Maintenance of Atlantic salmon (*Salmo salar*) at elevated temperature inhibits cytochrome P450 aromatase activity in isolated ovarian follicles

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Abstract

Atlantic salmon (*Salmo salar*) broodstock were transferred from natural (12–16 °C) to controlled temperatures of 14, 18 or 22 °C for 3 months during vitellogenesis. Fertility and survival were significantly reduced in eggs from broodstock held at 22 °C relative to 14 or 18 °C. Endocrine mechanisms were disrupted after only one month at 22 °C, as evidenced by decreased plasma vitellogenin (Vtg) and increased plasma testosterone (T) levels and, at later stages, decreased levels of plasma 17 β -estradiol (E₂). *In vitro* incubations of isolated ovarian follicles were carried out at monthly intervals, with follicles exposed to human chorionic gonadotropin, N, 2'-O-dibutyryl adenosine 3',5'-cyclic monophosphate, and the gonadal steroid precursors 17-hydroxyprogesterone, androstenedione and T. After one month of exposure to controlled temperature, T synthesis was generally enhanced in response to all treatments at all temperatures, but E₂ synthesis was inhibited at 22 °C, suggesting temperature impairment of cytochrome P450 aromatase (P450_{arom}) synthesis or activity. The effect became less marked as follicles matured suggesting that temperature sensitivity is stage dependent. The results of this study suggest that the inhibitory effects of elevated temperature on E₂ and Vtg synthesis, and subsequent egg development found in the present and earlier studies, arise at least partly, from temperature modulation of P450_{arom}.

Key words: Atlantic salmon; temperature; reproduction; vitellogenesis; 17 β -estradiol; aromatase; steroidogenesis

1. Introduction

It is well established that temperatures outside the optimum affect reproductive development and time of spawning in fish. Low temperature generally retards vitellogenesis and oocyte growth (Mackay and Lazier, 1993) but the effect of high temperature is more variable and depends on species, reproductive stage (Kagawa et al., 1983; Khan et al., 1999) and the regulatory factors involved at each stage (Van Der Kraak and Pankhurst, 1997). Studies on the cold-water teleosts rainbow trout (*Oncorhynchus mykiss*) (Pankhurst et al., 1996), Arctic charr (*Salvelinus alpinus* L.) (Gillet, 1991) and common wolffish (*Anarhichas lupus* L.) (Pavlov and Mokness, 1994), examining egg survival following exposure of broodstock to elevated temperature, suggested that effects were restricted to impairment of final oocyte maturation and ovulation. Similar effects have been demonstrated in Atlantic salmon (*Salmo salar* L.) (Taranger and Hansen, 1993; King and Pankhurst, 2000), but recently King et al. (2003) have shown that processes earlier in the reproductive cycle may also be affected. For example, plasma levels of 17 β -estradiol (E₂) and vitellogenin (Vtg) were reduced in Atlantic salmon that had experienced chronically elevated temperatures during vitellogenesis (King et al., 2003). This, coupled with observations of decreased egg quality and malformed chorionic membranes, suggested that elevated temperature during the vitellogenic phase was having a profound effect on fertility and egg survival (King et al., 2003). Studies with wolffish have also shown a trend towards reduced egg survival on exposure to increased temperature during vitellogenesis (Tveiten and Johnsen, 2001). Spring-spawning species also appear to be similarly compromised, with high temperature impairment of oocyte development during vitellogenesis being reported for the spring-spawning

perch (*Perca fluviatilis*) (Luksiene et al., 2000). Therefore, the effects of elevated temperature on reproduction during the vitellogenic period may be as severe as those experienced during later reproductive stages.

