Effect of thermal challenge on the expression of genes involved in ovarian steroidogenesis in Tasmanian Atlantic salmon (Salmo salar)

Author

Anderson, Kelli, Pankhurst, Ned, King, Harry, Elizur, Abigail

Published

2017

Journal Title

Aquaculture

Version

Accepted Manuscript (AM)

DOI

10.1016/j.aquaculture.2017.06.012

Effect of thermal challenge on the expression of genes involved in ovarian steroidogenesis in Tasmanian Atlantic salmon (*Salmo salar*).

Kelli Anderson[ab], Ned Pankhurst[c], Harry King[d], Abigail Elizur[a*]

[a] Genecology Research Centre, Faculty of Science, Health, Education and Engineering, University of the Sunshine Coast, Locked Bag 4 Maroochydore DC, Qld 4558, Australia. (Kelli: ando.kelli@gmail.com, Abigail: AElizur@usc.edu.au)

[b] Australian Seafood Cooperative Research Centre, Box 26, Mark Oliphant Building, Science 10 Park Adelaide, Laffer Drive, Bedford Park, SA, 5042, Australia

[c] Australian Rivers Institute, Griffith University, Gold Coast, Qld 4222, Australia. (Ned: N.Pankhurst@griffith.edu.au)

[d] Salmon Enterprises of Tasmania, P.O. Box 1, Wayatinah, Tas 7140, Australia

Current affiliation for Dr. King: Commonwealth Scientific and Industrial Research Organisation, GPO Box 1538, Hobart, Tas 7001, Australia (Harry: Harry.King@csiro.au)

*Author to whom all correspondence should be addressed, Phone: +61 7 5459 4813, Fax: +61 7 5430 2881, E-mail: AElizur@usc.edu.au

Acknowledgements

This work was supported by the Australian Fisheries Research and Development Corporation (grant 2008/217), the Australian Seafood Cooperative Research Centre (grants 2008/762 and

2010/719), and Salmon Enterprises of Tasmania Pty. Ltd. (SALTAS). Thanks are extended to SALTAS staff for assistance with fish husbandry and management. K. Anderson was supported by an Australian Post Graduate Award through the University of the Sunshine Coast.

Abstract

Thermal challenge typically results in lowered plasma 17β-estradiol and 17,20β-dihydroxy-4-pregnen-3-one, and is associated with poor reproductive performance in commercially important female Atlantic salmon. The aim of the present study was to gauge the thermal sensitivity of genes crucial to ovarian steroidogenesis, and determine how they fit into this endocrine response in reproductive fish of different ages. Exposure to higher-than-normal temperature (22 vs 14 °C) did not significantly impact the expression of forkhead transcription factor or follicle stimulating hormone receptor (*fshr*) during peak vitellogenesis, although, *fshr* was lower in thermally challenged maiden fish in the lead up to final oocyte maturation. For the first time, we have demonstrated the temperature-dependent down-regulation of ovarian steroidogenic acute regulatory protein and 3β-hydroxysteroid dehydrogenase in the month preceding ovulation, and now have a better understanding of the mechanism underlying the inability of thermally exposed fish to recover after temperature reduction prior to final oocyte maturation.

Keywords

Atlantic salmon; Temperature; Reproduction; Gene expression; Ovarian steroidogenesis

1.0 Introduction

There is significant concern over how climate change may impact farmed fish species, as the deleterious effects of temperature have been observed across a range of biological processes including reproduction (Pankhurst et al., 2011). This is especially relevant to Tasmanian Atlantic salmon (*Salmo salar*), a non-native but commercially important species currenlty farmed towards their upper limit of thermal tolerance (22-23.5 °C) (Pennell and Barton 1996).

In adult female *S. salar*, thermal exposure disrupts the endocrine process that produce 17β-estradiol (E2), which ultimately impairs reproductive development, and impacts can vary with stock age (Pankhurst and King 2010; Pankhurst et al., 2011; Anderson et al., 2012). However, the molecular mechanism(s) by which steroidogenic pathways are affected by elevated temperature are not properly understood, especially in adult fish. In female pejerrey (*Odontesthes bonariensis*), thermal inhibition of follicle stimulating hormone receptor (*fshr*) gene expression and a subsequent decrease in plasma E2 were observed in fish maintained at 23 and 27 versus 19 °C (Soria et al., 2008), suggesting that Fsh signal transduction had been affected. This seems likely considering that in *S. salar*, thermally-induced down regulation of p450 aromatase a (*cyp19a1a*) and p450 cholesterol side-chain cleavage protein (*cyp11a1*) occurs even when plasma Fsh levels are high (Anderson et al., 2012). This raises questions concerning other important factors 'upstream' of Cyp19a1a and Cyp11a1 such as steroidogenic acute regulatory protein (Star), as to our knowledge its thermal sensitivity has never been tested in fish or any other model. As such, it is not clear whether diminished *fshr* or *star* contribute to a general down-regulation of genes involved in the synthesis of E2 during periods of high temperature in *S. salar*.

