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Effect of thermal challenge on plasma gonadotropin levels and ovarian steroidogenesis in female maiden and repeat spawning Tasmanian Atlantic salmon (*Salmo salar*)

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Abstract

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

Key words: Atlantic salmon, temperature, reproduction, fertility, gonadal steroidogenesis, pituitary hormones, gene expression

1. Introduction

Reproductive development in fish is controlled by hypothalamic *gonadotropin releasing hormone* (Gnrh) and subsequent production and release of *follicle stimulating hormone* (Fsh) from the pituitary (Dickey and Swanson, 2000; Oppen-Berntsen et al., 1994). In response to Fsh, cholesterol is metabolised in a stepwise fashion to produce the gonadal steroids testosterone (T) and 17β -estradiol (E_2); the latter then stimulates the hepatic synthesis of *vitellogenins* (Vtg) and *zona pellucida* proteins (Zp) which are essential for oocyte development (reviewed by Lubzens et al., 2010). Exposure to higher-than-optimal temperatures can adversely affect the endocrine system and consequently impair reproductive development, as demonstrated for a variety of species (Pankhurst and King, 2010; Tveiten and Johnsen, 1999). In female pejerrey (*Odontesthes bonariensis*), short exposure to 23 or 27 °C resulted in a significantly lower level of gonadal *follicle stimulating hormone receptor* (*fshr*) mRNA and circulating E_2 relative to the 19 °C control group (Soria et al., 2008). The authors of the pejerrey study concluded that circulating E_2 was depressed as a result of thermal inhibition at the level of the gonad and pituitary (Soria et al., 2008). Furthermore, in an *in vitro* study, Watts et al. (2004) showed that conversion of T to E_2 in the gonad was inhibited at elevated temperature, and suggested this occurred through impairment of the steroidogenic enzyme *p450 aromatase a* (Cyp19a1a). Pankhurst et al. (2011) also found that plasma T levels were depressed at high temperature in Atlantic salmon (*Salmo salar*) suggesting that endocrine blockages also occur upstream of E_2 production. Thermal impairment of Cyp19a1a during sexual differentiation has been observed in other fish species including Japanese flounder (*Paralichthys olivaceus*) and Atlantic halibut (*Hippoglossus hippoglossus*) (Kitano et al., 1999; van Nes and Andersen, 2006). Additionally, in a further *in vitro* study, Watts et al. (2005) also showed a decrease in the number of high affinity estrogen receptors (Er) in cultured hepatic tissue from Atlantic salmon reared at 22 °C

relative to 18 and 14 °C. It is thought that the combination of depressed E₂ levels and reduced hepatic Er binding efficiency explains the impact on downstream processes such as vitellogenesis and zonagenesis described in thermally challenged fish (Pankhurst et al., 2011; Watts et al., 2005). Collectively, these studies suggest that thermal impairment is likely to occur at a range of levels in the reproductive endocrine cascade during reproductive development.

In farmed Tasmanian Atlantic salmon, high ambient water temperature accelerates growth rate and shortens production time relative to their Canadian ancestors (Jungalwalla, 1991). However, temperatures experienced by broodstock during summer can reach 22 °C (Battaglione et al., 2008) which is high enough to induce the endocrine anomalies described previously (King et al., 2003; Pankhurst et al., 2011). Regardless of the mechanisms by which E₂ and subsequent vitello- and zonagenesis are depressed in thermally exposed fish, the collective result of endocrine suppression is a reduction in reproductive performance. Fertility in Tasmanian Atlantic salmon was lower in fish reared at 22 °C during vitellogenesis compared to 14 and 18 °C (King et al., 2003). Other studies on Atlantic salmon and rainbow trout maintained at elevated temperature for a period of time prior to spawning have also shown subsequent reductions in embryo survival (Pankhurst et al., 1996; Taranger and Hansen, 1993). The majority of eggs produced by the Australian Atlantic salmon industry come from first-spawning (maiden, 2+ years old) fish (Jungalwalla, 1991). However, a recent study confirmed industry observations that maiden Atlantic salmon were more susceptible to exposure to high temperature than second-spawning (repeat, 3+ years old) in terms of egg fertility and embryo survival (Pankhurst et al., 2011). Because Tasmanian Atlantic salmon are routinely reared close to their upper limit of thermal tolerance, any further increase in temperature that occurs as a result of normal seasonal fluctuation or climate change will result in major production challenges (Pankhurst and King, 2010). In an

attempt to minimise exposure of broodstock to elevated temperature, the salmon industry in Australia is moving towards re-circulating systems that allow complete thermal regulation (Battaglione et al., 2008; Pankhurst and King, 2010). The apparently lower susceptibility of repeat spawning fish to thermal challenge may offer an additional option for the management of thermal insult, but assessment of this possibility is hindered by significant knowledge gaps in relation to pituitary and ovarian function in thermally challenged fish.

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

2. Materials and Methods

2.1 Fish maintenance

Seventy-seven maiden (first spawning 2+ year old fish) and 77 repeat (second spawning 3+ year old fish) cultured adult females were held at the Salmon Enterprises of Tasmania (SALTAS) Wayatinah Hatchery, Tasmania, Australia, in fresh water at natural temperature and photoperiod. Fish were maintained in either 200 (maidens) or 50 (repeats) m³ circular tanks at stocking densities of 12-18, and 24-36 kg m⁻³ for maidens and repeats, respectively from August 2007 until early January 2008 (Fig. 1). In January, 112 fish were transferred to temperature-controlled 4 m³ 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 (14 fish per tank, 2 tanks per experimental group, 8 tanks total, Fig. 1). Fish were not fed from the time of transfer to the temperature-controlled systems. All fish were maintained at their respective temperatures until early April when all fish were exposed to a temperature ramp down over 11 days to 8 °C to induce final oocyte maturation

and ovulation (King and Pankhurst, 2000). Maturation and ovulation data generated from the same fish utilised in the present study have been previously published in Pankhurst et al. (2011). This research activity was undertaken with approval from the Animal Ethics Committees of the University of the Sunshine Coast and Griffith University (approval numbers AN/A/07/35 and EAS/02/07/AEC respectively).

2.2 Sampling protocol

Fish from both maiden and repeat groups were sampled on August 31st and November 2nd 2007, and January 7th 2008 to cover the initiation of vitellogenesis for each age class. After introduction to the controlled temperature regimes, all 4 groups of fish were sampled on the February 14th, March 28th and April 25th 2008. Seven fish were sampled from each group at each sample time, leaving 7 fish from each treatment to proceed through to ovulation and fertilisation (ovulation, fertility and embryo survival data appear in Pankhurst et al., 2011).