Problems associated with thermal disruption of reproductive development have potentially more impact on cultured stocks of fish that are held near to the upper limits of their thermal tolerance range. This is the situation with culture of Atlantic salmon in Tasmania. The majority of vitellogenesis occurs between February and April (austral summer to autumn), with ovulation occurring during May to June, early in the austral winter (King, 2001). Recently, warm summers with inshore marine water temperatures of 19 – 20 °C for several weeks and occasional temperature spikes in excess of 22°C, and freshwater temperatures of 20–25 °C, coincided with poor egg quality and subsequent survival (King, 2001). Although concurrent experimental studies have identified thermal disruption of reproductive processes during both vitellogenesis and final maturation (King and Pankhurst, 2000; King et al., 2003) the cellular basis for these effects remains to be investigated.

The aim of the present study was to examine the possible mechanisms of thermal impairment of Atlantic salmon reproduction at the tissue level during vitellogenesis. *In vitro* follicle incubations were used to assess ovarian function as isolated follicles respond readily to steroid precursors and gonadotropins (GtHs) by releasing steroid hormones into the incubation medium (Pankhurst et al., 1996; Pankhurst, 1997; Pankhurst and Ripley, 2000). Specifically, the following effects on isolated ovarian follicles from fish held at elevated temperature were assessed: hormonal responsiveness to GtH; the integrity of intrafollicular signal transduction mechanisms (i.e. follicle responsiveness to dibutyryl cyclic AMP [dbcAMP]) and the functional capacity of steroidogenic enzymes in the steroid synthesis cascade from 17-

hydroxyprogesterone (17P) to E₂. *In vitro* studies allow assessment of cellular mechanisms but they can have the disadvantage of lacking biological relevance without the support of *in vivo* data. Accordingly, plasma levels of reproductive steroids and Vtg were measured simultaneously and oocyte fertility and survival were monitored following ovulation.

The application of information developed from these studies will be an essential component of amelioration strategies for managing the continued successful rearing of Atlantic salmon under warming environmental conditions. Problems that the Tasmanian salmon industry are detecting now may well be predictive for other areas where salmon (and other species) are farmed at their upper thermal limits, and for temperate and cold-water fish farming in general, given that global warming appears to be a reality.

2. Materials and methods

2.1. Fish husbandry and sample collection

Two-year old Atlantic salmon broodstock undergoing vitellogenesis were supplied by Salmon Enterprises of Tasmania Pty. Ltd. (SALTAS) and held at that company's hatchery on the Derwent river (Wayatinah, Tasmania). Seventy-five fish were transferred from natural temperature (~12-16°C) and photoperiod to controlled temperature regimes (initially 14°C) and simulated natural photoperiod on January 15, 2002, and distributed equally among three temperature treatments. One group of 25 fish was held at 14°C while the two other groups underwent temperature ramp-up to 18°C and 22°C respectively. Final holding temperatures were achieved on January 22.

At each temperature, the 25 fish were distributed among three 4 m³ Rathbun tanks (8, 8 and 9 fish per tank), supplied with partially recirculated water. Oxygen saturation was maintained at 100–120 % by the addition of gaseous oxygen via ceramic diffusers. Fish were not fed during the entire experimental period. Fish sampling took place on February 14th, March 14th and April 11th. At each sampling period 5 fish from each temperature group were removed from the tanks and killed by a blow to the head prior to tissue harvest for *in vitro* experiments. Fish were randomly selected from the tanks so that stocking density was reduced approximately equally across tanks. Blood was taken from either the caudal vessel or the duct of Cuvier (Lied et al., 1975) and collected into heparinised syringes (5 mg ml⁻¹ lithium heparin), and plasma was separated by centrifugation, aliquotted and stored at –20°C. Body weight and gonad weight were recorded and gonadosomatic index (GSI) [(gonad weight/body weight)x100] was calculated. Ovaries were removed into ice-cold Cortland salt solution supplemented with 1g l⁻¹ glucose, and follicles were dispersed with fine forceps. The diameter of 10 individual oocytes was measured from each fish using Vernier callipers. The remaining fish were used for egg fertility and survival data following ovulation during May and June.

