Effect of elevated summer temperatures on gonadal steroid production, vitellogenesis and egg quality in Tasmanian female Atlantic salmon (*Salmo salar* L.)

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To assess the effect of exposure to elevated water temperatures on vitellogenesis and egg quality in Tasmanian female Atlantic salmon (*Salmo salar* L.), groups of fish were maintained at 14, 18 and 22°C for 3 months from mid-summer (January). Blood plasma levels of 17 β -estradiol (E₂), testosterone (T), cortisol and vitellogenin (Vtg) were measured at regular intervals and in autumn (April) temperatures were reduced to 8°C to facilitate spawning and egg incubation. Maintenance at 22°C during vitellogenesis was associated with a general reduction in plasma E₂ levels and an early reduction in plasma Vtg levels relative to those observed in fish held at 14 and 18°C. Significantly reduced oocyte diameters in ova from fish held at 22°C (5.4 mm c.f. 5.7 mm) confirmed reduced maternal investment and an increase in the incidence of previously undescribed chorion damage suggested that zonagenesis may also have been impaired. As a result, the fertility and survival of ova from fish exposed to 22°C (69% and 42% respectively) were significantly reduced relative to those of ova from fish maintained at 14°C (93% and 86%) and 18°C (86% and 84%).

Key words: vitellogenesis; 17 β -estradiol; testosterone; vitellogenin; temperature; salmon.

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INTRODUCTION

Reproductive development in female salmonids commences many months or even years prior to final oocyte maturation (FOM) and ovulation (Billard, 1985). Vitellogenesis, the phase of rapid ovarian growth which is characterised by the hepatic production and ovarian uptake of the yolk protein precursor vitellogenin (Vtg) (reviewed by Specker & Sullivan, 1994), in turn occurs over several months of the female reproductive cycle (Billard, 1985). During vitellogenesis, individual oocytes may increase in size by several hundred-fold and Vtg can account for over 90% of final oocyte volume (reviewed by Tyler, 1991; Tyler *et al.*, 2000). Since salmonids are generally recognised as autumn and winter spawners (reviewed by Scott, 1990), much of the vitellogenic oocyte growth takes place during summer and early autumn (eg. Bromage & Cumaranatunga, 1988; Tyler *et al.*, 1990; Estay *et al.*, 1998; King & Pankhurst, 2003) when natural water temperatures tend to be elevated.

Many aspects of the reproductive development of fish including spermatogenesis, oogenesis, spermiation and ovulation, are known to be significantly influenced by environmental temperature with effects of abnormally elevated temperatures being strongly detrimental (reviewed by Van Der Kraak & Pankhurst, 1997). Exposure of salmonids to elevated temperature is known to inhibit FOM and ovulation in Arctic charr (*Salvelinus alpinus* L.) (Gillet, 1991), Atlantic salmon (*Salmo salar* L.) (Taranger & Hansen, 1993; King & Pankhurst, 2000) and rainbow trout (*Oncorhynchus mykiss* Walbaum) (Pankurst *et al.*, 1996; Pankhurst and Thomas, 1998). In contrast, the effects of chronically-elevated environmental temperature on vitellogenesis are poorly understood. Exposure of Atlantic salmon post-smolts to 10°C increased the hepatic vitellogenic output relative to that observed at 3°C (Korsgaard *et al.*, 1986). Similarly,

MacKay & Lazier (1993) reported a progressive increase in the rate and extent of Vtg production by rainbow trout held at 4, 9 and 15°C. Pankhurst *et al.* (1996) reported essentially unimpaired estrogen synthesis (and, by inference, vitellogenesis) in rainbow trout reared at temperatures as high as 21°C but Chmlevsky (2000) briefly described how gonadal development was arrested in the same species following exposure to 22-23°C. Thus, it is unclear at which point the apparent stimulatory effect of elevated temperature on vitellogenesis becomes an inhibitory one. There is limited information on the effects of temperature change on vitellogenesis in non-salmonids. In the common wolffish (*Anarhichas lupus* L.), Tveiten & Johnsen (2001) showed that exposure to elevated water temperatures (12°C) during vitellogenesis delayed ovarian steroidogenesis, although effects on vitellogenesis, inferred from differences in egg size, were less clear (Tveiten & Johnsen, 1999).

