Impacts of Fsh, Igf1, and high temperature on the expression of genes involved in steroidogenesis, cell communication, and apoptosis in isolated coho salmon previtellogenic ovarian follicles

Kelli Anderson[ab*], J. Adam Luckenbach[cd], Yoji Yamamoto[e], Abigail Elizur[a]

[a]Genecology Research Centre, University of the Sunshine Coast, Locked Bag 4 Maroochydore DC, Qld 4558, Australia

[b]Institute for Marine and Antarctic Studies, University of Tasmania Newnham Campus, Private Bag 1345, Launceston, Tas 7001, Australia

[c]Environmental and Fisheries Sciences Division, Northwest Fisheries Science Center, National Marine Fisheries Service, National Oceanic and Atmospheric Administration, 2725 Montlake Blvd E, Seattle, WA 98112, USA

[d]Center for Reproductive Biology, Washington State University, Pullman, WA 99164, USA

[e]School of Aquatic and Fishery Sciences, University of Washington, Seattle, WA 98195, USA

Current affiliation for Dr. Yamamoto: Department of Marine Biosciences, Tokyo University of Marine Science and Technology, Minato-ku, Tokyo 108-8477, Japan

*Author to whom all correspondence should be addressed, Phone: +613 6324 3807, E-mail: kelli.anderson@utas.edu.au

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Abstract

In salmonids, exposure to elevated temperature impairs oogenesis. As such, there is a need to understand the molecular mechanisms that underpin this process, and develop mitigation strategies that maintain or rescue reproductive development in broodstock. In this study, follicle stimulating hormone (Fsh) and/or insulin-like growth factor 1 (Igf1) treatment were assessed for their ability to promote reproductive function at 14 and 22 °C in ovarian follicles from coho salmon *in vitro*. Maintenance at 22 °C resulted in the downregulation of *fsh receptor*, 17α-*hydroxylase/C17,20-lyase* and *p450 aromatase a* (*cyp19a1a*), and *connexin 34.3* (*cx34.3*). While combined treatment with Fsh and Igf1 stimulated the expression of *cyp19a1a* at 14 °C, this treatment was not effective at 22 °C. Upregulation of *cx34.3* occurred in response to treatments that contained Igf1 regardless of temperature, and there is evidence to suggest that apoptosis was inhibited to some extent at 22 °C following combined treatment with Fsh and Igf1. This study demonstrates the thermal impairment of key reproductive genes, and highlights the potential for novel hormone treatments to rescue oogenesis in salmonids.

Keywords

Ovarian steroidogenesis; atresia; apoptosis; climate change; hormonal rescue

1.0 Introduction

It is apparent that climate change will affect the physiology of farmed and wild aquatic poikilotherms, including teleosts, such that their fitness may be compromised. This poses a challenge for the aquaculture of salmon on a global scale (Pankhurst and King, 2010), as higher-than-normal temperatures have a deleterious effect on reproductive function, and in some cases, salmon are already reared towards their upper limit of thermal tolerance (Pankhurst et al. 2011). As such, there

is a growing need to understand the molecular basis for reproductive impairment at high temperature, and develop mitigation strategies that maintain or rescue reproductive performance in changing climatic conditions.

Thermally-induced reproductive dysfunction is complex and appears to occur on multiple levels of the endocrine axis. For example, in female Atlantic salmon (*Salmo salar*), plasma levels of pituitary-derived follicle stimulating hormone (Fsh) were elevated in response to high temperature exposure (Anderson et al. 2012), however ovarian expression of the receptor (*fshr*) appeared to be unaffected during peak vitellogenesis (Anderson et al. 2017c). Similarly, there is a growing body of research demonstrating the thermal sensitivity of forkhead transcription factor L2 (*foxl2*) in non-salmonids (Li et al. 2015; Yamaguchi et al. 2007), which plays a role in the regulation of p450 aromatase a (*cyp19a1a*). However, there was no evidence to suggest that this gene is downregulated in thermally exposed female *S. salar* during vitellogenesis, even though thermal impairment of *cyp19a1a* consistently occurs in this species (Anderson et al. 2017c; Anderson et al. 2012). In addition to *cyp19a1a*, several other enzymes involved in ovarian steroidogenesis appear to be thermally sensitive, such as steroidogenic acute regulatory protein (*star*), 3β-hydroxysteroid dehydrogenase (*hsd3b*), and p450 cholesterol side-chain cleavage protein (*cyp11a1*) (Anderson et al. 2017c; Anderson et al. 2012). A collective dampening of ovarian enzyme gene expression leads to a decrease in 17β-estradiol (E2) production, and combined with a reduced hepatic E2 receptor binding affinity, impairs vitello- and zonagenesis (Anderson et al. 2017c; Watts et al. 2005). Impairment of these processes consistently leads to altered spawning dynamics, and reduced egg quality and embryo survival (Jobling et al. 1995; Pankhurst et al. 2011; Pankhurst et al. 1996; Vikingstad et al. 2016).

In salmonids, some level of ovarian atresia normally occurs during reproductive development as a means of regulating the recruitment of oocytes and total fecundity (Bromage and Cumaranatunga, 1988). However, exposure to elevated temperature promotes pre-spawning ovarian atresia in both

salmonids and non-salmonids, to the detriment of reproductive performance (Linares-Casenave et al. 2002; Miranda et al. 2013; Pankhurst et al. 2011). Despite this, the molecular mechanisms underpinning atresia at high temperature have not been well studied in fish, nor has the impact of high temperature on other essential processes such as cell-to-cell communication. Thus, simultaneously studying the effects of high temperature on steroidogenesis, atresia/apoptosis, and cell-to-cell communication will enable a more in-depth understanding of climate-induced impacts on both wild and farmed salmon.