2.2. Ovulation and fertility

Tank temperatures were progressively ramped-down to 8°C after the final sampling point in April. Previous experiments have shown that this is necessary for ovulation to occur (King and Pankhurst, 2000). Temperatures were reduced by 1, 0.7 or 0.4 °C per day for fish held at 22, 18 and 14 °C respectively. The final holding temperatures were achieved by 26th April. Fish were checked for ovulation at 3 – 4

day intervals from late May onwards. Oocyte stripping, fertilisation and incubation were as described in King et al. (2003). Fertilisation (%) was determined on the basis of first cell division after 120 degree-hours, visualised by treatment with a clearing solution (1:1:1 v/v methanol: acetic acid: water) for 2 min. Survival (%) of ova to the eyed stage was observed after 250 degree-days.

2.3. In vitro procedure

At each sampling time, individual follicles from two of the five fish from each holding temperature were isolated into 24-well tissue culture dishes at 5 follicles per well. Follicles were incubated with: 100 ng ml⁻¹ 17P, 20 ng ml⁻¹ androstenedione (A) or 10 ng ml⁻¹ testosterone (T) (all steroids were purchased from Sigma-Aldrich, Castle Hill, Australia), 100 IUml⁻¹ human chorionic gonadotropin (hCG: ICN Biochemicals Inc., Australia), 10 mM N, 2-0-dibutyryl adenosine 3,5-cyclic monophosphate (dbcAMP, Sigma-Aldrich, Castle Hill, Australia), in a final volume of 1 ml in Cortland solution containing glucose as before and supplemented with 1 g l⁻¹ of bovine serum albumin. The concentrations of hCG and dbcAMP were chosen from preliminary experiments with a range of dilutions, and the minimum dose for consistent elevation above base-line was chosen for subsequent experiments. Optimum steroid precursor concentrations were pre-determined from work with rainbow trout (*Oncorhynchus mykiss*) (Haddy and Pankhurst, 1998). 17P, A and T were dissolved in ethanol and dispensed in 10 µl volumes, and 10 µl of ethanol were also added to all other wells. Four replicate wells were used for each treatment and control wells contained supplemented Cortland solution and ethanol only. Plates were incubated at 14, 18 or 22 °C, to match adult holding temperature, or 10 °C irrespective of adult temperature. Our unpublished results have shown that 10 °C is the optimum

temperature for incubation of follicles from fish held at ambient temperatures (12–16 °C). However, matching incubation temperatures were also used in case there was a temperature shock effect in follicles from fish maintained at 18 and 22 °C. Follicles were incubated for 18 h (Haddy and Pankhurst, 1998) and then media were aspirated from each well and stored at -20°C.

2.4. Measurement of steroids

In vitro media were directly assayed for T and E₂ levels by RIA following methods described in Pankhurst and Carragher (1992). Wells that had been treated with T were edited out of the protocol for T determination. Plasma samples were assayed for T, E₂ and also for cortisol (as an indicator of stress status) following extraction with ethyl acetate (Pankhurst and Carragher, 1992). Extraction efficiency (mean recovery of ³H-labelled steroid from triplicates of a plasma pool) was determined as 63.6% for E₂, 84.24% for T and 86.05% for cortisol, and assay results were adjusted accordingly. All samples were measured in duplicate. Plasma assay detection limits were 0.3 ng ml⁻¹ for E₂ and T and 0.6 ng l⁻¹ for cortisol, and *in vitro* detection limits were 30 pg ml⁻¹ medium for both E₂ and T. Interassay variability (%CV) for the media samples, determined from repeat measures of an internal standard was 9% for E₂ (n=18) and 5 % for T (n=18). Plasma steroids were measured in a single assay. Possible cross-reactivity of precursor steroids has been previously assessed at a maximum of <0.01% (Pankhurst, 1997) and hence has a negligible effect on results.