For sampling, fish were netted from the holding tanks, terminally anaesthetised in Aqui-S™ (Crop & Food, New Zealand), and then blood was sampled by caudal puncture using pre-heparinised syringes fitted with 22 G needles. Blood was centrifuged at 12000 g for 5 min at room temperature, and plasma was stored frozen at -20 °C for later measurement of plasma hormones. Sections of ovary and liver were excised, transferred to 1-2 ml of RNA Later™ (Qiagen, Germany), kept at 4 °C overnight then stored at -80 °C to stabilise mRNA for later measurement of gene expression.

2.3 Plasma Fsh and Lh measurement

Plasma Fsh (all sampling points) and Lh (March and April sampling only) measurements were performed using an RIA developed for coho salmon (*Oncorhynchus kisutch*) by Swanson et al. (1989) with some modifications. Briefly, the assay utilised rabbit

antisera specific to the coho salmon Fsh or Lh beta subunit (lots #8621 and #38.5.92 respectively), and highly purified coho Fsh and Lh as the standards (Swanson et al., 1991). In these assays phosphate buffered saline (pH 7.4) was used instead of barbital and 500 µl polyethylene glycol (4 %) was included on day 4. All assays for a specific hormone were analysed in a single batch, and therefore inter-assay variation was not calculated. The cross reactivity of Fsh in the Lh assay and Lh in the Fsh assay was approximately 4.4 and 6 %, respectively (Swanson et al., 1989). ANCOVA was performed to determine whether parallelism was present between Atlantic salmon plasma and the respective purified coho standard that was serially diluted in PBS-BSA (triplicates) for Fsh and Lh. The detection limit (LOD) of the Fsh and Lh assays was approximately 0.6 and 0.5 ng.ml⁻¹ respectively.

2.4 Measurement of steroidogenic enzyme and era gene expression

Total RNA was isolated from 15 mg of hepatic tissue using the Illustra RNAspin Mini kit (GE Healthcare), and from 120 mg of ovarian tissue using TRIZOL[®] reagent (Invitrogen) according to each of the manufacturer's protocols. RNA yield and 260/280 purity ratio was determined using the NanoDrop 2000 (Thermo Scientific). An RNA integrity number (RIN) was determined for a random sample of hepatic and ovarian RNA (n = 48) using the 2100 bioanalyzer to establish RNA quality (Agilent). All RNA was stored at -80 °C until use.

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

qPCRs for *cyp19a1a*, *cholesterol side chain cleavage protein (cyp11a1)* and *era* were conducted on a Rotor-gene 6000 series thermal cycler (Qiagen) using the Platinum[®] SYBR[®]

Green qPCR SuperMix-UDG (Invitrogen) master mix and the following cycling conditions: 50 °C for 2 min; 95 °C for 2 min; 40 cycles of 95 °C for 15 s; 60 °C for 15 s, and 72 °C for 20 s (acquiring). At the end of cycle 40, a melt curve analysis was performed to confirm the amplification of a single product as follows: 90 s preconditioning step at 72 °C, followed by a temperature gradient up to 95 °C at 1 °C per 5 s. For *era* and *cyp19a1a*, the 10 µl qPCR reaction contained 5 µl SYBR mix, 200 nM each primer, 3.6 µl PCR grade water and 1 µl cDNA template. For *cyp11a1*, the reaction components were the same except only 2.6 µl of PCR grade water was added and the final concentration of MgCl₂ was 4 instead of 3 mM. For every gene analysed no-template controls (NTCs) and a calibrator sample of pooled cDNA were included to detect possible contamination, and control for in-between run variability, respectively.

Gene specific primers (GSPs) were designed from the Atlantic salmon *cyp19a1a*, *cyp11a1* and *era* mRNA sequences available on GenBank (AF436885, DQ361039 and X89959 respectively) to have an optimum annealing temperature of 60 °C using Primer3 software (<http://frodo.wi.mit.edu/primer3/>, Table 1). These qPCR assays were developed by using serially diluted cDNA to construct a validation curve for each primer set. 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. The GSPs were tested for specificity via melt curve analysis (as above) and the products were run on a 2 % agarose gel to establish amplicon size; gene identity was then confirmed through sequencing. During validation, negative reverse transcription controls and NTCs were analysed to ensure the absence of genomic DNA and other contamination. *Tata binding protein (tbp*, reference gene) qPCRs were performed using the cycling conditions above and the primers developed by Pankhurst et al. (2011, Table 1). *Tbp* showed consistently high stability under the experimental conditions Anderson and Elizur

(2011) and was therefore used as a reference gene for normalisation. The software Rest[®] 2008, V2.0.7 (Pfaffl et al., 2002) was used to normalise the data and calculate expression of key genes for each sample relative to the calibrator.

2.5 Statistical analysis

Circulating hormone levels were compared using one-way ANOVA coupled with Tukeys-b for post-hoc analysis and a P value of 0.05. Differences in relative gene expression levels were detected non-parametrically using the Kruskal-Wallis test coupled with Bonferroni's Correction to reduce the risk of type 1 error. The P value for significance prior to Bonferroni's correction was set at 0.05 for all non-parametric analyses. Values for fish with a non-detectable plasma concentration of Fsh were recorded as the lower limit of detection for the assay (0.6 ng.ml^{-1}) for statistical analysis. All analysis was performed using SPSS version 17.0.

3. Results

3.1 Plasma Fsh and Lh

Serial dilutions of Atlantic salmon plasma were parallel to the dilution curves obtained for purified coho salmon standards for Fsh and Lh as determined by ANCOVA (where a P value below 0.05 indicates a deviation from parallelism, the P values for Fsh and Lh were 0.62 and 0.34 respectively, Fig. 2). Circulating levels of Fsh were significantly higher in maiden than repeat spawning fish maintained at natural temperature in August and November but by January, the difference in Fsh plasma concentration had disappeared (Fig. 3). From August until February, the plasma concentration of Fsh did not exceed 4 ng.ml^{-1} in any fish. In February, there were no differences in plasma Fsh levels between temperatures within age classes, but levels were higher in repeats at $22 \text{ }^{\circ}\text{C}$ compared to maidens at $14 \text{ }^{\circ}\text{C}$.