Administration of hormones has been used for decades in teleosts to manipulate the endocrine system and ultimately control reproductive development and spawning at normal temperatures. In female Tasmanian *S. salar*, *in vivo* treatment with gonadotropin releasing hormone analogue (GnRHa) has shown promise in maintaining fertility and advancing ovulation at temperatures of up to 16 °C (King and Pankhurst, 2004). However, at higher temperatures that are relevant in the context of climate change, *in vivo* treatment with GnRHa or E2 during vitellogenesis did not maintain or improve egg quality (Anderson et al. 2017a, b). Treatment with other hormones has been examined *in vitro* and were able to enhance follicular function at normal temperatures. For example, in coho salmon (*Oncorhynchus kisutch*), Fsh-treatment stimulated ovarian steroidogenesis and increased the expression of anti-apoptotic factors (Luckenbach et al. 2011) and connexin 34.3 (*cx34.3*), a gap junction protein (Luckenbach et al. 2013). Similarly, gene expression and (short-term) enzyme activity of Cyp19a1a increased in response to Fsh in brown trout (*S. trutta*) ovarian follicles (Montserrat et al. 2004). In addition, treatment with insulin-like growth factor 1 (Igf1) promoted E2 and 17α-hydroxylase/C17,20-lyase (Cyp17a1) production in pre-ovulatory granulosa cells in *O. kisutch* (Maestro et al. 1997), and stimulated Cyp19a1a activity and expression in ovarian fragments from red seabream (*Pagrus major*) (Kagawa et al. 2003). There is also evidence to suggest that the action of Fsh may be amplified by the presence of Igf1 (Adashi et al. 1988). Whether these treatments have the same stimulatory effect at higher-than-normal temperatures, or could be used as an *in vivo* mitigation strategy in salmonids remains to be determined.

O. kisutch, a salmonid that is currently farmed in Chile, and to a lesser extent in Japan (Asche et al. 2013), has a synchronous pattern of oocyte development. While data are limited, there is evidence to suggest that the reproductive physiology of wild *O. kisutch* has been negatively impacted by elevated temperature in North America (Flett et al. 1996). In addition, *O. kisutch* has historically been a good model for studying the regulation of ovarian steroidogenesis *in vitro* (Luckenbach et al. 2011; Luckenbach et al. 2013), was therefore chosen as the model species in the current study. Due to the demonstrated stimulatory effects of treatment with Fsh and Igf1 on ovarian steroidogenesis, our aim was to determine whether Fsh and/or Igf1-treatment can be used to stimulate ovarian steroidogenesis and the expression of *cx34.3*, and dampen apoptotic processes at elevated temperature in *O. kisutch*. In doing so, the potential for using novel hormonal therapies to maintain reproductive function or rescue oogenesis in salmonids was evaluated *in vitro*. This approach was taken to gauge whether the treatments chosen show promise, and to improve our understanding of reproductive physiology at high temperature, without having to invest in a large scale/long term *in vivo* trial.

2.0 Methods

2.1 Fish and sampling

The coho salmon utilised in the current study were reared at the Northwest Fisheries Science Center (Seattle, WA, USA) in 10-15 °C recirculated fresh water and fed a standard ration (0.6-1.0% body weight/day) of a commercial diet (Skretting Feeds, Vancouver, BC, Canada), under simulated natural photoperiod (48°N) as previously described for this stock of fish (Yamamoto et al. 2011b). All fish were reared and handled according to the policies and guidelines of the University of Washington Institutional Animal Care and Use Committee (IACUC Protocol 2313-09).

In July, 6 age-2+ female salmon were captured and terminally anaesthetised using buffered tricaine methanesulfonate (0.05% MS-222, Argent Chemical, Redmond, WA) before body weight and fork length measurements were taken. Whole gonads were excised and weighed, then segments were collected into Bouin's fixative for histological analysis while the remaining tissue was temporarily stored in chilled Leibovitz's L-15 medium (Invitrogen, Carlsbad, CA). Gonadosomatic index (GSI) was calculated using the equation gonad weight/body weight x 100).

2.2 Ovarian histology

A segment of ovary from each fish was processed using standard paraffin histology to determine the stage of the follicles. Paraffin-embedded tissues were sectioned at 5 μm with a rotary microtome, stained with hematoxylin and eosin, and the follicles staged according to previously established characteristics (Campbell et al. 2006).

2.3 *In vitro* experimentation

The culture technique and reagents used in the current study have been described previously by Luckenbach et al. (2011). Briefly, ~60 mg pieces of ovary from each fish were sorted into 24-well polystyrene culture plates on ice containing 1 mL L-15 medium so that each fish was represented once in each treatment (n=6 fish/treatment). At the start of the experimental period, the medium in each culture well was replaced with 1 mL of fresh L-15 medium supplemented with 0.2% BSA, either with or without hormonal treatment at 14 or 22 °C with gentle shaking. Twenty-two degrees Celsius was chosen to represent the high temperature already experienced by some captive and farmed salmon in the northern and southern hemispheres during early reproductive development in summer (Flett et al. 1996; Pankhurst et al. 2011). Hormonal treatments were Fsh 100 ng/mL, Igf1 100 nM, or a combination of Fsh and Igf1 at the same concentrations. The Fsh concentration was

chosen based on previous dose-response work by Luckenbach et al. (2011), and Igf levels were based on work by Yamamoto et al. (2011a) and Baker et al. (2000). Human recombinant Igf1 (Bachem, Torrance, CA) was prepared according to Yamamoto et al. (2011b), and highly purified native Fsh was kindly provided by Dr. Penny Swanson (Swanson et al. 1991) and prepared for use as in Luckenbach et al. (2011). The control groups did not receive hormonal treatment, and tissue samples were collected and snap frozen in liquid nitrogen at time 0 (controls only), 3 h, and 24 h for downstream molecular analysis. At the same time, culture medium was collected for measurement of E2 via radioimmunoassay (RIA).

2.4 Measurement of medium E2

Duplicate medium samples from each well were heat treated at 80 °C for 1 h, centrifuged at 15,700 x g for 7 min, and supernatants were transferred to a fresh tube in a similar fashion to Schulz et al. (1994). Medium E2 levels were then quantified using a method established by Sower and Schreck (1982), and the average level for each well was used for subsequent analysis.

2.5 RNA isolation and cDNA synthesis

Total RNA was extracted from ovarian tissue using 1 ml of TriReagent (Sigma-Aldrich), then DNase-treated with TURBO DNA-free (Applied Biosystems) to digest DNA. RNA yield and 260/280 purity ratio were assessed via spectrometry (NanoDrop 1000), and cDNA was synthesised using 0.5 µg total RNA and SuperScript ll (Invitrogen) according to the manufacturer's specifications.

2.6 Primer design and qPCR

Gene specific primers previously designed for qPCR (Luckenbach et al. 2011; Luckenbach et al. 2013; Yamamoto et al. 2011a; Yamamoto et al. 2016) were used to quantify the expression of genes involved in steroidogenesis and reproductive development (*hsd3b*, *star*, *cyp11a1*, *cyp17a1*, *cyp19a1a*, *fshr*, and *luteinizing hormone receptor*, *lhcgr*), cell communication (*cx34.3*), and apoptosis (*caspase 3*, *casp3*, *caspase 8*, *casp8*, *Fas-associated death domain*, *fadd*, *lipopolysaccharide-induced tumour necrosis factor-α factor*, *litaf*) (Table 1). Expression of the candidate reference genes *TATA binding protein* (*tbp*) and *elongation factor 1α* (*eef1a*) was also quantified. Each qPCR reaction contained 150 nM each primer, 1x Power SYBR Green PCR master mix (Applied Biosystems), 0.5 ng cDNA template, and molecular grade water to a final volume of 12.5 µl. All samples were analysed on the same plate to eliminate between-run variation, and negative reverse transcriptase and no-template controls were included to detect possible contamination.