2.5. Measurement of plasma Vtg

Plasma Vtg levels were determined by competition ELISA developed for Atlantic salmon (Watts et al., 2003). Wells of microtitre plates were coated with 100 μ l volumes containing 2.5 μ g ml⁻¹ of a Vtg antigen in 50 mM sodium carbonate buffer (pH 9.6). In separate plates, termed 'dilution plates', standards were serially diluted in triplicate from 40 to 0.04 μ g ml⁻¹ in 20 mM Tris-HCl, 0.5 M NaCl, pH 7.2 (TBS) with 3% skim milk (TBSD). Plasma samples were diluted to 1/1000 in TBSD and 100 μ l added to triplicate wells. One hundred microlitres of salmon Vtg antiserum, diluted 1/500 in TBSD, were then added to each well to give final dilutions of 1:1000 for Atlantic salmon Vtg antisera, plasma dilutions of 1/2000 and standard curves from 0.02 to 20 μ g ml⁻¹. All plates were incubated overnight, with shaking, at 4°C. After incubation, antigen coated plates were washed four times with 300 μ l of TBS, and unoccupied sites on wells were blocked for 1 h at 37°C, with 300 μ l of 5% skim milk in TBS. Following blocking, wells were drained then 100 μ l were transferred from each well of the corresponding dilution plate to wells on antigen coated, blocked plates. Incubation was carried out for 1 h at 37°C, with shaking, and then plates were washed x4 as before. The complex was labelled with 100 μ l per well of donkey anti-sheep antiserum conjugated to alkaline phosphatase (Sigma-Aldrich, Castle Hill, Australia) diluted to 1:10,000 in TBSD. After 1 h incubation at 37°C wells were washed as before and then 100 μ l of 1mg ml⁻¹ para-nitrophenylphosphate in 0.01% MgCl₂, 10% diethanolamine buffer, pH 9.8, were added to each well. Following incubation for 1 h at 37°C absorbance was read at Abs₄₀₅. The assay detection limit was 0.04 mg ml⁻¹ and inter-assay variability (%CV) was determined as 13 % (n=3).

2.6. Statistical analysis

Means were compared by one-way ANOVA followed by Tukey's HSD test using SPSS 6 for Windows or Systat 8.0 for Windows. Proportion data were arcsin transformed prior to analysis. A confidence level of $p < 0.05$ was used throughout.

3. Results

3.1. Gonadal growth and ovulation

Mean body weight ranged from 3.58- 4.36 kg and did not differ significantly among temperature groups at any time (data not shown). There was no difference in GSI during February and March, but by April GSI was significantly lower in fish held at 22°C relative to those at 14°C and 18°C (Fig. 1). Just prior to ovulation, GSI was less at 22°C than at 18°C. Mean follicle diameter was also smaller at 22°C than at 14°C and 18°C in April and at ovulation (Fig. 1). Ovulation commenced first in fish maintained at 18°C on May 16, followed 4 days later by fish at 14°C (Table 1). The commencement of ovulation was delayed for a further three days in fish maintained at 22°C. All fish at 14°C had completed ovulation within 17 days, whereas those at 18°C and 22°C took 25 days to complete ovulation. Egg fertility was significantly reduced at 22°C relative to 14 and 18 °C with mean % fertility of 32, 93 and 89 respectively (Table 2). The same pattern was evident in egg survival (% survival of 86, 66 and 8 for 14, 18 and 22 °C respectively) (Table 2).