In March and April fish held at 22 °C had significantly higher levels of Fsh than fish at 14 °C irrespective of age. Additionally, in April repeat fish at 22 °C had significantly higher plasma levels of Fsh than maidens at 22 °C.

In March and April the mean (\pm SE) plasma levels of Lh for all fish were 0.97 ± 0.21 ng.ml⁻¹ and 0.94 ± 0.25 ng.ml⁻¹ respectively (Fig. 4). In March, Lh levels were significantly lower in maidens at 14 °C and repeats at 22 °C compared to maidens at 22 °C while repeats at 14 °C exhibited an intermediate concentration. In April, there was no significant difference in Lh concentration among the groups.

3.2 Relative gene expression of *cyp* genes and *era*

Gene expression profiles for *cyp19a1a* and *cyp11a1* followed a similar trend throughout the experiment (Fig. 5). In August, the relative level of expression of *cyp19a1a* was significantly higher in maiden than repeat fish. From November until January *cyp19a1a* gene expression steadily increased and there were no differences between maiden and repeat fish. In February, expression was suppressed in 22 °C repeats relative to 14 °C maidens, but there were no differences between temperatures within age classes. By March, thermal inhibition of *cyp19a1a* was present in repeat spawning fish at 22 °C relative to 14 °C repeats, and in April, expression was suppressed in both age classes at 22 °C relative to 14 °C.

In a similar fashion to *cyp19a1a*, *cyp11a1* gene expression was significantly higher in August in maiden fish compared to repeats (Fig. 5). From November until February, *cyp11a1* gene expression level increased in a time-dependent manner and was not significantly different between treatment groups, or as a result of thermal exposure (in February). In March *cyp11a1* gene expression was suppressed in repeats at 22 °C compared to repeats at 14 °C, and in April, both age classes showed reduced expression at 22 °C.

In August, relative *era* gene expression was significantly elevated in maiden fish compared to repeat fish reared at natural temperature (Fig. 6). By November, the difference in *era* gene expression between maiden and repeat fish had disappeared and by January, the level of gene expression had risen by approximately 2.5 fold in both groups. For the remaining months (February, March and April '08), there was no significant difference in *era* gene expression level as a result of thermal manipulation or broodstock age (Fig. 6).

4. Discussion

In the present study, RIAs originally developed to quantify coho salmon Fsh and Lh (Swanson et al., 1989) were used to assess gonadotropin levels in Atlantic salmon. Parallelism of assay binding curves for serially diluted Atlantic salmon plasma and purified coho salmon standards allowed measurement of gonadotropin-like immunoreactive material in the plasma of Atlantic salmon. This assay has also been used to quantify putative plasma gonadotropins in a northern hemisphere stock of Atlantic salmon (Olsen and Walther, 1993; Oppen-Berntsen et al., 1994). With the proviso that this is a heterologous assay, the immunoreactive constituents in Atlantic salmon plasma are hereafter termed Fsh and Lh, respectively. Circulating levels of Fsh were significantly lower in repeat compared to maiden fish in August and November which corresponds to the initial period of oocyte development in Tasmanian Atlantic salmon (King and Pankhurst, 2003). By January there was no significant difference in the circulating levels of Fsh as a result of stock age. In a previous study by Pankhurst et al. (2011) that used the same fish as the present study, the authors noted that somatic condition factor was lower in repeat than maiden fish at sampling points prior to January reflecting the fact that repeat spawning fish were recovering from the energetic demands of the previous reproductive season. It appears that this recovery may also be reflected in lower plasma Fsh levels. By January, repeat spawning fish have 'caught

up' and there are no subsequent differences in ovarian stage or development between maiden and repeat spawning fish (Pankhurst et al., 2011).

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

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

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

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

Lh levels during the late stages of vitellogenesis. However, it is possible that Lh levels were affected closer to the time of spawning given that ovulation was delayed in both maiden and repeat spawning fish maintained at 22 relative to 14 °C (Pankhurst et al., 2011).

Gonadal levels of *cyp19a1a* and *cyp11a1* gene expression were significantly lower in repeat compared to maiden fish in August. However, by November the relative expression levels in maiden and repeat fish were similar for both gonadal genes. In salmonids, Fsh is the regulating factor that promotes the synthesis of E₂ via an enzyme mediated steroidogenic pathway in the gonad (Oppen-Berntsen et al., 1994; Suzuki et al., 1988). It would appear that repeat spawning fish in the present study did not have sufficient stimulation by Fsh to produce the *cyp19a1a* and *cyp11a1* transcript levels observed in maiden fish early in reproductive development. For the same fish, Pankhurst et al. (2011) noted that condition factor, circulating E₂ levels and the gene expression levels of *vtg* and *zona pellucida b* were also lower in repeat fish during the initial stages of oocyte recruitment and development. As noted previously, this may reflect the fact that repeat fish were still recovering from the energetic demands of the previous spawning season.

By February, there was evidence that temperature was driving the down-regulation of *cyp19a1a* gene expression. For the same groups of fish, Pankhurst et al. (2011) found that E₂ levels were lower at 22 °C than 14 °C while plasma T levels remained unaffected during February. The modest reduction in *cyp19a1a* gene expression at high temperature could have contributed those lower E₂ levels. In March and April, after exposure to differential thermal regimes for approximately 2 and 3 months respectively, gene expression was down-regulated for *cyp19a1a* in fish reared at 22 °C regardless of age. The thermal sensitivity of *cyp19a1a* has been confirmed in a range of other species. Nineteen month old red seabream (*Pagrus major*) showed suppression of *cyp19a1a* gene expression at 25 and 20 °C relative to 15 °C after 4 and 8 weeks respectively (Lim et al., 2003). Similarly, *cyp19a1a* gene expression was

reduced in Atlantic halibut larvae reared at 13 °C relative to 7 °C (van Nes and Andersen, 2006). Most studies involving the thermal inhibition of aromatase address issues surrounding sex change/reversal in juvenile fish (reviewed in Devlin and Nagahama, 2002). However, we have shown here that adult fish also show aromatase inhibition as a result of thermal challenge, as was suggested previously for Atlantic salmon (Pankhurst et al., 2011; Watts et al., 2004).