Thermal impairment of *cyp19a1a* and *cyp11a1* have been observed during vitellogenesis in adult *S. salar* exposed to 22 vs 14 °C (Anderson et al., 2012). Consequently, plasma E2, vitellogenesis, zonagenesis, and reproductive performance indicators were lower at the higher temperature, and younger first-time-spawning (maiden) fish were more severely impacted than second-time-spawning (repeat) fish (Pankhurst et al., 2011). There is growing evidence to suggest that forkhead transcription factor (Foxl2) plays a role in transcriptional regulation of *cyp19a1a* and 17α-hydroxylase/C17,20-lyase (*cyp17a*) (Nakamoto et al., 2006; Wang et al., 2007; Zhou et al., 2007), and is sensitive to thermal exposure (Yamaguchi et al., 2007). Therefore it is possible that in adult salmon that ovarian steroidogenesis is influenced by the expression of transcription factors such as Foxl2, and their potential role in reproductive dysfunction at higher-than-normal temperatures requires investigation.

In the lead up to ovulation, Star, Cyp11a1 and 3β-hydroxysteroid dehydrogenase (3β-Hsd) are all crucial for the production of 17α-hydroxyprogesterone in ovarian follicles, which is the base molecule for maturation-inducing steroid (17,20β-dihydroxy-4-pregnen-3-one, MIS) (Nagahama and Yamashita 2008). Previous studies in *S. salar* and rainbow trout (*O. mykiss*) have demonstrated thermal suppression of MIS, and a subsequent impairment of final oocyte maturation (FOM), ovulation and egg quality (King and Pankhurst 2004; Pankhurst and Thomas 1998). While *cyp11a1* is known to be thermally sensitive in adult *S. salar* in the months preceding ovulation (Anderson et al., 2012), currently no data is available for *star* or *3β-hsd* in salmonids or any other fish species.

The aim of the present study was to characterise the expression profiles of genes involved in ovarian steroidogenesis throughout the reproductive season of female *S. salar*, and determine whether their expression is thermally sensitive at temperatures relevant to the Tasmanian salmon industry. For two of the genes studied (Fshr and Foxl2), there is a growing body of research suggesting that thermal sensitivity of gene expression is likely. For 3β-Hsd and Star,

the present study will provide a first insight into how these genes respond to thermal insult in a fish model over an entire reproductive season.

2.0 Materials and Methods

2.1 Fish maintenance

Seventy-seven maiden (first spawning 2+ year old) and 77 repeat (second spawning 3+ year old) cultured adult female Atlantic salmon were held at the Salmon Enterprises of Tasmania Wayatinah Hatchery, Tasmania, Australia, in fresh water at natural temperature and photoperiod from August 2007 until early January 2008 (Fig. 1). During this period, maiden (2.2 – 4 kg) and repeat fish (4 – 9 kg) were maintained in either 200 or 50 m^3 circular tanks at a stocking density of 12 - 18, and 24 - 36 $kg.m^{-3}$ respectively. Note that there were no significant differences in body weight within each group, however repeat spawners were consistently larger than maidens (Pankhurst et al., 2011).

In January, 112 fish were transferred to temperature-controlled 4 m^3 Rathbun tanks under simulated natural photoperiod according to the following treatment groups; maiden 14 °C, maiden 22 °C, repeat 14 °C and repeat 22 °C. Each experimental group (n=28) was split between 2 tanks (14 fish per tank) giving a total of 8 tanks. Fish were not fed from the time of transfer to the temperature controlled systems in January consistent with hatchery practice for management of this stock.

All fish were maintained at the nominated temperature (14 or 22 °C) until early April when they were exposed to a temperature ramp down over 11 days to 8 °C (Fig. 1) to induce final oocyte maturation and ovulation as in King and Pankhurst (2000). This research activity was

undertaken with approval from the Animal Ethics Committee of the University of the Sunshine Coast (approval number AN/A/07).