3.2. Plasma steroids and vitellogenin

There was no difference in plasma E₂ levels among temperature groups in February but, during March and April, fish held at 22°C had significantly lower plasma E₂ levels than fish at 14°C. Within each temperature group plasma E₂ levels increased over time from initial values of 6.8, 6.5 and 5.2 ng ml⁻¹ at 14, 18 and 22 °C respectively in February, to 32.1, 19.6 and 13.6 ng ml⁻¹ by April. In contrast to plasma E₂, plasma T levels in February were greater at 22°C than at 18°C and 14°C, but in March and April there were no significant differences among temperature groups. Plasma T levels also increased over time from 3.1 (14°C), 3.5 (18°C) and 4.9 ng ml⁻¹ (22°C) in February to 19.5, 15.6 and 18.6 ng ml⁻¹ in April (Fig. 2). Plasma Vtg levels in fish at 22°C were significantly lower than at 14°C and 18°C in February, but there were no differences among the different temperature groups at later stages (Fig 2). Overall plasma Vtg levels ranged between 5.0 and 23.0 mg ml⁻¹ and showed a trend of increase in April, relative to February and March. Plasma cortisol levels ranged between 9–16 ng ml⁻¹ and were not significantly different among the different temperature groups during March and April. However, in February cortisol levels in the fish held at 18°C were significantly greater relative to the other two groups, with mean levels of 6, 17 and 8 ng ml⁻¹ at 14, 18 and 22 °C respectively (data not shown). There was no evidence of depressed T, E₂ or Vtg levels in fish showing elevated cortisol levels.

3.3. In vitro incubations

Preliminary examination showed that all incubations were active in producing steroids and that there was no temperature shock effect associated with incubation at

10°C. Accordingly, 10°C incubations were used for all subsequent data analysis to standardise degree-hour incubation times.

In February, treatment with 17P, A, T and hCG stimulated E₂ production in follicles from fish previously held at 14 °C, whereas dbcAMP did not (Fig. 3). There was no difference between steroid treatments, but in one fish 17P and A were more effective than hCG. The pattern at 18°C was similar to that at 14°C with the exception that all steroids were more effective than hCG. In follicles from fish held at 22 °C, E₂ production was markedly reduced, with all steroids and hCG stimulating a small increase in one fish, but no treatment being effective in the other. In contrast to E₂, T production was significantly greater in follicles from fish at 14 °C in response to dbcAMP but not hCG (Fig 3). Both 17P and A also generated increases in T production, with 17P being more effective than A. The stimulatory effect of dbcAMP was absent in follicles from 18 °C fish, but other effects were the same as at 14 °C. In follicles from 22 °C fish all treatments elevated T levels, with 17P and A being more effective than hCG and dbcAMP in one fish.

In March, follicles from both fish previously held at 14°C produced E₂ in response to treatment with 17P, T was effective in follicles from one fish, but treatment with A, hCG and dbcAMP had no significant effect (Fig. 4). Follicles from both fish held at 18°C responded to treatment with 17P, A and hCG, but follicles from only one fish responded to T and, again, dbcAMP did not elicit a response. In follicles from fish at 22°C, E₂ production in response to treatment with 17P, A and T was similar to that at 18°C, but follicles of neither fish responded to hCG. In common with the other temperature groups there was no response to dbcAMP. Follicles from both fish held at 14°C produced T in response to 17P, A and dbcAMP, but as in February, there was no significant response to treatment with hCG. In follicles from both fish at 18°C, T

production was increased following treatment with 17P, hCG and dbcAMP, but follicles from only one fish responded to treatment with A. At 22°C, treatment with 17P elevated T production in both sets of follicles but only one responded to A. There was no significant increase in T production following treatment with hCG or dbcAMP in follicles of either fish held at 22°C.

In April, follicles from both fish held at 14°C produced E₂ in response to 17P and hCG; follicles from one fish responded to treatment with A and T but there was no response to treatment with dbcAMP (Fig. 5). Follicles from fish at 18°C were less responsive than those at 14°C. Follicles from one fish only responded to A, and one to hCG but there were no other significant effects. Follicles from both fish at 22°C responded to A and one to 17P, T and hCG, but there was no significant response to dbcAMP by follicles from either fish, as seen in previous months, and in one fish E₂ production in response to dbcAMP was significantly less than basal. Follicles from both fish at 14°C produced T in response to dbcAMP and one in response to hCG, but there was no significant response to treatment with 17P or A. Follicles from both fish at 18°C responded to treatment with 17P and dbcAMP, but there was no significant response to A or hCG. Stimulation with dbcAMP was the most effective treatment at both 14°C and 18°C. Follicles from both 22°C fish responded to treatment with 17P and A, follicles from one fish responded to treatment with hCG, and the other to dbcAMP, but these treatments were less effective than 17P in each fish.

