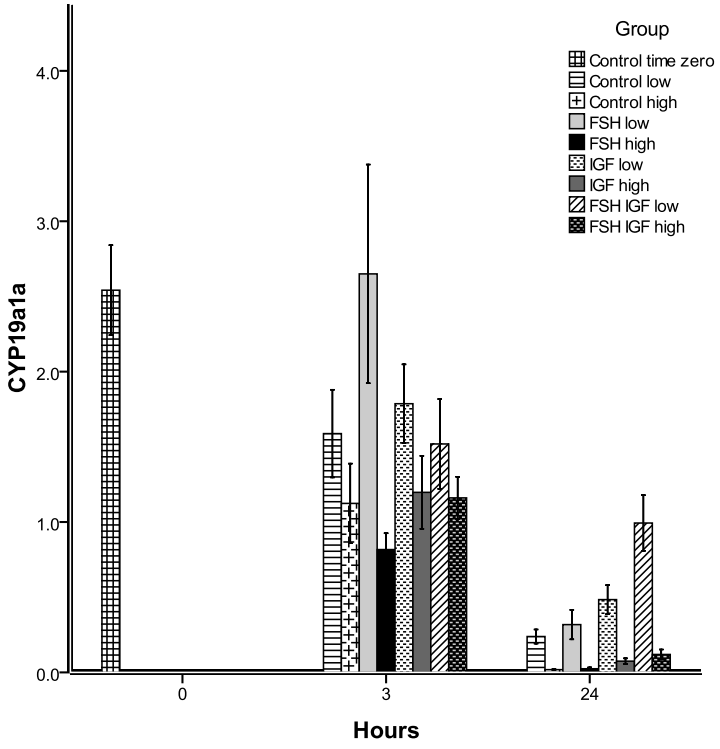

Figure 7. Raw (un-normalised) CYP19a1a gene expression in isolated ovarian follicles treated with FSH, Igf1 or a combination of FSH and Igf1 then incubated at 14 or 22 °C for 3 or 24 h. 'High' and 'Low' refer to 22 and 14 °C respectfully.

Elevated temperature may have had an initial stimulatory affect on the relative gene expression of StAR in control fragments at 3 h; however the affect is transitional and temperature dependant down regulation was seen in this group at 24 h (Fig. 8). At 3 h there is evidence that FSH upregulated StAR gene expression at low temperature and to a lesser extent at high temperature, though FSH treatment had no affect at 24 h. As with CYP19a1a, incubation at 22 °C resulted in dramatically lower StAR gene expression by 24 h regardless of hormonal treatment. In the teleost ovary, StAR is responsible for transporting cholesterol (which is an essential T precursor) across the inner mitochondrial membrane and is therefore considered to be the rate limiting step in T

production (Lubzens et al., 2010). If StAR expression in compromised *in vivo* as it is *in vitro* in isolated coho follicles, then this could have implications for the rate of steroidogenesis, E_2 production, and therefore reproductive performance in female Atlantic salmon reared under thermal pressure.

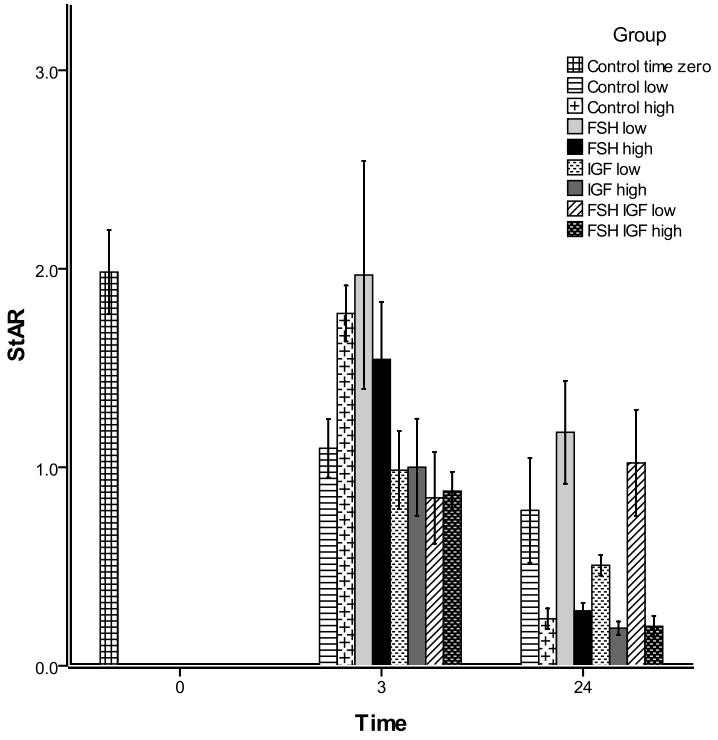

Figure 8. Raw (un-normalised) StAR gene expression in isolated ovarian follicles treated with FSH, Igf1 or a combination of FSH and Igf1 then incubated at 14 or 22 °C for 3 or 24 h. 'High' and 'Low' refer to 22 and 14 °C respectfully.