2.2 Sampling protocol

Ovaries were dissected from sacrificed fish (terminally anaesthetised in Aqi-S ™), from both age groups on August 31st and November 2nd 2007, and January 7th 2008 to cover the initiation of vitellogenesis at ambient temperature (Fig. 1). After introduction to the controlled temperature regimes, all 4 groups of fish were sampled on February 14th, March 28th and April 25th 2008 (Fig. 1). Seven fish were sampled from each group at each sample time, leaving 7 fish from each treatment to proceed through to ovulation and fertilisation.

2.3 RNA isolation and cDNA synthesis

Total RNA was isolated from 120 mg of ovarian tissue using TRIZOL® reagent (Invitrogen) according to the manufacturer's protocol. RNA yields and purity ratios (260/230: range 1.89 - 2.1 and 260/280: range 1.75 - 1.93) were determined using the NanoDrop 2000 (Thermo Scientific). An RNA integrity number was determined for a random sample of ovarian RNA (n=24, range 8.8 - 9.3) using the 2100 bioanalyzer to establish RNA quality (Agilent). One microgram of ovary-derived RNA was then used to synthesise cDNA for use in real-time quantitative PCR (qPCR) using the QuantiTect® reverse transcription kit (Qiagen).

2.4 Partial isolation of Foxl2 gene

To amplify a fragment of the Foxl2 gene from *S. salar*, Foxl2 mRNA nucleotide sequences from *Paralichthys olivaceus* (AB303854), *Oreochromis aureus* (AM232738), *Oncorhynchus mykiss* (NM_001124485) were aligned using ClustalW2 (http://www.ebi.ac.uk/) and primers were designed from conserved regions (F1: GAG GAT GAC GCA ATG GCC, R3: AGT GCA TCA TGG ASA GCT CCG). One microliter of ovary derived cDNA template and the appropriate amount of molecular grade H_2O were added to each PCR reaction to give a final concentration of 1× PCR buffer, 0.2 mM each dNTP, 2 mM MgCl2, 0.2 µM of each primer and 1 unit TAQ (Fisher Biotec). Thermal cycling consisted of an initial denaturation step at 94 °C for 1 min; followed by 34 cycles of 94 °C for 30 s, 57 °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) according to the manufacturer's instructions. The resultant Foxl2 sequence was submitted to GenBank (accession JX184084).

2.5 Measurement of gene expression

Primers used to quantify Fshr, Foxl2, 3β-Hsd and Star gene expression were designed using Primer3 software (Table 1). qPCRs were conducted on a Rotor-gene 6000 series thermal cycler (Qiagen) using the Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen) master mix and the cycling conditions: 50 °C - 2 min; 95 °C - 2 min; 40 cycles of 95 °C - 15 s; 60 °C - 15 s, and 72 °C - 20 s (acquiring). Following amplification, a melt curve analysis was performed to confirm the amplification of a single product as follows: 90 s - 72 °C, followed by a temperature gradient up to 95 °C at 1 °C per 5 s. Each 10 µl qPCR reaction contained 5 µl SYBR mix, 200 nM each primer, 4 µl PCR grade water and 0.6 µl cDNA template. For every gene analysed no-template controls and a calibrator sample of pooled

cDNA were included to detect possible contamination, and control for in-between run variability, respectively.

For each primer set, a validation curve was constructed using serially diluted cDNA. The reaction efficiencies (Table 1) were automatically calculated from these curves by Rotor-gene software version 1.7.87 using the equation: $E = [10^{(-1/M)}] - 1$, where E equals efficiency and M is equal to slope.

Similar to what was observed for *tbp* in Atlantic salmon hepatic tissue (Anderson and Elizur 2012), Kruskal-Wallis analysis coupled with Bonferroni's correction (performed on a month-by-month basis) revealed no significant differences in *tbp* transcript abundance during the experimental period. In addition, no significant correlation was found between Cq value and sample point by Kendall's tau correlation analysis at $p \leq 0.01$. Thus, *tbp* showed consistently high stability under the experimental conditions and was used as a reference gene for normalisation using Rest© 2009 (Pfaffl et al., 2002).

2.6 Statistical analysis

Differences in relative gene expression levels were detected non-parametrically using the Kruskal–Wallis test coupled with Bonferroni's Correction (starting P was set to 0.05). Values for fish whose level of gene expression was outside the region of linear amplification were recorded as the lower limit of detection for the assay (LOD, Table 1) for statistical and graphical purposes. All analysis was performed using SPSS version 19.0.