4. Discussion

Maintenance at elevated temperature for three months during peak vitellogenesis delayed ovulation and reduced subsequent reproductive performance in Atlantic

salmon in the present study. Ramping temperature down to 8°C for 1 month prior to the time of normal ovulation was necessary to ensure ovulation in fish from all temperature groups, but ovulation was delayed in the 22°C group and oocyte survival was dramatically reduced at this temperature relative to 14°C and 18°C. These results support and confirm those reported in previous work by King and Pankhurst (2000), demonstrating that, for Atlantic salmon, temperature elevation during vitellogenesis is likely to have as severe an effect on reproductive development as temperature elevation at later developmental stages. High temperature disturbance of plasma steroid and Vtg levels observed in the present study also mirrored the results reported by King et al. (2003). As a major function of E₂ is to stimulate hepatic synthesis of Vtg (Specker and Sullivan, 1994), the reduction in plasma Vtg levels at 22°C in February, before there was any significant effect on plasma E₂, suggests that Vtg level may be a sensitive indicator of early reproductive impairment. However, the fact that Vtg levels did not appear to be affected by high temperature in March and April, suggests that, at later stages, Vtg may accumulate in the plasma, particularly if there is impairment of oocyte uptake mechanisms and/or damage to zona radiata (z.r.) proteins. Z.r. protein synthesis has been shown to be acutely sensitive to E₂ levels (Hyllner et al., 1994) and may have been affected by reduced plasma E₂ levels at 22°C in the present study. An effect at this level would explain the reduced GSI and oocyte size among fish held at 22°C, despite apparently normal Vtg levels during March and April. As plasma Vtg was depressed after only one month at 22°C, there is evidence that temperature related damage occurred rapidly, and this raises the question as to whether temperature spikes may be equally as severe in effect as prolonged exposure to temperatures at or above 22°C.

Reduction in plasma E₂ levels with concomitant increase in plasma T implies inhibition of cytochrome P450-aromatase (P450_{arom}), which catalyses the T to E₂ conversion step (Simpson et al., 1994). Support for this conjecture was provided by the present *in vitro* studies. In February, follicles from fish that had been held at 22°C failed to produce substantial amounts of E₂ in response to any treatment, but were highly responsive to all treatments in terms of T production. However, follicles of fish with prior temperature histories of 14°C and 18°C were highly responsive to treatment with steroid precursors and hCG in terms of E₂ synthesis at this time. These results suggest that GtH-receptor binding, subsequent signal transduction and steroidogenic enzyme activity up to T production were intact and functional at all temperatures in February. However, T synthesis with concomitant low levels of E₂ production suggest that thermal impairment of P450_{arom} had occurred at the follicular level.

The temperature sensitivity of P450_{arom} has been well documented in several species within the context of sex determination and gonadal sex differentiation. P450_{arom} is the key enzyme for ovarian differentiation and it has been shown in reptiles that incubation temperature determines sex by controlling activity of steroidogenic enzymes in eggs (Crews, 1996), in particular P450_{arom} (Jeyasuria and Place, 1998). High environmental temperature during the period of sex determination usually results in increased male: female ratio in most species studied, although in some species this is reversed (for reviews see Jalabert et al., 2000; Baroiller and D'Cotta, 2001; Devlin and Nagahama, 2002). In addition, sex reversal during critical periods of gonadal development in thermosensitive species is linked to P450_{arom} activity. Tilapia (*Oreochromis niloticus*) female progeny which were functionally masculinised by exposure to high temperature were shown to have decreased P450_{arom} mRNA expression (D'Cotta et al., 2000). Treatment with P450_{arom} inhibitors have