Preliminary studies on female Atlantic salmon showed impairment of ovarian steroidogenesis was also apparent during the late stages of vitellogenesis in fish held at elevated temperature (King & Pankhurst, 2000). Plasma levels of both 17 β -estradiol (E₂) and its precursor testosterone (T) were significantly reduced in fish held at 16°C relative to levels in fish held at 6 and 11°C (King & Pankhurst, 2000). Because the primary function of E₂ is the stimulation of hepatic synthesis and ovarian sequestration of Vtg (Specker & Sullivan, 1994), King & Pankhurst (2000) concluded that the reduced viability observed in ova from fish maintained at 16°C might have resulted in part from impairment of vitellogenesis.

During a study of the natural reproductive development of female Atlantic salmon in Tasmania, King & Pankhurst (2003) reported that oocyte size and plasma levels of E₂, T and Vtg changed little during the hottest months of the austral summer (December –

February), then showed rapid increases in March. This suggested that there was possibly temperature-related impairment of reproductive development during summer. Accordingly, the present study was conducted to assess the effect of elevated summer temperature on vitellogenesis and egg quality in Tasmanian female Atlantic salmon exposed from the early stages of vitellogenesis. Groups of female Atlantic salmon were maintained at temperatures of 14, 18 and 22°C for 3 months from mid-summer onwards. At the end of the exposure period, temperatures were reduced to 8°C to facilitate FOM, ovulation and subsequent egg incubation. Blood samples were collected at regular intervals during the period of temperature manipulation, and plasma levels of E₂, T, cortisol and Vtg were measured to assess the relationship between endocrine status and Vtg production, and differences in reproductive development and gamete quality.

MATERIALS AND METHODS

HUSBANDRY, TEMPERATURE AND PHOTOPERIOD REGIMES

Ninety sexually maturing 2 year-old female Atlantic salmon (mean weight 4.6 kg) were transported from a marine cage-farm operated by Aquatas Pty. Ltd., (Margate, Tasmania) to SALTAS Freshwater Operations (Wayatinah, Tasmania) in mid November 1998 and were maintained in a single circular 44 m³ outdoor tank supplied with river water ($\sim 15 \text{ l.s}^{-1}$) at natural temperature ($\sim 13\text{-}15^\circ\text{C}$ at time of transfer). Fish were fed to satiation on a commercial broodstock ration (Pivot Aquaculture Ltd., Cambridge, Tasmania). On 12 January 1999, fish were transferred from natural conditions (diurnal temperature range 15-20°C) to 3 sets of 3 temperature-controlled 4 m³ Rathbun tanks (10 fish per tank) supplied with partially recirculated water ($\sim 1.5 \text{ l.s}^{-1}$ per tank) initially set at a temperature of 14°C. Biological filtration and water

exchanges of 50% per day were employed to prevent the accumulation of toxic metabolites. Oxygen saturation was maintained at 100-120% by the addition of gaseous oxygen via ceramic diffusers. Twenty-four hours after transfer, the temperature of one group of tanks was increased to 18°C and that of a second group to 22°C at a maximum rate of 1°C.day⁻¹. The third group was left at 14°C. Following transfer to temperature-controlled conditions, fish were unfed and exposed to a simulated natural photoperiod (42° S). In order to facilitate final oocyte maturation and ovulation, the water temperatures of all three tank groups were reduced after 13 April 1999, at a maximum rate of 2°C.day⁻¹, such that all three groups reached a temperature of 8°C on April 20, the date by which fish under natural conditions would have been expected to have completed vitellogenesis (King & Pankhurst, 2000, 2003).