3.0 Results

The relative gene expression level of *fshr* steadily increased from August until mid-vitellogenesis, and then decreased into late vitellogenesis for fish reared at both temperatures (Fig. 2). From August until January, there was no difference in the mean relative expression level of *star* and *fshr* between maiden and repeat-spawning salmon at natural temperature (Fig. 2). There were no differences in *fshr* gene expression with the exception of February where expression was higher in maidens than repeats at 22 °C, and in April where expression was lower in maidens at 22 °C relative to all other groups. After approximately 1 month at 14 or 22 °C, the expression of *star* was significantly higher in maiden than repeat fish at both temperatures, this difference was not present in March, then in April thermal suppression of *star* was evident regardless of age class.

Gene expression levels of *3β-hsd* were lower in maiden than repeat-spawning fish in January at ambient temperature and February at 14 °C (Fig. 2). In April, *3β-hsd* gene expression was lower in fish reared at 22 °C relative to 14 °C irrespective of age-class. For foxl2, gene expression levels were significantly higher in repeat-spawning fish during August relative to maidens at ambient temperature. From November gene expression in both age groups was similar and increased until mid-vitellogenesis, from then on gene expression was relatively stable regardless of temperature (Fig. 2).

4.0 Discussion

4.1 Early vitellogenesis

Oocyte development and developmental trajectories were similar among maiden and repeat-spawning female Atlantic salmon, although differences in somatic condition have been observed for repeat-spawning fish during early vitellogenesis (Pankhurst et al., 2011). The

lower somatic condition of repeat-spawners may reflect a need for these fish to recover from the energetic demands of the previous spawning season (Pankhurst et al., 2011). As a result, early development in repeats is typically characterised by a lag period where levels of some endocrine factors (plasma Fsh, E2 and vitellogenin - Vtg), and ovarian/hepatic transcripts (*vtg*, zona pellucida b - *zpb*, *cyp19a1a*, *cyp11a1* and estrogen receptor alpha - *erα*) are depressed relative to maidens (Pankhurst et al., 2011; Anderson et al., 2012). In the present study (which used the same set of tissue samples as the two aforementioned studies), there were no significant differences in gene expression for ovarian *fshr*, *3β-hsd* or *star* between groups. As such, it appears that transcription of these genes is not sensitive to differences in somatic condition or fish age.

For the first time we have observed a clear age-dependent difference in gene expression levels of *foxl2* where expression was significantly higher in repeat-spawning fish during early vitellogenesis in August. As the expression of most reproductive genes tested to date are downregulated in repeat-spawning fish during that time (Pankhurst et al., 2011; Anderson et al., 2012), the contrasting gene expression pattern for *foxl2* may represent a mechanism by which lagging fish can begin to 'catch up' in terms of augmenting endocrine processes. Having said that, the observed increase in *foxl2* was not enough to offset the lower transcription levels of ovarian *cyp19a1* or subsequent lack of production of E2 observed during August (Pankhurst et al., 2011; Anderson et al., 2012).

4.2 Peak vitellogenesis

Consistent with previous observations in other species, ovarian *fshr* levels in the present study increased as oocyte development progressed, peaked at mid-vitellogenesis and then decreased as FOM approached (Kwok et al., 2005; Kobayashi et al., 2008). A previous study

by Anderson and colleagues (2012), suggested that levels of *fshr* may be depressed in *S. salar*, due to the down regulation of ovarian *cyp11a1* and *cyp19a1a* in the presence of elevated plasma FSH at high temperature. However, in the present study, *fshr* levels were unaffected by elevated temperature during the period corresponding to vitellogenic growth. This finding is not consistent with those of Soria et al., (2008), or with Yamaguchi et al., (2007) who found that ovarian *fshr* gene expression was reduced in adult *O. Bonariensis* and juvenile Japanese flounder (*P. olivaceus*) respectively at elevated temperature. An alternate explanation for the lack of downstream gene stimulation by the high levels of Fsh observed by Anderson et al., (2012) is that Fshr ligand binding affinity may have been negatively impacted, which would affect signal transduction as observed for hepatic erα in both tilapia (*Oreochromis aureus*) and *S. salar* (Tan et al., 1999; Watts et al., 2005). At present, no information is currently available on the effects of temperature on Fshr binding affinity in teleosts or other organisms.

The transportation of cholesterol across the inner mitochondrial membrane by Star is considered to be the key rate-limiting step of ovarian steroidogenesis (Reviewed by Lubzens et al., 2010). In the present study, *star* expression was higher in maiden than repeat female *S. salar*, irrespective of temperature, during vitellogenesis in February. This pattern of expression is contrary to those observed for downstream genes (zona pellucida c - *zpc*, *zpb*, *vtg*, *cyp19a1a*, *cyp11a1* and *erα*) in our previous work (Pankhurst et al., 2011; Anderson et al., 2012). Interestingly, King et al., (2007) discovered that February corresponds to a period of heightened thermal sensitivity, and as such is a critical time for oocyte development at elevated temperature. Thus, over-expression of *star* during February may reflect an (albeit unsuccessful) attempt to compensate for downstream endocrine suppression during a known critical window.