been shown to have the same effect as high temperature inhibition. One hundred percent of all-female populations of rainbow trout, and 73.5% of female tilapia treated with the steroidal aromatase inhibitor 1,4,6-androstatriene-3-17-dione became masculinised (Guigen et al., 1999). A non-steroidal aromatase inhibitor, fadrozole, had a similar effect in masculinising genetic female tilapia (Kwon et al., 2000; Nakamura et al., 2000). Treatment of coho salmon (*Oncorhynchus kisutch*) with fadrozole (Afonso et al., 1999) produced effects very similar to those seen in the present study in response to elevated temperature. Plasma E₂ levels fell and plasma T levels increased after a single injection of fadrozole and multiple injections resulted in decreased oocyte diameter and GSI, and increased incidence of follicle atresia (Afonso et al., 1999).

Although the strongest inhibitory effect of temperature on *in vitro* follicular E₂ synthesis was found during February in the present study, plasma E₂ levels were not significantly reduced at this time. In contrast, plasma E₂ levels in March and April were reduced at 22°C but there was a much less marked effect on *in vitro* E₂ production. *In vitro* work with rainbow trout (Pankhurst et al., 1996) showed that even though *in vitro* capacity to synthesise T and E₂ was impaired at 18°C, plasma levels remained unchanged over 2 months. Plasma steroid levels reflect the sum of the processes of secretion, metabolism and excretion and may not mirror the exact effect found at tissue level *in vitro*. This means that *in vitro* outcomes must be interpreted with caution in terms of their likely significance to *in vivo* events. However, there can be increased confidence that *in vitro* events mimic *in vivo* mechanisms if the same directional effect is seen *in vitro* and *in vivo* as in the present study, albeit with temporal separation.

There was evidence in the present study that follicle responsiveness changed with oocyte growth, and that this temporal shift was further modified by temperature. In February, steroid precursors were generally more effective than hCG in stimulating an E₂ response at 14°C and 18°C, and a T response at all temperatures. By April the pattern was different and stimulation with hCG generally produced a higher E₂ and T response than steroid precursors at the lower temperatures, but not at the higher temperature. Steroid production following hCG stimulation probably requires *de novo* synthesis of steroid converting enzymes (Planas et al., 1995), and reduced stimulation by hCG at 22°C in the present study suggests that this mechanism is impaired, although whether this is at the level of receptor-ligand interaction or cAMP activation is unclear. Pankhurst (1997) hypothesised that two methods of steroidogenic regulation operated in teleosts; one primarily via levels of enzyme activity (in salmonids) and the other primarily via substrate limitation (in non-salmonids). The present study demonstrated that both methods of control are likely to operate in Atlantic salmon, depending on the oocyte stage, with the more mature oocytes showing greater capacity to respond to gonadotropin stimulation, but earlier stage follicles showing evidence of substrate limitation.

Stage effects were also evident in response to treatment with dbcAMP, although factors other than temperature also appeared to have an impact. T production was generally high in response to dbcAMP stimulation, and the strongest response occurred at 22°C during February, but during April the strongest stimulation of T production was most apparent at 14°C. However, E₂ levels were never significantly above basal at any time or temperature in response to dbcAMP stimulation. This effect is difficult to explain, as in other studies dbcAMP is reported to stimulate E₂ production in a dose dependent manner by mid-vitellogenic follicles (Srivastava and

Van der Kraak, 1994). It is possible that high levels of dbcAMP mimicked the naturally high cAMP levels that occur due to receptor binding by GtH II, which results in inhibition of E₂ production and/or P450_{arom} activity, during final maturation (Planas et al., 1997). This is consistent with the normal fall in plasma E₂ in association with the periovulatory rise in GtH II in salmonids (Planas et al., 1997). It is also possible that post-translational modification of P450_{arom} might occur under conditions of high intracellular cAMP. Post-translational modification of other P450 cytochrome enzymes occurs due to phosphorylation events initiated by high cAMP levels (Oesch-Bartlomowicz et al., 2001; Oesch-Bartlomowicz and Oesch, 2002). As Atlantic salmon ovarian P450_{arom} has serine residues present (Andersson et al., 2001) and as serine residues are particularly susceptible to phosphorylation (Oesch-Bartlomowicz et al., 1998), an effect of this type is possible in salmon.