FISH SAMPLING AND HANDLING

Twenty-four hours after the temperature-controlled groups had reached the correct holding temperatures of 14, 18 or 22°C, all fish from each of the three temperatures were anaesthetised (25p.p.m. Aqui-s, Crop & Food, New Zealand) and tagged by placing visible implant tags (VI Tags, Northwest Marine Technology Inc, Shaw Island, WA) in the left adipose eyelid. Fish were also weighed to confirm that, as a result of random assignment, there were no significant differences in the mean weights of the 3 treatment groups (overall mean weight 5.2 kg). Blood samples were taken from each fish by puncture of the duct of Cuvier (Lied *et al.*, 1975) using heparinized (lithium heparin) syringes and 22G needles. After centrifugation, the resulting plasma was stored at -20°C prior to analysis of steroid hormone and vitellogenin levels. Anaesthesia and blood sampling was repeated at 3 weekly intervals until mid April 1999. Ovulation checks of un-anaesthetised fish were commenced at the start of the recognised spawning season (late April) and continued at 3-4 day intervals until all surviving fish had

ovulated (aside from 3 fish which escaped from one tank at 14°C, there were no treatment-related differences in mortality which was $n = 3, 3$ and 2 at $14, 18$ and 22°C respectively).

OVA FERTILISATION AND INCUBATION

Fish that expressed ova in response to the gentle application of pressure to the abdomen were killed by a blow to the head, towelled dry and ova were expressed into a stainless steel sieve. In order to facilitate ova collection, a 2cm cut was made at the genital papilla. Ova were transferred to a stainless steel bowl and fertilised using milt from 3-4 naturally spermiating males sourced from commercial broodstocks. Sperm pooling was used to avoid sperm quality problems as individual male milt quality was not checked. Ova and milt were gently mixed and 500 ml water was added to ensure sperm activation. After 2 mins, ova were rinsed with clean water then left to water-harden for 60 mins. After water-hardening, sample batches (1000-1500) of ova from each female were removed and incubated at 8°C in mesh baskets ($4.0 \times 10.0 \times 12.0$ cm) placed in Heath vertical incubator trays (Marisource Inc., Tacoma, WA). Sub-samples of ova from each female were collected after water-hardening and fertilisation (%) was determined on the basis of first cell division after 120 degree-hours, visualised by treatment with a clearing and fixing solution (5:4:6:85 v/v formalin: acetic acid: glycerine: 1% NaCl) and the diameters of 10 fixed oocytes from each female were later measured (± 0.01 mm) using digital vernier callipers. Eyed ova survival (%) was observed directly after 250 degree-days of incubation.

PLASMA HORMONE AND VTG MEASUREMENT

Plasma Steroids

Plasma levels of E₂, T and cortisol were measured by radioimmunoassay following extraction with ethyl acetate using the reagents and protocols described by Pankhurst & Carragher (1992). Extraction efficiency (mean recovery of ³H-labelled steroid from triplicates of a plasma pool) was 59.2-66.5, 85.3-90.6% and 94.3-100% for E₂, T and cortisol respectively and values for each steroid were adjusted accordingly. Interassay variability measured using aliquots of a pooled internal standard was (%CV(n)) 12.4(10) and 10.8(10), 12.2(8) for E₂, T and cortisol respectively.

Plasma Vtg

Plasma levels of Vtg were measured by Enzyme-linked Immunosorbent Assay (ELISA) using the reagents and protocol described by Watts *et al.* (2002). Interassay variability measured using aliquots of a pooled internal standard was (%CV(n)) 11.5 (20).

CHORION MORPHOLOGY

At stripping, a sub-sample (n=10-20) of unfertilised ova from each ovulated female was placed in Bouin's fixative and after 48h, fixed ova were transferred to 70% ethanol. Fixed ova stored in 70% ethanol were cut into quarters with a scalpel blade, and the chorion from one of the quarters was dehydrated in 90% ethanol for 30 mins, and then 100% ethanol for 30 mins, prior to critical point drying in a BAL-TEC CPD-030 critical point dryer. The dried samples were fixed to scanning electron microscope (SEM) stubs (5 samples per stub) with double-sided carbon tape to allow exposure of the outer surface of the chorion to the electron beam. The stubs were then coated with gold in a

Union FL9496 Blazer splutter coater and chorion samples were observed at approximately 150x magnification using an Electro Scan Environmental SEM.