Table 1. qPCR Primers

Gene	****Direction***	***Sequence 5' → 3'***
3ß-hydroxysteroid dehydrogenase	F	CCT TCA TCT ACA CCA GCA GCA TC
	R	TAC AAC ACA TCC CCG TTC CG
steroidogenic acute regulatory protein	F	GGG ACT TCG TTA GTG TTC GCT G
	R	TGG TCT TGT TGG GGT CAT CG
p450 cholesterol side-chain cleavage protein	F	TCA TGG TGC ACA ACT TCA ACA C
	R	GTT CCT GTA GTC TCT GTA TGA
17α-hydroxylase/C17,20-lyase	F	AGA GAC AAG CTG CTT CAG AA
	R	GCC CAT TTT AGG ACT GTT GAC G
p450 aromatase a	F	ACC CGC ACC TAC TTC GCT AAA G
	R	TGC TCT CCT GTG TTT CTG CTG G
follicle stimulating hormone receptor	F	GAC GCA CAT CAG AGT GTT TCC C
	R	GTA GAA CCC TCA GTC CAG TGT TGC

luteinizing hormone receptor	F	TAT CCA TTC TCT GGA ACC TTG G
	R	CTT GGT CCC ATT AAA GGC ATA G
connexin 34.3	F	ACT ACC TGT ATG GCT TCA CCC T
	R	CTG GAT CAT CTG GTC TTT GTT C
caspase 3	F	AAT GAA CTA TCC CAG CCT TGG AC
	R	GCT TTC CCA CCA GCG TTT TG
caspase 8	F	TGT GCC TGC TGT CTC GTA TC
	R	TCC AGG CGT TTC CTA TTG AG
fas-associated death domain	F	AAA CTT GGA CCT GAC AAC ACG G
	R	TCA TTA GGG TGC TTC TGT GCG
lipopolysaccharide-induced tumour necrosis factor-α factor	F	TTC CAG TGC AGA CTC ATT GC
	R	AGC AGG CAA CAG CCA TAG AT
elongation factor 1α	F	CCC CTG GAC ACA GAG ATT TCA TC
	R	AGA GTC ACA CCG TTG GCG TTA C
TATA-box-binding protein	F	TCC CCA ACC TGT GAC GAA CA
	R	GTC TGT CCT GAG CCC CCT GA

*F = forward, R = reverse

qPCRs were conducted in 384-well plates on an ABI 7700 Sequence Detector using the standard cycling conditions outlined by Luckenbach et al. (2011), and a melt curve was included post-amplification to confirm the presence of a single product. *TATA binding protein* was used for target gene normalisation, as consistent with previous studies on salmon, gene expression was stable at ambient and elevated temperatures and expression did not change in response to hormonal treatment (Anderson et al. 2017b; Anderson et al. 2017c; Anderson and Elizur, 2012).

2.7 Statistical analysis

Means for experimental groups were compared within time points via one-way ANOVA coupled with Tukey's post hoc analysis (IBM SPSS statistics v24). All figures were produced using ggplot2 v3.0.0 and cowplot v0.9.3 in RStudio v3.5.1.

3.0 Results

3.1 Morphometric data and ovarian histology

For the 6 fish sampled, mean (± SEM) body weight was 595.75 ± 35.16 g, and mean (± SEM) fork length was 36.42 ± 0.81 cm. Mean paired ovary weight (± SEM) was 7.45 ± 0.42 g and GSI was 1.25 ± 0.02. Ovarian follicles from 5 fish were in the lipid droplet/early yolk granule stage, while those of 1 fish were in lipid droplet stage and did not have noticeable yolk granule accumulation.

3.2 Candidate reference genes

The lipid droplet stage follicles did not have outlying gene expression or medium E2 levels relative to lipid droplet/early yolk granule stage follicles. As such, all samples from each treatment were used for statistical and graphical purposes in the following sections.

Statistically significant differences in *eef1a* expression were present within the 3 and 24 h time points ($p = 0.012$ and 0.046, respectively) (Figure 1). In contrast, there were no statistical differences in ovarian *tbp* levels between experimental groups within the 3 or 24 h time points ($p = 0.765$ and 0.809, respectively), and *tbp* was therefore used as the reference gene for normalisation of target gene expression.

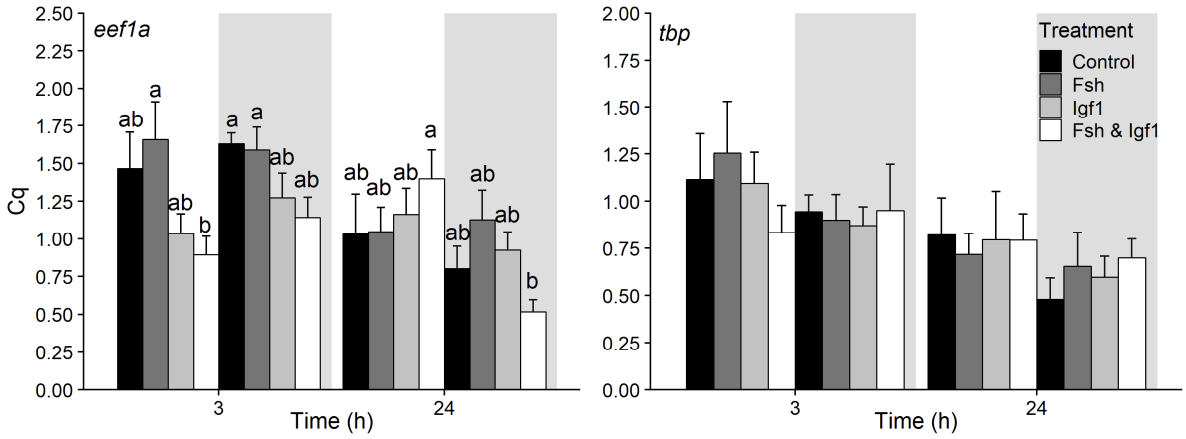


Figure 1. Quantification cycle (Cq) for the candidate reference genes *elongation factor 1α* (*ef1a*) and *TATA binding protein* (*tbp*) in isolated follicles from *O. kisutch* at 14 (white panels) or 22 °C (grey panels) with or without hormonal treatment. Different superscripts between groups at each sampling point denote significantly different means ($p \leq 0.05$).