The molecular mechanism(s) by which gonadal steroidogenic enzymes are affected by elevated temperature are not properly understood, especially in adult fish. Various studies have shown that *forkhead transcription factor* (Foxl2) is involved in transcriptional regulation of *cyp19a1a* in female fish (Nakamoto et al., 2006; Wang et al., 2007) and is sensitive to thermal exposure. Yamaguchi et al. (2007) showed that high water temperature had a negative influence on *foxl2* gene expression in Japanese flounder during sexual differentiation. The Foxl2 gene is not only expressed in juvenile fish; Nakamoto et al. (2006) and Zhou et al. (2007) detected *foxl2* gene expression in both previtellogenic and vitellogenic follicles of adult medaka (*Oryzias latipes*). Therefore, thermal inhibition of gonadal *foxl2* gene expression may have occurred in the present study, with the result that subsequent regulation of Cyp19a1a did not take place normally. If this is the case, other gonadal steroidogenic enzymes may also have been affected, as Foxl2 has been implicated as a regulator of *17 α -hydroxylase/C17,20-lyase* (Cyp17a) in medaka (Zhou et al., 2007). Pankhurst et al. (2011) found that T levels were lower in thermally exposed fish during March (maiden and repeat) and April (maiden only) suggesting that the same effect was present. Another possibility is that Fsh signal transduction, and subsequent steroidogenesis was partially inhibited because *fshr* gene expression was thermally impaired. The fact that the higher level of circulating Fsh observed during March and April in the present study did not stimulate increased *cyp11a1* and *cyp19a1a* gene expression at 22 °C, is consistent with

this possibility. In female adult pejerrey, 8 days of exposure to high temperature caused a significant reduction in *fshr* gene expression during vitellogenesis (Soria et al., 2008). In a similar fashion, maintenance at high temperature resulted in lower *fshr* gene expression levels in Japanese flounder during sex differentiation (Yamaguchi et al., 2007). A change in *fshr* gene expression is likely to affect downstream Cyp19a1a activation through its role in transducing modulation of intracellular cAMP levels (Montserrat et al., 2004; Planas et al., 1997). The Cyp19a1a gene contains cAMP responsive elements (CREs) in several species (Kazeto et al., 2001; Wong et al., 2006), and cAMP has been shown to stimulate gonadal *cyp19a1a* gene expression *in vitro* (Yamaguchi et al., 2007).

In the present study, *cyp11a1* gene expression was suppressed in thermally challenged repeat spawning fish during March and in both maidens and repeats in April. Cyp11a1 catalyses the side chain cleavage of cholesterol in the synthesis of pregnenolone and is considered to be the first rate-limiting step in the gonadal biosynthesis of T, and subsequently E₂ (reviewed in Payne and Hales, 2004). Pankhurst et al. (2011) reported that for the same fish used in the present study, plasma T levels were generally reduced at 22 °C in maiden and repeat spawners during mid to late vitellogenesis. It therefore seems likely that inhibition of *cyp11a1* gene expression was contributing to the lower level of circulating T reported. There is little information on Cyp11a1 regulation in teleosts; however, a recent *in vitro* study by Luckenbach et al. (2011) found that exposure of coho salmon ovarian follicles to Fsh caused a moderate increase in *cyp11a1* gene expression. In mammals, stimulation by Fsh causes an increase in cAMP which up-regulates the synthesis of steroidogenic cytochrome P450 enzymes through CREs (Payne and Hales, 2004) as has been shown previously for rodent and bovine Cyp11a1 (Sher et al., 2007; Waterman, 1994). This may suggest that thermally suppressed *fshr* gene expression and subsequent reduction in Fsh signal transduction is partly responsible for the suppressed *cyp11a1* gene expression observed in the present study and the

lower plasma T levels (Pankhurst et al., 2011) among fish held at 22 °C. Regardless of the molecular basis for reduced *cyp19a1a* and *cyp11a1* gene expression, the collective result of reduced steroidogenic enzyme gene expression is significantly lower circulating levels of E₂ (maidens and repeats) and Vtg (maidens only), and gene expression levels of *vtg* and *zp* (maidens and repeats) in fish reared at 22 °C (Pankhurst et al., 2011). In maiden-spawning fish, such changes in endocrine status are associated with a significant reduction in egg fertility and embryo survival (Pankhurst et al., 2011). Repeat spawning fish appear to be more resilient to high temperature in terms of egg fertility and embryo survival, although this was not reflected by obvious age class related differences in the endocrine data from our present or previous study (Pankhurst et al., 2011).

In the present study hepatic *era* gene expression was 3-times higher in maidens than repeats during August. As noted previously, the onset of vitellogenesis is retarded in repeat spawning fish (Pankhurst et al., 2011) and the lower expression of *era* may contribute to this effect. After August, there was no difference in relative *era* gene expression between any groups at any sampling point for the duration of the experiment. Similarly, in a study by Pawlowski et al. (2000), hepatic tissue from rainbow trout was maintained *in vitro* at either 14 or 18 °C, and after 12 and 24 hours of exposure to elevated temperature no difference in *er* gene expression was observed. In Atlantic halibut reared at 7, 10 or 13 °C during sexual differentiation, there was no clear temperature related pattern in *er* gene expression during 120 days of thermal management (van Nes and Andersen, 2006). While it appears that temperature does not modify the expression of *era* in adult female Atlantic salmon, the effects of high temperature on other estrogen subtypes, estrogen receptor beta (Erβ) or the novel isoform Erα2, are currently unknown. For male medaka, it has been suggested that each Er subtype specifically regulates a particular Vtg gene (Yamaguchi et al., 2009). For example, *vtga* mRNA levels were regulated via Erα only; in contrast, *vtgb* mRNA levels were

influenced by both $Er\beta$ and $Er\alpha$ although their roles in initiation and enhancement of transcription seem to be different (Yamaguchi et al., 2009). Therefore, it is possible that Ers other than $Er\alpha$ are thermally sensitive which could affect the expression of other Vtg genes not measured by Pankhurst et al. (2011). Furthermore, an *in vitro* study by Watts et al. (2005) revealed that Ers in liver tissue from Atlantic salmon reared at 22 °C during vitellogenesis had a significant reduction in E_2 binding affinity compared to Ers from fish maintained at 14 or 18 °C. Reduced Er binding affinity due to thermal challenge has also been reported for other teleost species such as tilapia (*Oreochromis aureus*) (Tan et al., 1999). While we did not determine whether Er affinity was impaired in the present study, diminished Er binding affinity could have potentiated the effects of lower circulating E_2 , and therefore could have contributed to the lower expression of hepatic genes observed by Pankhurst et al. (2011).