STATISTICAL ANALYSIS

Where appropriate, steroid, Vtg and egg quality data were analysed by one-way ANOVA and Tukey's HSD test using the SYSTAT 8.0 for Windows computer package. The same package was used to derive linear correlation coefficients between parameters and to construct contingency tables for the comparison of treatment-related differences in egg external morphology. Prior to analysis, proportion data were normalised by arcsin transformation.

RESULTS

Ovulations in fish from all three temperature regimes commenced simultaneously on 18 May (Fig. 1). The 14 and 22°C treatment groups completed ovulation on 6 June, whereas fish from the 18°C treatment did not complete ovulation for a further 8 days. A level of 80% ovulation was first exceeded by fish from the 22°C treatment on 24 May, whereas similar progress was only achieved by the 14 and 18°C treatment groups after a further 7 and 17 days respectively.

The mean diameters of ova from fish from the 14 and 18°C temperature treatments were not significantly different (~ 5.7 mm, Table I). However, ova from fish which had been held at 22°C were significantly smaller (~ 5.4 mm) than those from fish held at 14 and 18°C (Table I). Mean fertility exceeded 85% in ova from fish which had been held at 14 and 18°C but was significantly lower (below 70%) in ova from fish maintained at 22°C (Table I). Similarly, mean eyed-egg survival was greater than 80% in ova from fish held at 14 or 18°C, whereas ova from fish held at 22°C displayed a significantly lower mean survival of only ~ 42% (Table I).

Plasma E₂ levels in fish held at 22°C were significantly lower than in fish held at 14°C at four out of five sample points (Fig. 2), and significantly lower than at 18°C at the first two sample points (Fig. 2). E₂ levels at 18°C were also significantly lower than those at 14°C at the final sample point (Fig. 2). In contrast, plasma T levels in fish held at 22°C tended to be higher than those in fish held at either 14 or 18°C at three out of five sample points (Fig. 3). At the final sample, plasma T levels were significantly lower in fish held at 18 and 22°C than in fish maintained at 14°C (Fig. 3).

Plasma cortisol levels were initially high in all treatments and declined as sampling progressed (Fig. 3). Cortisol levels in fish held at 22°C were higher than those in fish held at 14°C for the first four samples, and values in fish held at 18°C were also higher than those in fish held at 14°C in four out of five samples (Fig. 3). Plasma Vtg levels were significantly higher at 14°C than 22°C for the first three samples, after which there was a marked increase in plasma Vtg levels in fish held at 22°C (Fig. 4). Plasma Vtg levels at 18°C were also lower than those at 14°C in three out of five samples.

Comparison of E₂, Vtg and cortisol data revealed that, plasma Vtg levels displayed a weak but significant positive correlation with plasma E₂ levels at the first ($R = 0.42$, Bartlett χ^2 -Statistic = 16.7, d.f. = 1, $P < 0.001$) and second samples ($R = 0.33$, Bartlett χ^2 -Statistic = 8.8, d.f. = 1, $P < 0.01$). Similarly, plasma Vtg levels displayed a weak but significant negative correlation with plasma cortisol levels at the first ($R = -0.48$, Bartlett χ^2 -Statistic = 19.8, d.f. = 1, $P < 0.001$) and second samples ($R = -0.44$, Bartlett χ^2 -Statistic = 16.1, d.f. = 1, $P < 0.001$) but there was no significant relationship between plasma E₂ and cortisol levels at any sample time.