After 3 h of incubation, relative expression of 3β-HSD was stimulated by temperature while hormonal treatment did not appear to have an affect (Fig. 9). However, by 24 h, ovarian fragments that were incubated at 22 °C had a much lower level of relative 3β-HSD than their 14 °C equivalent. These results show that 3β-HSD gene expression could potentially be compromised in thermally challenged female Atlantic salmon; and since 3β-HSD catalyses the production of various intermediates critical to the production of T during steroidogenesis (Payne and Hales, 2004, Lubzens et al., 2010), endocrine dysfunction at this level could significantly impact T production and have negative downstream effects.

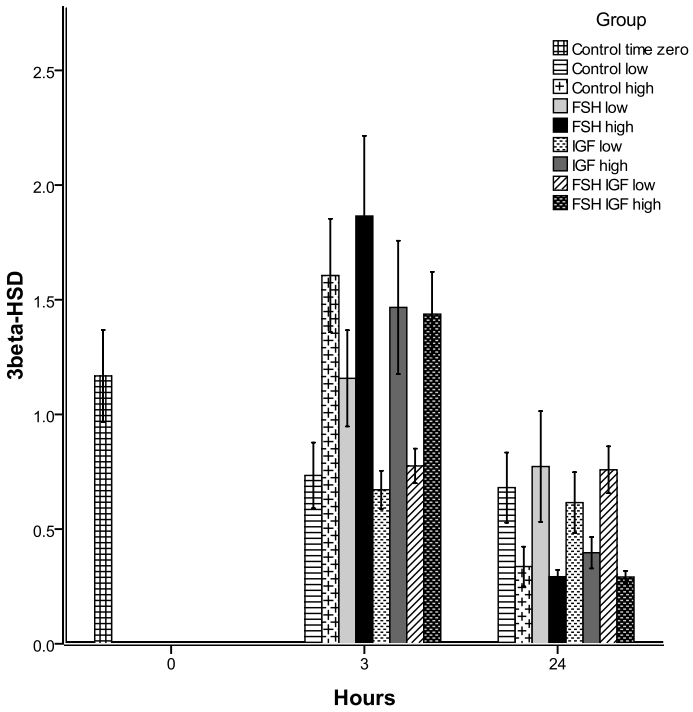

Figure 9. Raw (un-normalised) 3β-HSD gene expression in isolated ovarian follicles treated with FSH, Igf1 or a combination of FSH and Igf1 then incubated at 14 or 22 °C for 3 or 24 h. 'High' and 'Low' refer to 22 and 14 °C respectfully.

The negative effects of elevated temperature on relative gene expression were observed at 3 and 24 h for CYP11a1 (Fig. 10) and 24 h for CYP17a (Fig. 11). Again, some evidence is presented here that FSH stimulates genes expression of both CYP11a1 and CYP17a at 3h though the effect appears to be greater for CYP17a. However, FSH did not stimulate the expression of either gene at 24 h. Generally speaking, during T production enzymes in the P450/CYP family catalyse the cleavage and hydroxylation of precursor molecules (Payne and Hales, 2004). So in a similar way to StAR and 3β-HSD, if expression of CYP11a1 and CYP17a is deficient, downstream events could be impacted and ultimately reproductive performance may decline.

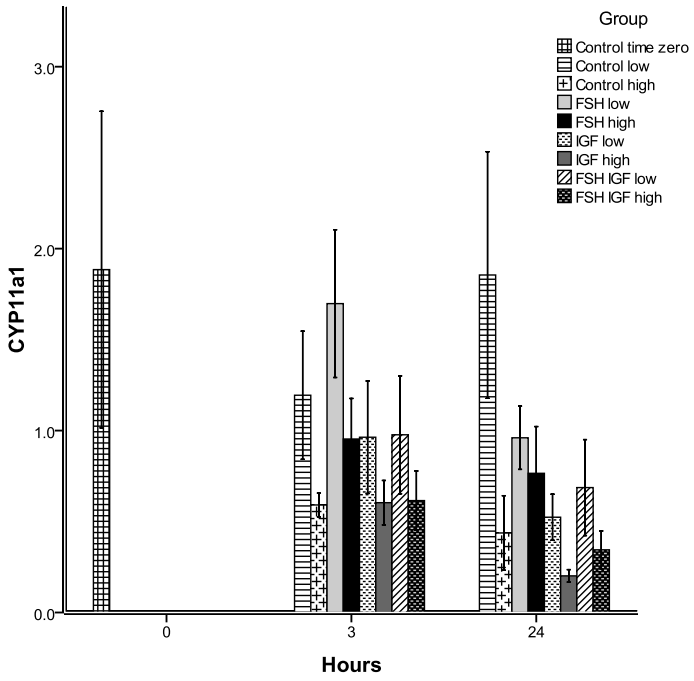

Figure 10. Raw (un-normalised) CYP11a1 gene expression in isolated ovarian follicles treated with FSH, Igf1 or a combination of FSH and Igf1 then incubated at 14 or 22 °C for 3 or 24 h. 'High' and 'Low' refer to 22 and 14 °C respectfully.