Information regarding the effects of abnormally raised temperature on the circulating levels of Fsh and Lh, and the gene expression levels of *cyp11a1*, *cyp19a1a* and *era* for maiden and repeat female Atlantic salmon was not previously available. Here, for the first time, we have demonstrated that thermal challenge inhibits the transcription of *cyp11a1* and *cyp19a1a* which results in impaired T, E_2 , *vtg*, Vtg and *zp* synthesis and these are associated with reduced egg quality as reported in Pankhurst et al. (2011). We have also shown that Fsh levels are elevated from mid-vitellogenesis in fish held at high temperature, possibly due to a lack of negative feedback by E_2 in combination with a general increase in metabolism. However, the relatively high levels of Fsh were unable to stimulate steroidogenesis, perhaps due to other thermal impairments at multiple levels of the endocrine cascade controlling reproductive development. During the later stages of vitellogenesis, we have provided evidence that Lh levels are mostly unaffected by temperature up to 1 month prior to spawning. For circulating Fsh and Lh, and expression of the genes studied, the effects of

thermal challenge do not appear to discriminate between fish of different ages. Therefore it is unlikely that the basis for increased thermal resilience of repeat spawning fish lies at the level of gonadal steroidogenesis, instead it may be a function of increased egg size, and increased ability of repeat fish to sequester factors that promote egg viability as suggested by Pankhurst et al. (2011).

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Table 1. qPCR primers and sequence information

Gene name	Primer name	Sequence (5'→3')	Prod. size	E*	Source seq.
Cyp19a1a	Cyp19F1	TTC CAT CCC GTG GTG GAC TT	106 bp	0.982	AF436885
	Cyp19R1	TGC GGC CCA TGT TCA GAA T			
Cyp11a1	Cyp11F1	GGC GTT CCA ACA GGG TGA TT	106 bp	0.949	DQ361039
	Cyp11R1	ACT CGG GCC ACA AAG TCC TG			
Era	EraF1	AAG CAT GCC GCC TCA GAA AG	150 bp	1.003	X89959
	EraR2	TCC TGT GCT CCA GGT CAC CA			
Tbp	TbpF1	TCC CCA ACC TGT GAC GAA CA	117 bp	0.981	BT059217
	TbpR1	GTC TGT CCT GAG CCC CCT GA			

*E = efficiency, bp = base pairs,

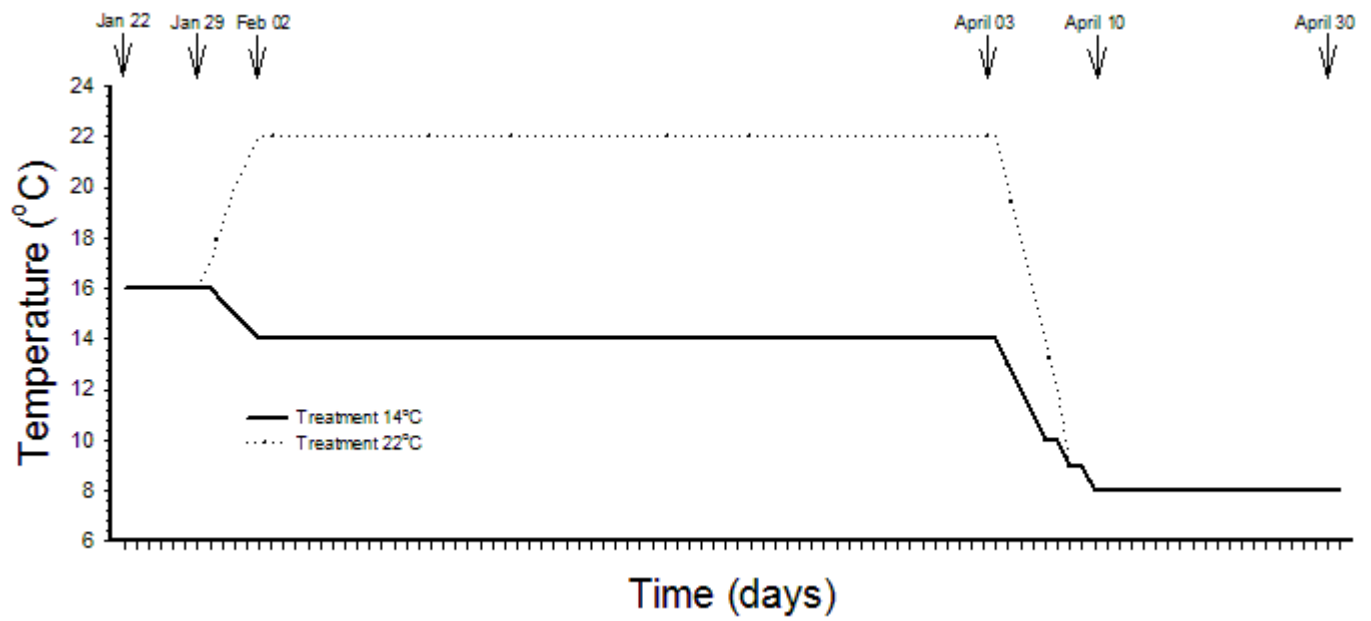


Fig. 1. Thermal treatment regimes for Atlantic salmon maiden and repeat spawners in 14 °C and 22 °C treatments.