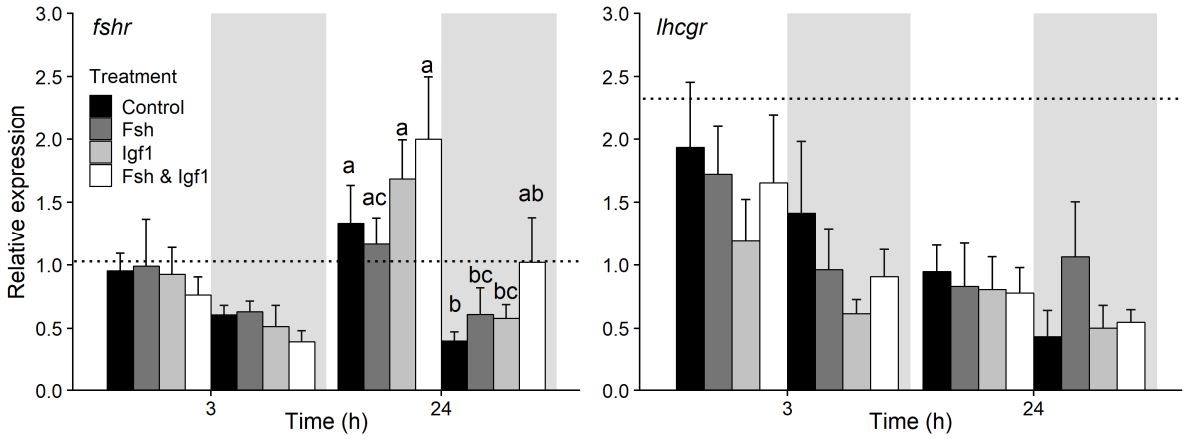
3.3 Impact of culture conditions on basal gene expression

In control groups at 14 °C, the basal level of expression for several genes appeared be lower at 24 and/or 3 h relative to time zero, including *star*, *cyp17a1*, *cyp19a1a*, *lhcgr*, *cx34.3*, *casp3*, and *casp8* (Figures 2, 3, 4, 5). However, the basal expression of some genes appeared to be unaffected by the culture conditions as demonstrated by relatively stable expression for the 14 °C control groups over time for *fshr*, *hsd3b*, and *fadd* (Figures 2, 3, 5).

3.4 Effect of elevated temperature on target gene expression

The expression of ovarian *fshr* at 24 h was significantly lower in the 22 than the 14 °C control group, and there was no significant impact on *lhcgr* (Figure 2). Elevated temperature had a short-term stimulatory effect on *hsd3b* at 3 h, but significantly reduced the expression of *cyp19a1a, cyp17a1,* and *cx34.3* by 24 h (Figures 3, 4). Expression of *cyp11a1* may have been negatively influenced by

228 elevated temperature at 24 h, however differences in expression were not significant (Figure 3). The
229 expression of genes involved in apoptosis was unaffected by temperature within each time point
230 (Figure 5).



231

232 **Figure 2.** Relative gene expression levels (mean + SE, n=6) for gonadal *follicle stimulating hormone receptor*
233 (*fshr*) and *luteinizing hormone receptor* (*lhcgr*) at 14 (white panels) or 22 °C (grey panels). Gene expression
234 levels were normalised against *TATA-box-binding protein*, and the broken horizontal line represents the mean
235 relative expression level of the control group at time zero. Different superscripts between groups at each
236 sampling point denote significantly different means (p≤0.05).

237

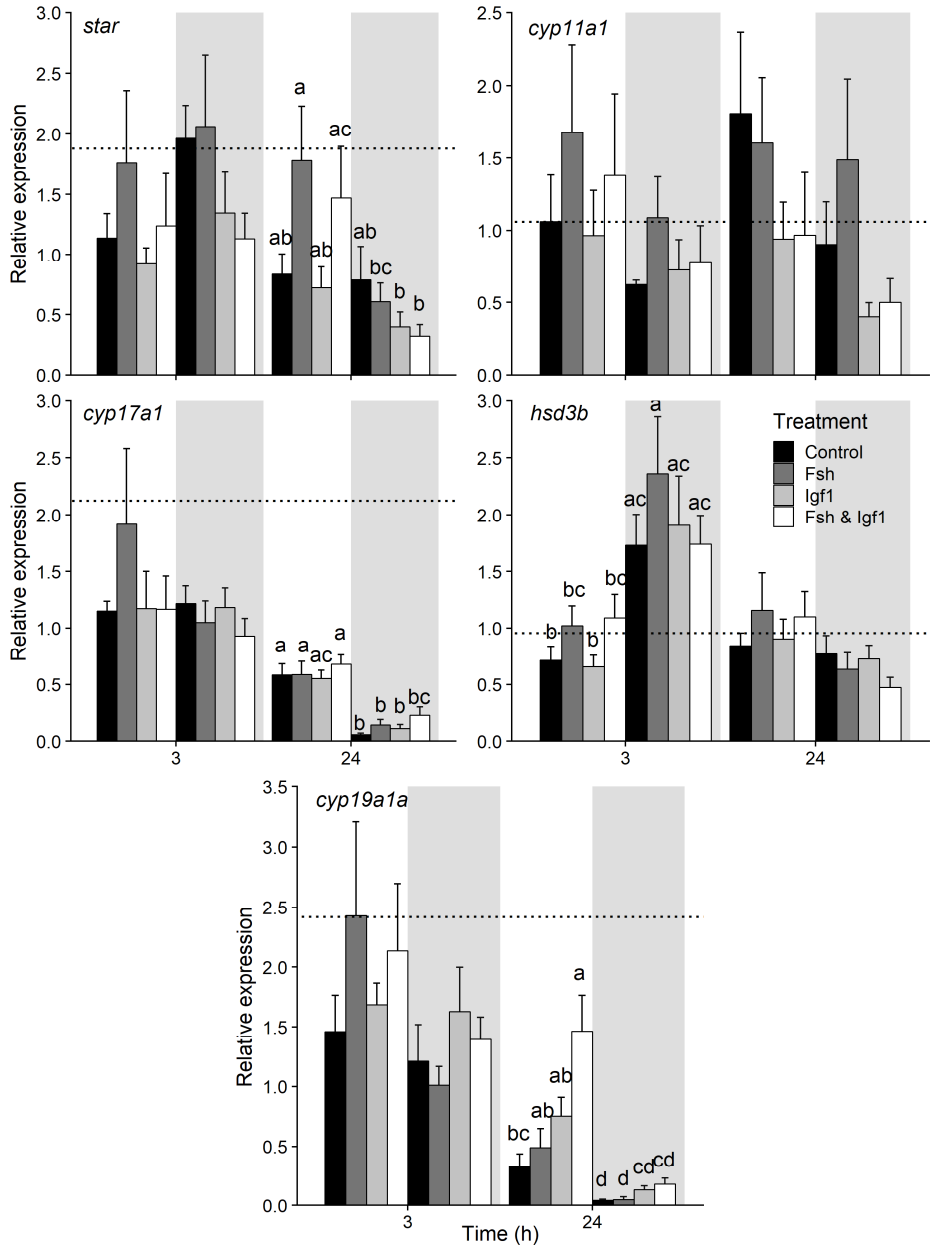
238 **3.5 Effect of hormonal treatment on target gene expression**