Foxl2 stimulates expression of Cyp19a1a by binding to its promoter, and is therefore able to alter the rate of T to E_2 conversion during ovarian steroidogenesis (Wang et al., 2007; Yamaguchi and Kitano 2008). In the present study, *foxl2* in maiden and repeat-spawning fish was not significantly influenced by exposure to higher temperature. An unexpected finding considering the pronounced thermal inhibition of both E_2 and *cyp19a1a* levels in adult female salmon during vitellogenesis (Pankhurst et al., 2011; Anderson et al., 2012), and the thermal sensitivity of *foxl2* in juvenile teleosts (Lim et al., 2003; Yamaguchi et al., 2007; Baroiller et al., 2009). While temperature-dependant sex determination (TSD) has been demonstrated to some extent in juvenile salmonids, namely *S. salar* (King et al., 2012), *O. nerka* (Azuma et al., 2004) and *O. mykiss* (Magerhans et al., 2009), the molecular mechanism(s) driving TSD are yet to be confirmed for salmonids. As such, for *S. salar* it's possible that *foxl2* down-regulation is an important aspect of TSD in juveniles as observed for other species, or alternatively, *foxl2* is not thermally sensitive in adult or juvenile life stages unlike other species. In any case, *foxl2* does not appear to be thermally sensitive in adult female *S. salar*, and the basis for *cyp19a1a* inhibition remains unclear.

4.3 Post vitellogenesis/FOM

Follicular Maturational Competence (FMC) can be defined as a follicle's ability to respond to gonadotropin and subsequently resume meiosis in the lead up to ovulation. In *O. mykiss*, fish that displayed a high level of FMC had significantly higher levels of *fshr* during FOM than those that were less competent (Bobe et al., 2003). The physiological significance of such a finding is unclear (Bobe et al., 2003), however it is interesting to note that in the present study maiden fish maintained at 22 °C had a significantly lower abundance of *fshr* transcripts than any other group in the lead up to FOM during April. Ovulation in that group of fish was

delayed relative to other groups (Pankhurst et al., 2011). Thus, role of *fshr* in the later stages of this process remains to be elucidated, and may be important in the context of ovulation.

For female *S. salar* maintained at 16 °C, temperature reduction to 11 °C in early May resulted in a heightened sensitivity to maturation-inducing hormones, and an advancement of ovulation relative to fish that were maintained exclusively at 16 °C (King and Pankhurst 2004). The authors of that study concluded that temperature reduction is a useful tool for stimulating the synthesis of MIS and subsequently restoring maturational competence in thermally exposed female salmon (King and Pankhurst 2004). In the present study, temperature reduction to 8 °C in April resulted in a dramatic increase in *star* expression in all fish previously maintained at 14 °C but not 22 °C. As cholesterol is a potentially limiting substrate in the gonadal synthesis of MIS (Lubzens et al., 2010) and is essential for oocyte maturation and ovulation, an increase in *star* expression may be important for attaining maturational competence after temperature reduction. This seems plausible, as fish previously maintained at 22 °C had stable *star* expression, and ovulation was delayed in those fish relative to those held at 14 °C, where a sharp increase in *star* was apparent after temperature reduction (Pankhurst et al., 2011).

Previous studies in *S. salar* and *O. mykiss* have demonstrated thermal suppression of MIS, and a subsequent impairment of FOM, ovulation and egg quality (Pankhurst and Thomas 1998; King and Pankhurst 2004). During ovarian steroidogenesis, the enzyme 3β-Hsd plays an important role in the creation of multiple intermediate substrates required for the synthesis of MIS (Lubzens et al., 2010) and subsequent maturation and ovulation. In the present study, we demonstrate for the first time, thermal suppression of *3β-hsd* in ovarian tissue from maiden and repeat-spawning fish in April prior to FOM. As such, it is possible that the reduced reproductive performance observed in other studies on *S. salar* occurred at least in part due to down regulation of *3β-hsd*, and a subsequent shortfall in the quantity of substrate

available for the production of MIS. Furthermore, *3β-hsd* levels rose sharply in fish previously maintained at 14 °C but not 22 °C between March and April after temperature reduction to 8 °C. This data suggests that the upregulation of *3β-hsd* (and *star*) plays an important role in the attainment of maturational competency and ovulation in *S. salar* after temperature reduction in the period prior to spawning.