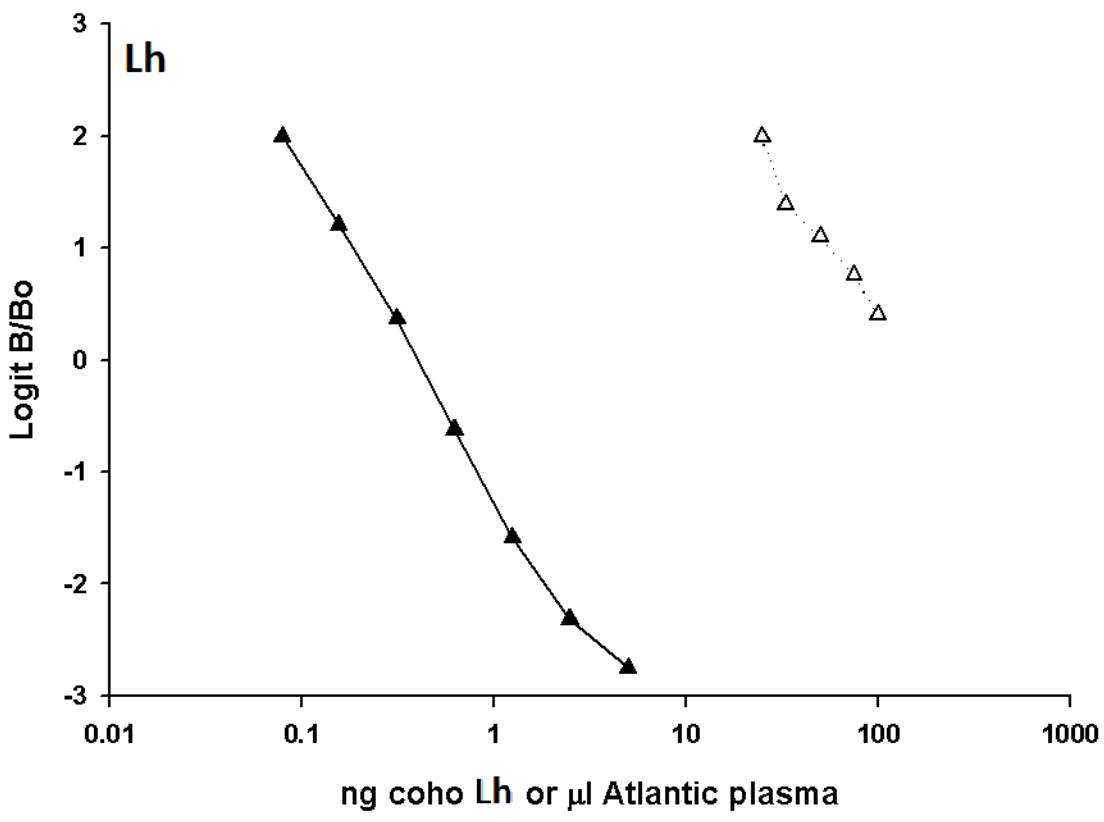
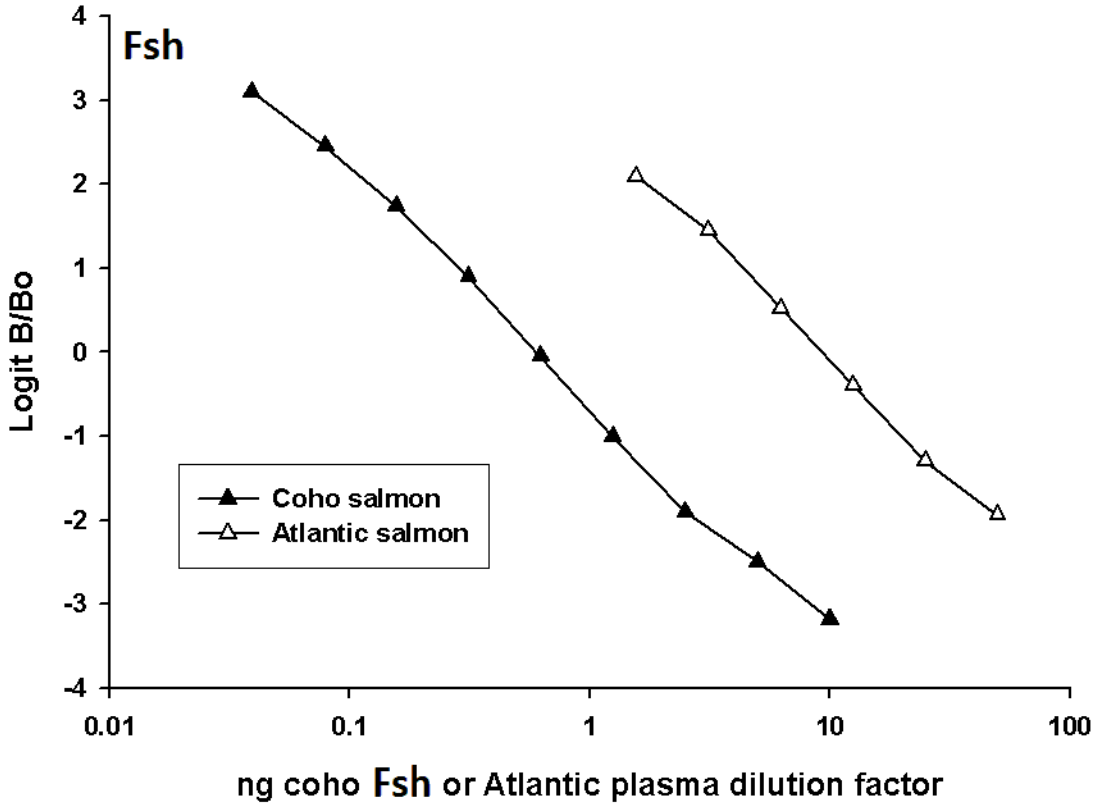


Fig. 2. Parallelism between serially diluted purified coho salmon standards, and Atlantic salmon plasma diluted in PBS-BSA for follicle stimulating hormone (Fsh) or luteinizing hormone (Lh).