239 Treatment with Fsh/Igf1 may have stimulated the expression of *fshr* to some extent at 22 °C, though
240 the difference between these groups was not significantly different (Figure 2). Treatment with a
241 combination of Fsh and Igf1 at 14 °C resulted in an increase in *cyp19a1a* mRNA at 24 h, but this
242 effect was not significant at 22 °C (Figure 3). At 24 h, *cx34.3* expression was significantly higher in
243 Igf1-treated follicles at 14 and 22 °C, and Igf1/Fsh-treated follicles at 22 °C (Figure 4).

244 Treatment with Igf1 and Fsh/Igf1 resulted in significantly lower levels of *litaf* at 22 °C at 24 h, and
245 while the effect was similar at 14 °C the difference was not statistically significant (Figure 5).
246 Similarly, at 22 °C expression of *casp3* was significantly reduced in the Fsh/Igf1 group at 24 h, and

247 *casp8* expression was lower to some extent (non-significant) at 24 h in the 22 °C Igf1 and Fsh/Igf1

248 groups.



249

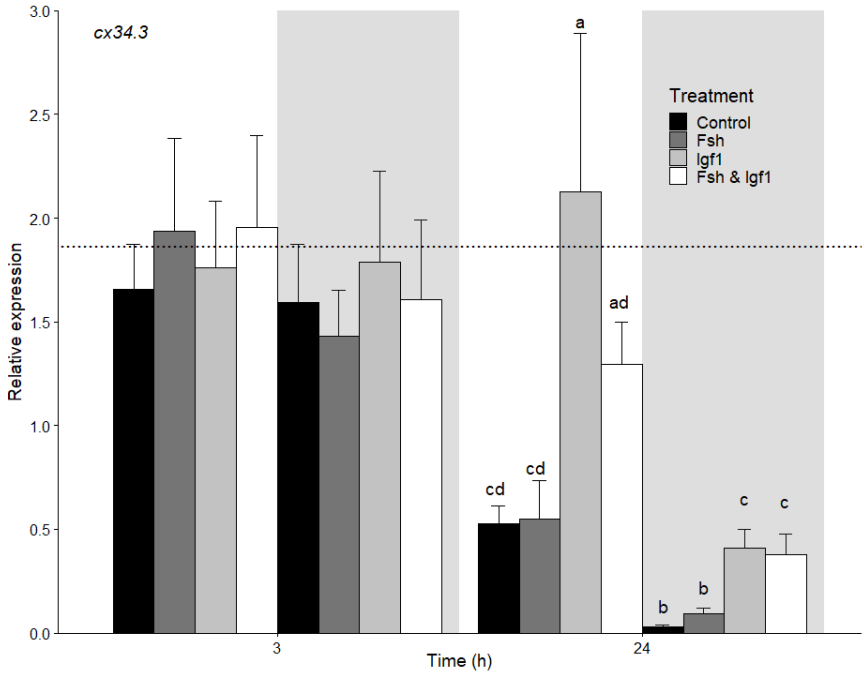
250 **Figure 3.** Relative gene expression levels (mean + SE, n=6) for genes involved in ovarian steroidogenesis:

251 *steroidogenic acute regulatory protein* (*star*), *p450 cholesterol side-chain cleavage protein* (*cyp11a1*), *17α-*

252 *hydroxylase/C17,20-lyase* (*cyp17a1*), *3β-hydroxysteroid dehydrogenase* (*hsd3b*), and *p450 aromatase a*

253 (*cyp19a1a*) at 14 (white panels) or 22 °C (grey panels). Other details as for Figure 2.

254



255

256 **Figure 4.** Relative gene expression levels (mean + SE, n=6) for gonadal and *connexin 34.3* (*cx34.3*) at 14 (white

257 panels) or 22 °C (grey panels). Other details as for Figure 2.