4.4 Conclusion

Contrary to expectations, exposure to higher-than-normal temperature did not significantly impact the expression of Foxl2 or Fshr during peak vitellogenesis. For the first time, we have demonstrated the temperature-dependent down-regulation of ovarian *star* and *3β-hsd* in adult fish, and now have a better understanding of the mechanism underlying the inability of thermally exposed fish to recover after temperature reduction prior to FOM. Furthermore, we highlight that the role of *fshr* in FOM and ovulation needs to be elucidated. Thus, we have added to a growing body of research demonstrating the complex mediation of reproductive endocrine factors by elevated temperature in salmonids.

References

Anderson, K., Swanson, P., Pankhurst, N., 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 334-337:205-212.

Anderson, K., Elizur, A., 2012. Hepatic reference gene selection in adult and juvenile female Atlantic salmon reared under thermal challenge. BMC Res. Notes 5. 10.1186/1756-0500-5-21

Azuma, T., Takeda, K., Doi, T., Muto, K., Akutsu, M., Sawada, M., Adachi, S., 2004. The influence of temperature on sex determination in sockeye salmon *Oncorhynchus nerka*. Aquaculture 234:461-473.

Baroiller, JF., D'Cotta, H., Bezault, E., Wessels, S., Hoerstgen-Schwark, G., 2009. Tilapia sex determination: where temperature and genetics meet. Comp. Biochem. and Physiol. 153:30-38.

Bobe, J., Maugars, G., Nguyen, T., Rime, H., Jalabert, B., 2003. Rainbow trout follicular maturational competence acquisition is associated with an increased expression of follicle stimulating

hormone receptor and insulin-like growth factor 2 messenger RNAs. Mol. Reprod. Devel. 66:46-53.
King, HR., Pankhurst, NW., 2000. Ovulation of Tasmanian Atlantic salmon maintained at elevated temperatures: implications of climate change for sustainable industry development. In: Norberg B, Kjesbu OS, Taranger GL, Andersson E, and Stefansson SO, eds. 6th International Symposium on the Reproductive Physiology of Fish. Bergen, 396-398.
King, HR., Pankhurst, NW., 2004. Effect of short-term temperature reduction on ovulation and LHRHa responsiveness in female Atlantic salmon (*Salmo salar*) maintained at elevated water temperatures. Aquaculture 238:421-436.
King, HR., Pankhurst, NW., Watts, M., 2007. Reproductive sensitivity to elevated water temperatures in female Atlantic salmon is heightened at certain stages of vitellogenesis. J. Fish Biol. 70:190-205.
King, HR., Ruff, N., Evans, B., Elliott, N., 2012. Evidence that sex differentiation in Atlantic salmon is thermolabile. International Conference on Integrative Salmonid Biology. Oslo.
Kobayashi, T., Pakarinen, P., Torgersen, J., Huhtaniemi, I., Andersen, Ø., 2008. The gonadotropin receptors FSH-R and LH-R of Atlantic halibut (*Hippoglossus hippoglossus*)—2. Differential follicle expression and asynchronous oogenesis. Gen. Comp. Endocrinol. 156:595-602.
Kwok, H-F., So, W-K., Wang, Y., Ge, W., 2005. Zebrafish gonadotropins and their receptors: I. Cloning and characterization of zebrafish follicle-stimulating hormone and luteinizing hormone receptors- evidence for their distinct functions in follicle development. Biol. Reprod. 72:1370-1381.
Lim, B-S., Kagawa, H., Gen, K., Okuzawa, K., 2003. Effects of water temperature on the gonadal development and expression of steroidogenic enzymes in the gonad of juvenile red seabream, *Pagrus major*. Fish Physiol. Biochem. 28:161-162.
Lubzens, E., Young, G., Bobe, J., Cerdà, J., 2010. Oogenesis in teleosts: how fish eggs are formed. Gen. Comp. Endocrinol. 165:367-389.
Magerhans, A., Müller-Belecke, A., Hörstgen-Schwark, G., 2009. Effect of rearing temperatures post hatching on sex ratios of rainbow trout (*Oncorhynchus mykiss*) populations. Aquaculture 294:25-29.
Nagahama, Y., Yamashita, M., 2008. Regulation of oocyte maturation in fish. Dev. Growth Differ. 50:195-219.
Nakamoto, M., Matsuda, M., Wang, D-S., Nagahama, Y., Shibata, N., 2006. Molecular cloning and analysis of gonadal expression of Foxl2 in the medaka, Oryzias latipes. Biochem. Biophys. Res. Commun. 344:353-361.
Pankhurst, NW., King, HR., 2010. Temperature and salmonid reproduction: implications for aquaculture. J. Fish Biol. 76:69-85.
Pankhurst, NW., King, HR., Anderson, KC., Elizur, A., Pankhurst, PM., Ruff, N., 2011. Thermal impairment is differentially expressed in maiden and repeat spawning Atlantic salmon. Aquaculture 316:77-87.
Pankhurst, NW., Thomas, PM., 1998. Maintenance at elevated temperature delays the steroidogenic and ovulatory responsiveness of rainbow trout *Oncorhynchus mykiss* to luteinizing hormone releasing hormone analogue. Aquaculture 166:163-177.
Pennell, W., Barton, BA., 1996. Principles of salmonid culture. Developments in aquaculture and fisheries science. Amsterdam, The Netherlands: Elsevier.
Pfaffl, MW., Horgan, GW., Dempfle, L., 2002. Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res. 30:e36.
Soria, FN., Strüssmann, CA., Miranda, LA., 2008. High water temperatures impair the reproductive ability of the pejerrey fish *Odontesthes bonariensis*: effects on the hypophyseal-gonadal axis. Physiol. Biochem. Zool. 81:898-905.
Tan, NS., Frecer, V., Lam, TJ., Ding JL., 1999. Temperature dependence of estrogen binding: importance of a subzone in the ligand binding domain of a novel piscine estrogen receptor. Biochim. Biophys. Acta – Mol. Cell. Res. 1452:103-120.
Wang, D-S., Kobayashi, T., Zhou, L-Y., Paul-Prasanth, B., Ijiri, S., Sakai, F., Okubo, K., Morohashi, K-I., Nagahama, Y., 2007. Foxl2 up-regulates aromatase gene transcription in a female-