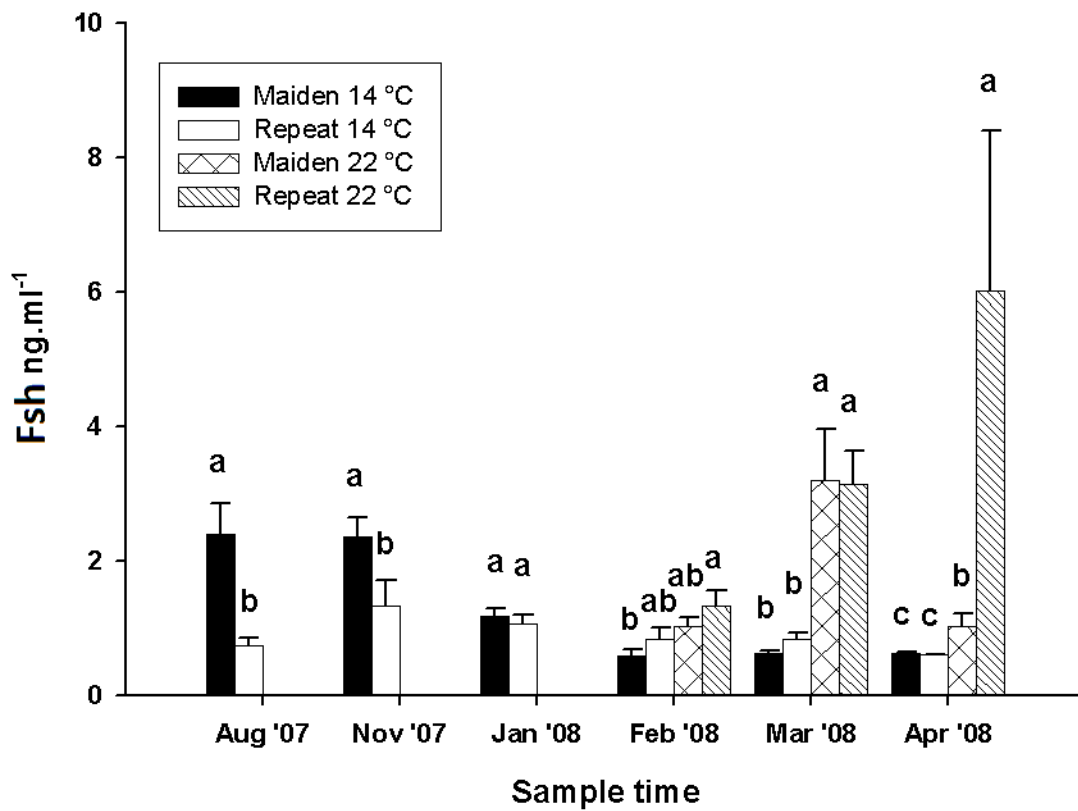


Fig. 3. Plasma levels (mean + SE, n=7) of *follicle stimulating hormone* (Fsh) in maiden and repeat spawning Atlantic salmon held at either 14 or 22 °C. Fsh levels below the LOD are displayed as 0.6 ng.ml⁻¹. Different superscripts between groups at each sampling point denote significantly different means (p ≤ 0.05).

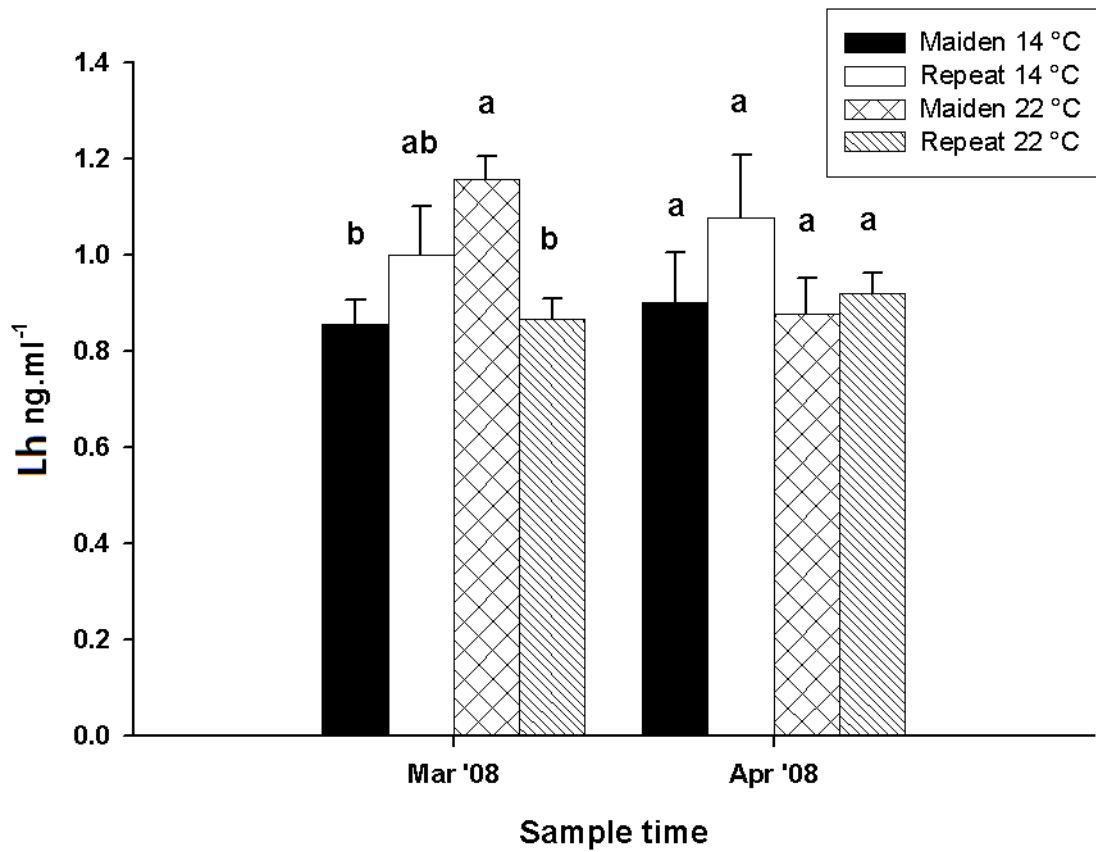


Fig. 4. Plasma levels (mean + SE, n = 7) of *luteinizing hormone* (Lh) in maiden and repeat spawning Atlantic salmon held at either 14 or 22 °C. Different superscripts between groups at each sampling point denote significantly different means ($p \leq 0.05$).