258

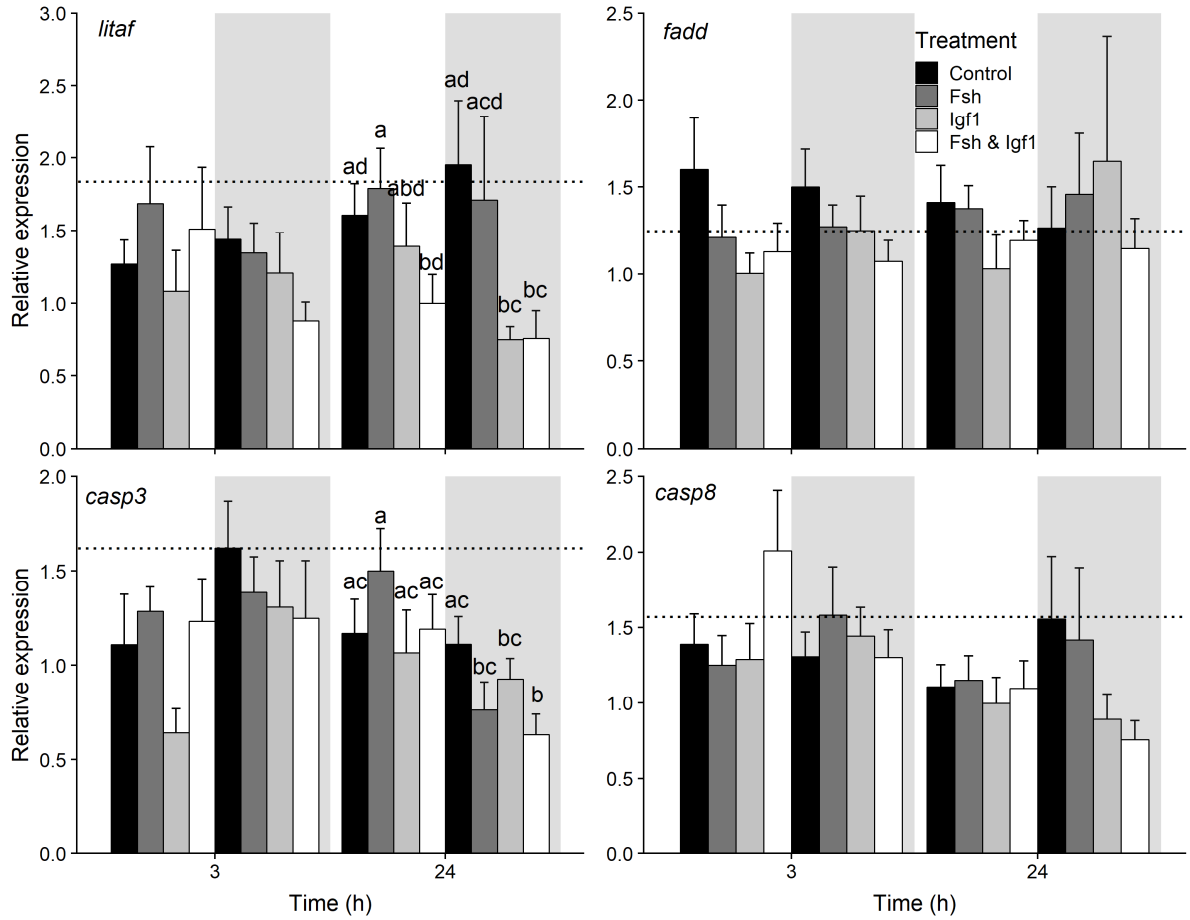


Figure 5. Relative gene expression levels (mean + SE, n=6) for gonadal *lipopolysaccharide-induced tumour necrosis factor-α factor* (*litaf*), *Fas-associated death domain* (*fadd*), *caspase 3* (*casp3*), and *caspase 8* (*casp8*) at 14 (white panels) or 22 °C (grey panels) with or without hormonal treatment. Other details as for Figure 2.

3.6 Medium E2

Medium E2 levels appeared to be similar between the 14 and 22 °C control groups at 3 and 24 h (Figure 6). Medium E2 levels were significantly elevated above those of the respective controls in groups treated with Fsh and Fsh/Igf1, but not Igf1, irrespective of temperature at both time points.

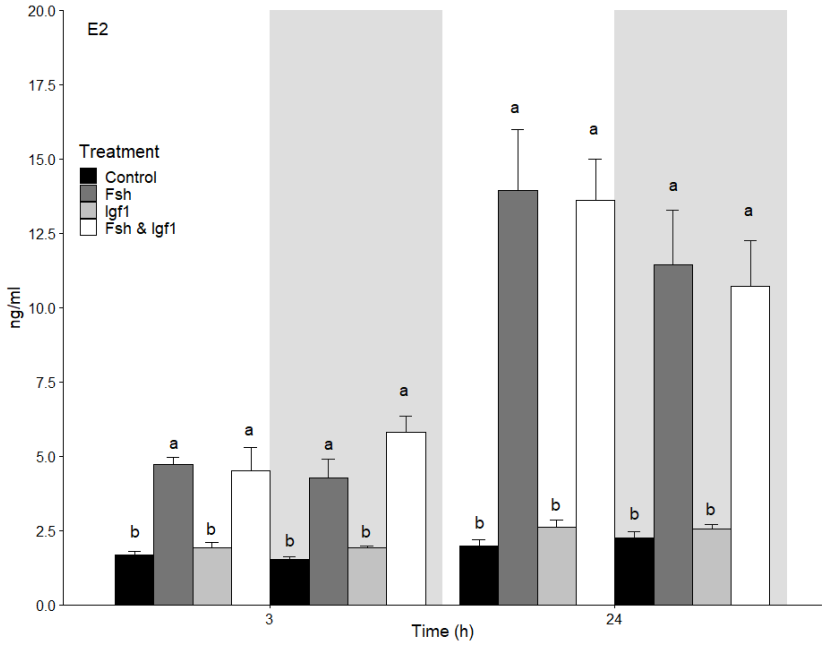


Figure 6. Concentration of medium 17β-estradiol (E2) at 14 (white panels) or 22 °C (grey panels) with or without hormonal treatment. Statistical details as for Figure 2.

4.0 Discussion

Present and predicted climate-driven changes in reproductive physiology pose a unique challenge to those wishing to culture salmonids and other fish species. Despite this, significant knowledge gaps exist concerning the molecular mechanisms underpinning reduced reproductive performance in fish, and research regarding the use hormonal treatments as a mitigation strategy is in its infancy. As such, the aim of the current study was to assess the usefulness of novel hormonal therapies to enhance or maintain the ovarian function of *O. kisutch in vitro* at elevated temperature. To this end, ovarian fragments composed of lipid droplet/early yolk granule stage follicles were maintained at either 14 or 22 °C, with or without Fsh and/or Igf1 treatment, and ovarian steroidogenesis and apoptotic processes were assessed at 0, 3 and 24 h. The work presented here is the first to report on the physiological response of coho salmon ovarian fragments to elevated temperature.

Ovarian expression of *fshr* was significantly downregulated at 22 °C at 24 h, which appears to be consistent with thermal exposure studies during sex differentiation (Yamaguchi et al. 2007), and vitellogenesis (Soria et al. 2008) in non-salmonids. However, the only available data for salmonids (*S. salar*) indicate that *fshr* is not thermally sensitive during vitellogenesis for fish maintained at 22 versus 16 °C (Anderson et al. 2017c), or in fish maintained 2 °C above versus 2 °C below simulated natural temperature (maximum 18.1 versus 15.4 °C, respectively) (Taranger et al. 2015). On the other hand, temperature-dependent impairment of *fshr* gene expression has been detected during the spawning season in *S. salar* (Taranger et al. 2015). Thus, it is possible that the differences in *fshr* sensitivity may be species-specific or may be related to the stage of gonadal development.