specific manner by binding to the promoter as well as interacting with Ad4 binding protein/steroidogenic factor 1. Mol. Endocrinol. 21:712-725.
Watts, M., Pankhurst, NW., King, HR., Geraghty, DP., 2005. Differential effects of temperature and maturity stage on hepatic estrogen receptor characteristics of Atlantic salmon. Comp. Biochem. Physiol. Part A 140:377-383.
Yamaguchi, T., Kitano, T., 2008. The mechanism of transcriptional regulation of P450 aromatase gene by Foxl2 in Japanese flounder (*Paralichthys olivaceus*). Cybium 32:74-76.
Yamaguchi, T., Yamaguchi, S., Hirai, T., Kitano, T., 2007. Follicle-stimulating hormone signalling and Foxl2 are involved in transcriptional regulation of aromatase gene during gonadal sex differentiation in Japanese flounder, Paralichthys olivaceus. Biochem. Biophys. Res. Commun. 359:935-940.
Zhou, L-Y., Wang, D-S., Shibata, Y., Paul-Prasanth, B., Suzuki, A., Nagahama, Y., 2007. Characterization, expression and transcriptional regulation of P450c17-I and -II in the medaka, *Oryzias latipes*. Biochem. Biophys. Res. Commun. 362:619-625.

Table 1. qPCR primers

Gene name	Primer direction	Sequence (5'→ 3')	Prod. size	E*	LOD	Source sequence(s)
Fshr	F	TGG ATC CAA ATG TCG CCC AA	129	0.978	0.05	NM_001123610
	R	CCA TGC CTG GTC TTG GGT TC				
Foxl2	F	GCG GCG AGA GAA AAG GGA AT	125	0.986	0.1	JX184084
	R	CTG GAA ATG GGT CGG TGG AG				
3β-Hsd	F	CCA ACG GAG ACC CCA TCA TC	97	1.004	0.06	ES555811, AF468975
	R	ACC TGC TCG GCC TCC TTC TT				
Star	F	CCG ATG ACC CCA ACA AGA CC	113	0.996	0.1	DQ415678
	R	GGC AAA GTC CAC CTG CGT CT				
Tbp	F	TCC CCA ACC TGT GAC GAA CA	117	0.981	N/A	BT059217 (Pankhurst et al., 2011)
	R	GTC TGT CCT GAG CCC CCT GA				

*E = efficiency, F = forward, R = reverse, LOD = limit of detection in relative expression units, product size is in base pairs