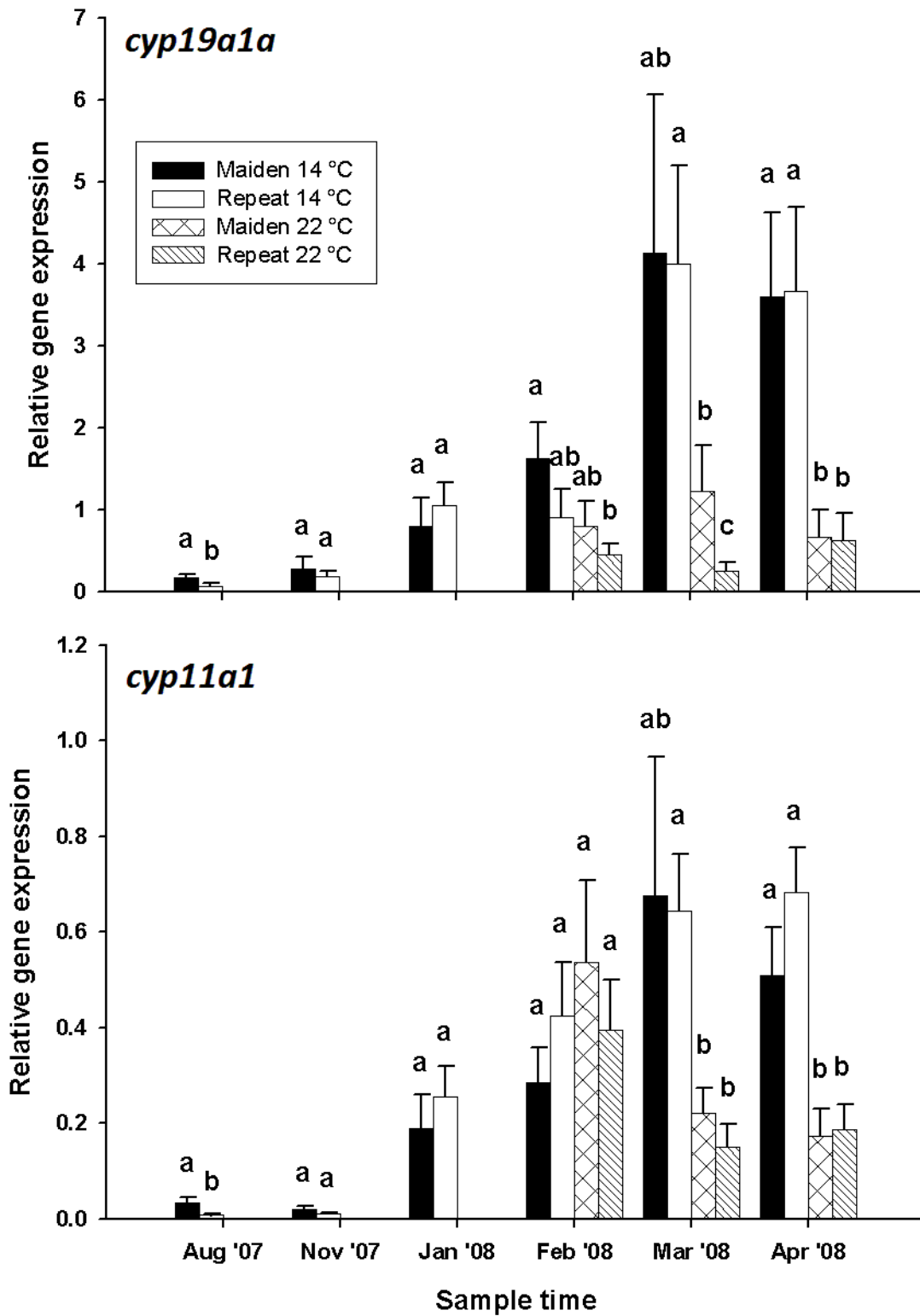


Fig. 5. Relative gene expression levels (mean + SE, n = 7) of gonadal *p450 aromatase a* (*cyp19a1a*) and gonadal *cholesterol side chain cleavage protein* (*cyp11a1*) in maiden or repeat spawning fish held at 14 °C or

22 °C. Gene expression levels were normalised against *tata binding protein* expression. Different superscripts between groups at each sampling point denote significantly different means ($p \leq 0.05$).

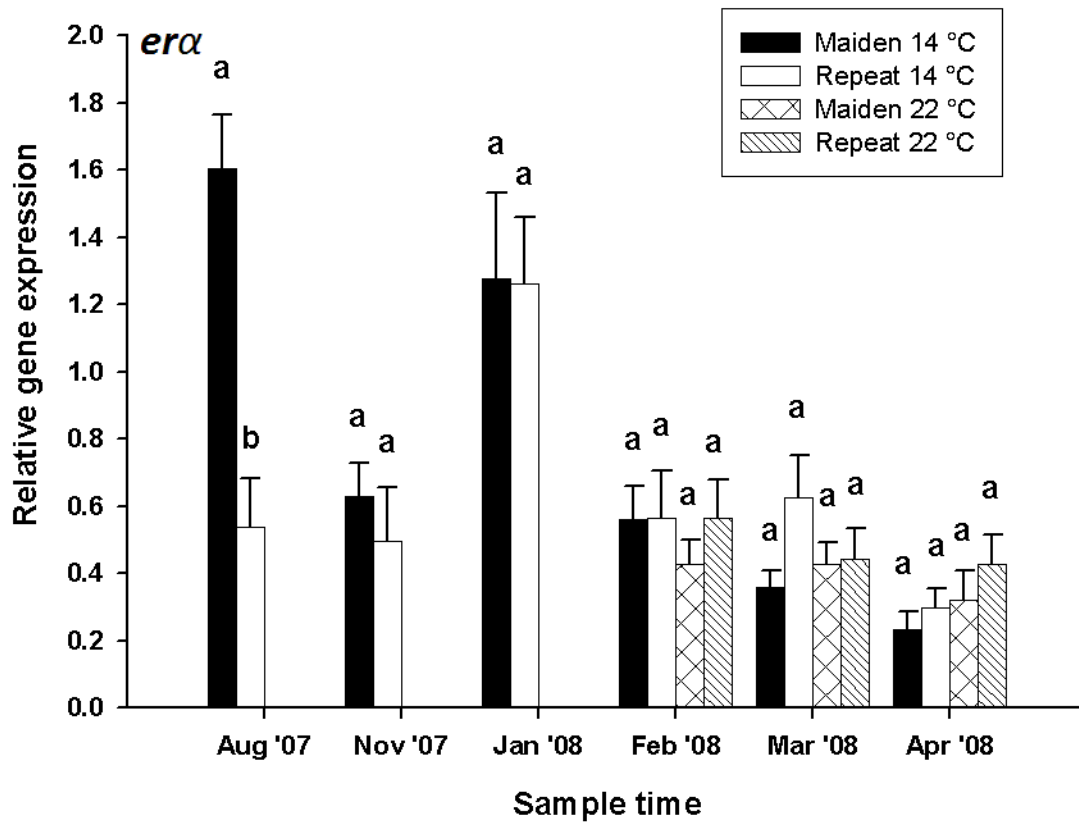


Fig. 6. Relative hepatic gene expression levels (mean + SE, $n = 7$) of *estrogen receptor alpha* (*era*) in maiden or repeat spawning fish held at 14 °C or 22 °C. Gene expression levels were normalised against *tata binding protein* expression. Different superscripts between groups at each sampling point denote significantly different means ($p \leq 0.05$).