Downstream of *fshr*, elevated temperature did not significantly affect the expression of *star* or *cyp11a1* after 24 h of exposure, an unexpected result given the evidence for the thermal sensitivity of these genes in *S. salar* (Anderson et al. 2017c; Anderson et al. 2012). However, it should be noted that in *S. salar*, *star* mRNA levels were lower during late summer (Anderson et al. 2017c), a period of vitellogenic growth that appears to be particularly sensitive to elevated temperature (King et al. 2007), while *cyp11a1* was only reduced in the two months preceding ovulation (Anderson et al. 2012). As such, there is evidence to suggest that transcriptional responses vary according to the stage of oocyte development and timing of thermal exposure, and earlier stage oocytes may be relatively robust in terms of gene expression for some enzymes. This statement comes with the caveat that *cyp11a1* expression was quite variable, and there was a (non-significant) tendency for *cyp11a1* expression to be lower at the higher temperature at 24 h.

hsd3b and *cyp17a1* are enzymes responsible for the conversion of intermediate substrates in the biosynthesis of E2. The only available data describing the thermal sensitivity of these genes in a teleost comes from a study in *S. salar*, where inhibition of ovarian *hsd3b* was present in the month preceding ovulation, but not during early or mid vitellogenesis following exposure to 22 °C (Anderson et al. 2017c). In the current study, there was no evidence to suggest that expression of

this gene was inhibited during short term exposure to elevated temperature. In fact, a short-term stimulatory effect was observed at 3 but not 24 h. Therefore, it is likely that thermal sensitivity is closely linked with the stage of oocyte development and length of exposure in salmonids, as previously demonstrated during vitellogenesis for *S. salar* by King et al. (2007). In contrast, for the first time we report that *cyp17a1* was severely impacted by elevated temperature after 24 h of exposure in a teleost species, and while the exact mechanism remains unknown, dysfunction occurred independent of the brain and pituitary. Temperature-dependant down regulation of *cyp17a1* might be explained to some extent by dampening of *foxl2* expression, as this gene has been shown to play a role in regulating *cyp17a1* in medaka (*Oryzias latipes*) and is thermally sensitive in some species (Li et al. 2015; Yamaguchi et al. 2007; Zhou et al. 2007). However, whether this is the case for *O. kisutch* remains to be seen.

Impairment of ovarian *cyp19a1a* expression at elevated temperatures has gained attention for its importance in adult fish that exhibit reduced E2 levels in response to high temperature (Anderson et al. 2012; Miranda et al. 2013). Consistent with previous work, expression of ovarian *cyp19a1a* was severely impaired after 24 h of exposure to elevated temperature, however, medium E2 levels were unaffected at both time points. While it is possible that the reduction in *cyp19a1a* expression may have been driven to some extent by impairment of *foxl2*, as observed in non-salmonids (Li et al. 2015; Yamaguchi et al. 2007), this phenomenon has not been previously studied in lipid droplet/early yolk granule stage ovaries in any fish species, and was not present in thermally challenged *S. salar* during the vitellogenic growth period (Anderson et al. 2017c). The simultaneous stability of medium E2 levels and reduction in *cyp19a1a* suggests a (presumably) short-term decoupling between enzyme transcript levels and protein synthesis at high temperature, which is similar to what has been observed at normal temperatures in this species (Luckenbach et al. 2011).

At 14 and 22 °C, treatment with Fsh and/or Igf1 did not significantly impact the expression of *fshr*, *star*, *cyp11a1*, *cyp17a1*, or *hsd3b* at any time point. While inhibition of *fshr* gene expression by Fsh

has been previously demonstrated in early secondary growth follicles from *O. kisutch* (Luckenbach et al. 2011), this effect was transient, and the dose used was five times higher than that of the current study. Similarly, significant upregulation of *star* was only achieved with the highest dose of Fsh (500 ng/mL), and only occurred after 72 and 36 h for *cyp11a1* and *cyp17a1*, respectively, at a normal temperature (Luckenbach et al. 2011). In *O. mykiss*, Fsh treatment at 100 ng/mL was able to induce the expression of *star*, *cyp11a1*, and *hsd3b* in late/postvitellogenic but not midvitellogenic follicles (Nakamura et al. 2016). Thus, a high dose may be required to stimulate the expression of some enzymes *in vivo* but may come at the expense of downregulating other important regulatory elements such as *fshr*, and the effectiveness of treatment will be strongly stage-dependent. In the current study, the combined Fsh/Igf1 treatment appeared to be beneficial with significant upregulation of *cyp19a1a* relative to the control at 24 h of exposure. However, this effect was only observed at 14 °C, suggesting to some extent that the effectiveness of hormonal therapy may be reduced at high temperatures *in vivo*.

At 14 °C, treatment with Fsh stimulated the production of E2, which agrees with previous *in vitro* observations for a range of salmonids (Montserrat et al. 2004; Nakamura et al. 2016) including *O. kisutch* (Luckenbach et al. 2011). However, medium E2 was significantly elevated by the Fsh and Fsh/Igf1 treatments at 22 °C despite downregulation of *fshr*, *cyp17a1*, and *cyp19a1a*. This phenomenon could be partially explained by an Fsh-dependent increase in aromatase activity, as observed in mammals, yet Fsh does not appear to elicit the same effect in salmonids (Miwa et al. 1994; Montserrat et al. 2004). In fact, incubation of *S. trutta* follicles with the same concentration of Fsh used in the current study inhibited Cyp19a1a activity after 1 h of exposure (Montserrat et al. 2004). Alternatively, exposure to elevated temperature could have increased enzyme activity, thereby acting as a compensatory mechanism in the production of E2. However, if this were the case, any benefits would likely be short-term, as the longer-term *in vivo* studies consistently demonstrate the negative impacts of thermal challenge on E2 synthesis. Furthermore, there is no evidence to

suggest that the 'rate limiting' step of cholesterol transportation across the inner mitochondrial membrane was negatively impacted, as *star* expression remained intact at the higher temperature.

Expression of *lhcgr* was unaffected by exposure to elevated temperature and hormonal treatment in the current study. This is in line with observations from northern and southern hemisphere stocks of *S. salar* that were exposed to elevated temperature *in vivo*. For example, *lhcgr* expression was not significantly impacted by higher-than-normal temperature during the several months preceding spawning (Taranger et al. 2015), and circulating levels of Lh were not negatively impacted following exposure to 22 °C during mid and late vitellogenesis (Anderson et al. 2012). While the thermal sensitivity of *lhcgr* has been previously demonstrated for other species, such as pejerrey (*Odontesthes bonariensis*) (Elisio et al. 2012), it appears that *lhcgr* may be relatively robust across multiple developmental stages for salmonids at the temperatures tested. In addition, it is unlikely that the action of Fsh was exhibited through Lhcgr, as previous work in *O. kisutch* has demonstrated that Fsh and Lhcgr do not interact (Miwa et al. 1994).