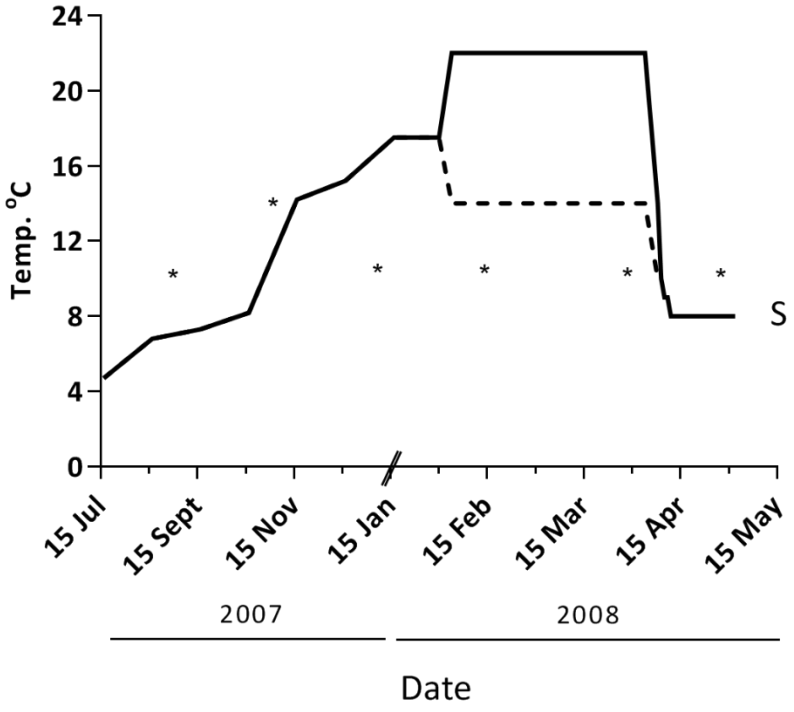

Fig. 1. Thermal treatment regimes for maiden and repeat *S. salar*, including the period of ambient temperature before introduction to 14 and 22 °C treatments. Stars indicate sample points and S = approximate time of earliest spawning. Break in the x-axis indicates a change in scale.

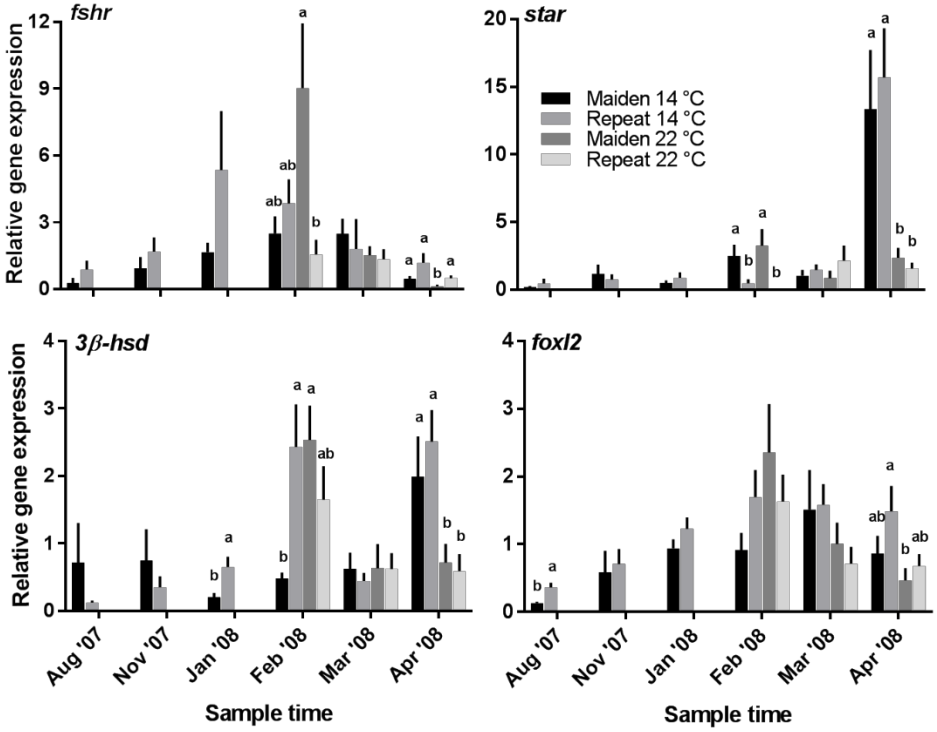

Fig. 2. Relative gene expression levels (mean ± SE, n=7) of *gonadal steroidogenic acute regulatory protein* (*star*), *forkhead transcription factor* (*foxl2*), *follicle stimulating hormone receptor* (*fshr*) and *3β-hydroxysteroid dehydrogenase* (*3β-hsd*) in maiden or repeat spawning fish held at 14 or 22 °C. Gene expression levels were normalised against *TATA-box-binding protein* expression. Different superscripts between groups at each sampling point denote significantly different means ($p \leq 0.05$).