In conclusion, the present study documents the first direct evidence of temperature impairment of steroidogenesis in ovarian follicles of Atlantic salmon. The *in vivo* results we found from fish in the present study are in agreement with previous studies on Atlantic salmon (King and Pankhurst, 2000; King et al., 2003), indicating that abnormally elevated temperature has a consistent detrimental effect on reproductive development, and this effect is exercised by thermal challenge during the period of vitellogenesis. At least part of this effect appears to be generated by the inhibition of P450_{arom} activity. Because P450_{arom} impairment by elevated summer and autumn temperatures will inhibit reproduction in Atlantic salmon by disrupting E₂-dependent reproductive processes, the ability of broodstock to produce viable eggs is highly susceptible to climatic variation. This may have severe implications for the commercial production of Atlantic salmon worldwide in the face of substantive global

warming. At this stage it remains unclear if the effects of thermal challenge during vitellogenesis are reversible and this will be the focus of continuing studies.

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Figure 1: Gonadosomatic index (GSI) and follicle diameters (mean + SE) of Atlantic salmon maintained at 14, 18 or 22 °C (n=5 for February, March and April and n=10 at final sample near ovulation). Differing superscripts within a group indicate significantly different means ($p<0.05$).

Figure 2: Plasma levels (mean + SE, n=5) of testosterone (T), 17 β -estradiol (E₂), and vitellogenin (Vtg) in Atlantic salmon maintained at 14, 18 or 22 °C and sampled during February, March and April (n=5). Differing superscripts within a group indicate significantly different means ($p<0.05$).

Figure 3: E₂ and T production by isolated ovarian follicles of Atlantic salmon in February in response to incubation with Cortland salt solution (CSS) alone or containing 100 ng ml⁻¹ 17P; 20 ng ml⁻¹ A; 10 ng ml⁻¹ T; 100 IU ml⁻¹ hCG, or 10 mM dbcAMP. Values are mean + SE (n=4). 'a' and 'b' denote duplicate fish at each temperature. Different superscripts (comparisons between treatments for each fish) indicate significantly different means ($p<0.05$).

Figure 4: E₂ and T production by isolated ovarian follicles of Atlantic salmon in March. Other details as for Figure 3.

Figure 5: E₂ and T production by isolated ovarian follicles of Atlantic salmon in April. Other details as for Figure 3.

Table 1. Cumulative percent ovulation in Atlantic salmon maintained at 14, 18 or 22 °C

Date	Temperature Regime (°C)		
	14	18	22
16 May 02	0	22	0
20 May 02	8	44	0
23 May 02	25	56	10
27 May 02	67	56	40
30 May 02	92	78	50
3 June 02	92	89	50
6 June 02	100	89	70
10 June 02		100	90
13 June 02			90
17 June 02			100

Table 2: Percent fertility and survival to eyed stage from ova of Atlantic salmon maintained at 14, 18 or 22 °C. Figures with the same superscript are not significantly different ($p>0.05$)

Parameter	Temperature Regime (°C)		
	14	18	22
Egg Fertility (%)	92.71 ^a	88.50 ^a	32.42 ^b
Upper 95%CL	95.21	93.40	50.22
Lower 95%CL	89.73	82.46	16.93
Egg Survival (%)	86.48 ^a	66.06 ^a	7.98 ^b
Upper 95%CL	93.49	90.19	25.61
Lower 95%CL	77.45	40.41	0.18