Connexins are the building blocks of gap junctions (GJs), which facilitate cell-to-cell communication throughout oocyte development (Bruzzone et al. 1996). In teleosts, the number of GJs present within the follicle varies with the stage of development, and GJs/connexins have been implicated in facilitating steroidogenesis and in attainment of maturational competence (Patiño and Kagawa, 1999; Yamamoto et al. 2011b; Yamamoto et al. 2007; York et al. 1993). In the current study, *cx34.3* was significantly downregulated following exposure to high temperature, and expression was restored to baseline (control 14 °C) levels following treatment with Igf1 and Fsh/Igf1 at 22 °C. To our knowledge, this is the first study to suggest that cell-to-cell communication via GJs could be impaired by elevated temperatures due to the downregulation of *cx34.3*, and downregulation of *cx34.3* may play a part in the observed dampening of follicular processes. Previous work on *O. kisutch* has demonstrated that connexins expressed in the granulosa are positively regulated by Fsh and Igf1 in lipid droplet stage follicles (Yamamoto et al. 2011b). While treatment with Fsh did not influence *cx34.3* expression at

any temperature in the current study, Igf1 had a stimulatory effect at 14 and 22 °C, indicating that the pathway for upregulation of *cx34.3* by Igf1 is intact to some extent at elevated temperature. Studies in fish on the hormonal regulation of connexins are lacking, though E2 is able to modulate the expression of some connexins in Atlantic croaker (*Micropogonias undulatus*) in a concentration-dependent fashion *in vitro* (Chang et al. 2000). In the current study, medium E2 levels were elevated in the Fsh and Fsh/Igf1 treatment groups regardless of temperature, yet *cx34.3* levels were similar between groups with significantly different E2 levels (i.e. control and Fsh-treated, 14 °C at 24 h). Thus, it appears unlikely that the presence of E2 at the range of concentrations measured influenced the expression of *cx34.3*.

Ovarian atresia is the process by which follicles degenerate and are resorbed if they fail to complete maturation. In fish, apoptosis plays a role in atresia and it has been suggested that the upregulation of several genes, namely Fadd, Casp8, and Casp3 could be early indicators of ovarian apoptosis in fasted *O. kisutch* (Yamamoto et al. 2011a). Casp3 is the main 'effector caspase' in the death receptor-mediated and mitochondria-mediated apoptosis pathways, while Casp8 is an 'initiator caspase' that primarily plays a role in death receptor-mediated apoptosis (Johnson and Bridgham, 2002). The transcription factor Litaf, and Fadd which is involved in death receptor-mediated apoptosis were also analysed. The relative expression levels of *litaf*, *fadd*, *casp3*, and *casp8* were not significantly impacted by exposure to high temperature, and expression of these genes was stable for the duration of the experiment. Given the demonstrated ability of thermal challenge to induce atresia in adult fish prior to spawning (Linares-Casenave et al. 2002; Pankhurst et al. 2011), and promote apoptosis/gonadal degeneration in subadult fish (Ito et al. 2008), the lack of response in terms of gene expression could potentially be explained by multiple hypotheses. First, the simplest explanation may be that the duration of thermal exposure may not have been long enough to elicit a response in the lipid droplet stage follicles tested. This is plausible, as *in vitro* susceptibility to ovarian apoptosis appears to increase as oocytes mature in *O. mykiss*, with susceptibility being

greatest in postovulatory follicles (Wood and van der Kraak, 2001), and in *S. salar* the incidence of ovarian atresia tends to increase with prolonged (months) exposure to elevated temperature (Pankhurst et al. 2011). Second, while apoptosis appears to play a role in atresia in *O. kisutch* (Yamamoto et al. 2011a; Yamamoto et al. 2016), some studies have suggested that autophagy plays a larger part (Thomé et al. 2009) and apoptosis is more evident towards the later stages of follicular degeneration (Morais et al. 2012). Since the temporal coordination of the processes driving atresia have not been studied in *O. kisutch* and have not been well studied in pre-vitellogenic or early secondary growth follicles, the relative importance of apoptosis in the earlier stages of atresia in lipid droplet stage follicles is currently unclear.

Due to the impact of exposure to elevated temperature on incidence of ovarian atresia, it was hypothesised that treatment with hormones that dampen apoptotic pathways/promote cell survival, namely Fsh and Igf1 (Markstrom et al. 2002), may help to maintain follicular health at high temperature. While the expression of apoptosis-related genes was not affected by high temperature, *litaf* was downregulated following treatment with Igf1 and Igf1/Fsh at 22 °C relative to the controls at 14 and 22 °C. Similarly, the combined treatment of Fsh/Igf1 resulted in a significant downregulation of *casp3* at the higher temperature, and there was a non-significant tendency towards decreased *casp8* following Igf1 and Fsh/Igf1 treatment at 22 °C. While short-term treatment with Igf1 or Fsh/Igf1 may provide some level of protection in terms of promoting cell survival, this was not reflected by the expression patterns of genes involved in ovarian steroidogenesis. Thus, it is currently unclear what benefit female fish reared at elevated temperature would receive *in vivo* from such treatments, though there may be some net advantage due to the dampening of apoptosis and subsequent higher frequency of surviving follicles. In addition, the possible implications of the relatively rapid transition to 22 °C used in this study must also be considered, as temperature change in natural or farm environments may occur more gradually, and elicit different physiological effects *in vivo*.

5.0 Conclusion

The current work is the first to study the impact of Fsh and/or Igf1 treatment on the expression of genes involved in follicular steroidogenesis, cell-to-cell communication and apoptosis at elevated temperature in a salmonid species. For most steroidogenic genes analysed (*e.g. cyp19a1a*), the negative effects of high temperature appear conserved among fish species from different taxonomic groups, though there is evidence to suggest that the stage of oocyte development is important when considering the likelihood of thermal impairment (*e.g. fshr*). While combined treatment with Fsh and Igf1 was able to stimulate the expression of *cyp19a1a*, this effect was only significant at 14 °C which implies that the effectiveness of hormonal therapies may be limited to some extent at high temperatures in the context of steroidogenesis. On the other hand, genes involved in cell-to-cell communication and apoptosis were up and downregulated at 22 °C, respectively, in response to treatments containing Igf1. Thus, *in vivo* treatments containing Igf1 warrant future investigation, as there may be some benefit in terms of follicular function and survival at high temperatures, which could in turn help to maintain oogenesis and reproductive performance.

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