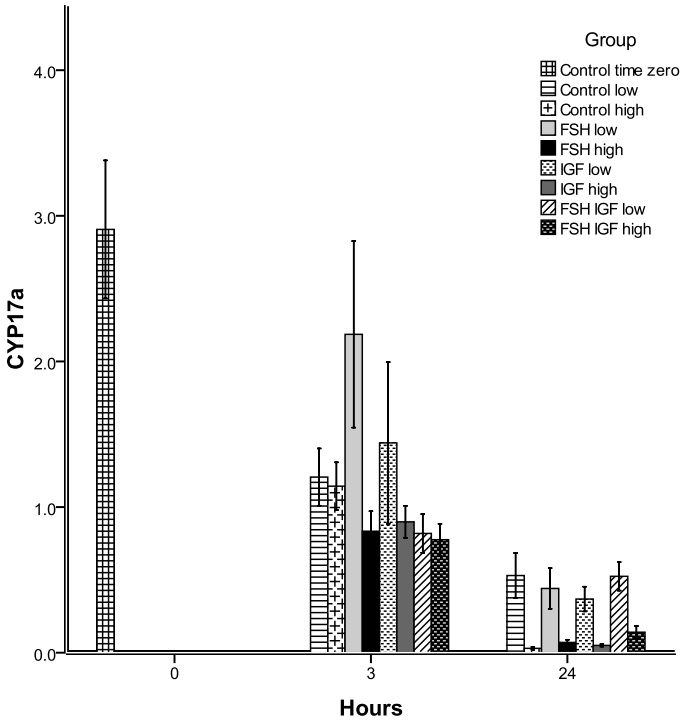

Figure 11. Raw (un-normalised) CYP17a gene expression in isolated ovarian follicles treated with FSH, Igf1 or a combination of FSH and Igf1 then incubated at 14 or 22 °C for 3 or 24 h. 'High' and 'Low' refer to 22 and 14 °C respectfully.

Collectively, the results show that elevated temperature suppresses the relative expression of several enzymes that play critical roles in steroidogenesis in incubated coho ovarian tissue. In Tasmanian Atlantic salmon, it has been shown that thermal challenge can cause a reduction in circulating T levels at some points during reproductive development (King et al., 2003). Therefore, this study has revealed a suite of candidate genes that may contribute to low T levels and therefore reproductive performance in Atlantic salmon reared at 22 °C. Treatment with FSH appeared to temporarily stimulate the expression of some genes incubated at 14 °C but did not promote gene expression at all in 22 °C follicles. This could indicate that gene expression of FSH receptor (FSHr) is impaired, or the binding affinity of the receptor is reduced by thermal insult which would limit the effectiveness of hormonal therapy both *in vitro* and *in vivo* due to impaired signal transduction.

Benefits and adoption

Whilst in Seattle, I worked closely with Dr. Penny Swanson (RIA work), Drs. Adam Luckenbach and Yoji Yamamoto (*in vitro* work) who are very experienced and accomplished scientists. That allowed me to develop new 'hands on' skill sets in the areas on immunology and in vitro culture, as well as broaden my knowledge base by learning more about the GTH system and steroidogenesis.

Now that the FSH and LH assays have been tested on a southern hemisphere stock of Atlantic salmon and a working relationship has been formed between myself and Dr. Swanson, there may be opportunities for collaborative work in the future that utilises immunological technology and would benefit the Australian aquaculture sector.

As I am coming to the end of my PhD, the results obtained while in Seattle will be prepared for publication and form an integral part of my PhD thesis. It is my goal, that all of insights gained into the affect of thermal insult and hormonal therapy on the physiology of Atlantic (and coho) salmon will be made publically available to the Australian and international scientific community through publication.

Further development

Treatment with GnRH did not stimulate the release of FSH during reproductive development in female Atlantic salmon reared at 22 °C (experiment 2). However, female pink salmon treated with a combination of GnRH and T larger ovaries and had oocytes displaying signs of more advanced vitellogenesis compared to control and GnRH (only) treated fish (Crossin et al., 2010). This means that trialling a similar approach which aims improving reproductive performance in Atlantic salmon may be warranted as GnRH and T can act synergistically *in vivo*.

While E_2 therapy was not effective at enhancing reproductive performance (Pankhurst., unpublished data) or promoting circulating levels of FSH in thermally challenged female Atlantic salmon, mixed results have also been reported in the literature. For example, Nile tilapia treated with various does of E_2 displayed differential responses in terms of plasma FSH levels *in vivo* (Levavi-Sivan et al., 2006).

For this reason exploring the potential for hormonal rescue by E_2 therapy at a variety of concentrations at 22 °C may be worth while.

As a result of the *in vitro* trial, a suite of candidate genes have been identified that may be dysfunctional in thermally exposed female Atlantic salmon. To determine whether these genes are defective and would therefore help drive poor reproductive performance, the relative expression of various HSD and CYP genes will be analysed for fish from experiment 1 (preliminary qPCR assay development has already begun). By reanalysing these ovarian samples from experiment 1 for HSD and CYP genes, I hope to gain further insight into reproductive dysfunction caused by thermal insult in female Atlantic salmon which will ultimately help to pave the way for effective management protocols that improve reproductive performance. It may also be beneficial to analysed ovarian FSHr gene expression *in vivo*, as the expression some genes was temporarily stimulated *in vitro* by FSH treatment at 14 °C but not 22 °C suggesting reduced signal transduction at high temperature.

Planned outcomes

Whilst in Seattle, I received training in diagnostic techniques which is in line with objective 1. Circulating levels of FSH and LH were successfully measured by RIA during my trip to Seattle; however, an ELISA was not developed as stated in objective 2. Doing the ELISA required the use of recombinant FSH; unfortunately, the FSH produced failed to bind the antibody and could therefore not be used as an ELISA standard. Dr. Swanson will continue to develop this assay in my absence and therefore it may be available in the near future.

As *in vivo* treatment with GnRH (experiment 2) and E_2 (experiment 3) failed to promote the release of FSH into the blood and stimulate the endocrine system of female Atlantic salmon reared at 22 °C, it can be concluded that treatment with these hormones at the doses explored can not be employed as a means of improving reproductive performance. For this reason, other means of hormonal and/or thermal manipulation must be explored before an effective alternate broodstock management protocol can be incorporated into industry practice.

During my stay, an *in vitro* trial was performed which was not part of the original CRC travel grant application. However, this trial has identified potential points of endocrine dysfunction in thermally stressed female Atlantic salmon and helped to fill knowledge gaps concerning the physiological response of salmon to environmental conditions.

Conclusion

The purpose of the research was to determine whether the endocrine profiles of FSH and LH changed as a result of thermal exposure, stock age or hormonal treatment in female Atlantic salmon. Results from experiment 1 and 2 indicate that it is unlikely that plasma FSH is affected by stock age however the same can not be said for temperature. There is some evidence to suggest that fish reared at 22 °C have higher levels of circulating FSH compared to fish reared at 14 °C and this is probably due to a lack of negative feedback in 22 °C fish that have compromised E_2 levels.

Additionally, implantation with GnRH (experiment 2) and E_2 (experiment 3) failed to stimulate the production of FSH or LH at 22 °C *in vivo* which demonstrates the complexity of the hormonal cascade controlling reproductive development. No obvious differences were observed in circulating levels of LH for experiments 1-3, though the preovulatory surge of LH was not captured by our sampling schedule.

The relative expression of several genes involved in steroidogenesis was down regulated by increased temperature in isolated ovarian fragments from coho salmon. Interestingly, treatment with FSH appeared to temporarily stimulate the expression of some genes incubated at 14 °C but did not promote gene expression at all in 22 °C follicles. This could indicate that gene expression of FSH receptor (FSHr) is impaired, or the binding affinity of the receptor is reduced by thermal insult which would limit the effectiveness of hormonal therapy both *in vitro* and *in vivo*. From this trial, a suite of candidate genes that may contribute to low T levels and therefore reproductive performance in Atlantic salmon reared at 22 °C have been identified.

These results combined with our previous work (Pankhurst., unpublished) demonstrate that dysfunction at multiple levels in the endocrine cascade controlling reproductive development in thermally challenged female Atlantic salmon is likely. This work also shows that formulating management strategies that improve reproductive performance in Atlantic salmon reared at elevated temperature is no easy feat. However, as we are learning more about how reproductive physiology changes with various therapeutic, biological (i.e. age) and environmental conditions, we becoming better positioned to make informed choices that will benefit the aquaculture industry in Australia.

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Appendix 1: Intellectual property

It is the intention of the author that all results be made publically available to the aquaculture and wider scientific community through publication (and word of mouth where applicable).