Examination of chorion morphology revealed a number of differing egg surface characteristics. The majority of samples exhibited a chorion surface where the pore

channel plugs were clearly visible (Fig. 4a and b). The open micropyle of the ovum was also visible in a number of samples (Fig. 4c). However, in addition to the normal features above, holes were observed in the chorion of many samples (Fig. 4d). These holes appeared to have originated from blister-like eruptions on the surface of the chorion resulting in raised craters of 2-10 μm in diameter (Fig. 4d). Closer examination revealed that the holes were irregular in diameter and that they were partially obstructed by fibrous material (Fig. 4e).

Among fish maintained at 14°C, 27.3% of females produced ova that contained abnormal holes (Table II) and the mean incidence of holes in those egg batches was 32.6%. At 18°C, 60.9% of females produced ova with holes and the mean incidence of holes was 31.9% (Table II), while at 22°C, 38.5% of females produced ova with holes at a mean incidence of 38.4% (Table II). The proportion of females which produced damaged ova was significantly higher at 18°C than at 14°C, whereas, the proportion of females at 22°C which produced damaged ova did not differ significantly from that at either 14 or 18°C (Table II). Among damaged batches of ova there were no significant differences in the mean incidence of holes (Table II).

There were weak but significant negative correlations between percent survival of ova to the eyed stage and incidence of holes for ova from fish maintained at 14°C ($R = -0.63$, Bartlett χ^2 -Statistic = 9.8, d.f. = 1, $P < 0.01$) and at 18°C ($R = -0.47$, Bartlett χ^2 -Statistic = 5.2, d.f. = 1, $P < 0.05$) whereas, for ova from fish held at 22°C, there was no significant correlation between the incidence of chorion damage and the survival of ova.

DISCUSSION

Exposure to elevated temperature during vitellogenesis was associated with significant reductions of both the fertility and the survival of ova from female Atlantic salmon. Mean fertility of ova from fish held at 22°C was less than 70% and subsequent survival to the eyed stage was only ~ 40% whereas, values for both parameters exceeded 80% for fish held at 14 and 18°C. While there have been no reports of directly comparable studies in other salmonids, Chmielevsky (2000) observed arrested gonadal development in rainbow trout held at 22-23°C. In addition, the present results are broadly consistent with those reported by Pankhurst *et al.* (1996) in rainbow trout, where maintenance at 18 and 21°C for the 3 months prior to ovulation was associated with failure to produce viable ova. Similarly, in a study of coho salmon (*Oncorhynchus kisutch* Walbaum) from the Great Lakes of North America, Flett *et al.* (1996) reported highly variable fertility (0 to > 80%) and low survival to hatch (42%) in ova from females which had been exposed to elevated temperatures during the 5 months preceding spawning. Furthermore, in an examination of reproductive performance in the common wolffish which more closely resembled the design of the present study (albeit at a lower range of temperatures), Tveiten & Johnsen (1999) observed a trend of declining egg survival in association with increasing holding temperature during vitellogenesis.

Bromage *et al.* (1992, 1993) suggested that reproductive phasing in salmonids is almost totally dependent on photoperiod, whereas temperature is typically understood to have a permissive role, inhibiting maturation at its upper and lower extremes. However, the present observation of accelerated and more synchronous ovulation following temperature reduction in fish previously held at 22°C also indicates the involvement of temperature in cueing maturation in female Atlantic salmon, and suggests that the

magnitude and/or rate of temperature reduction may assist in entraining an endogenous reproductive rhythm in a manner similar to that reported for changes in photoperiod (eg, Porter *et al.*, 2000; Randall *et al.*, 2000). Taranger *et al.* (2000) also reported that exposure to reduced water temperatures (approximately 5°C below natural) both synchronised and advanced ovulation in Atlantic salmon. Duncan *et al.* (2000) reported that the timing of ovulation in female Atlantic salmon was less photoperiod-labile than in rainbow trout and concluded that a temperature-sensitive mechanism was partially responsible for the control of maturation in female salmon.

In general, the patterns of E₂ and T production observed during the present study agree with those presented by King & Pankhurst (2003). In mid January, both steroids were recorded at levels of ~2-3 ng.ml⁻¹ and levels increased approximately 7 to 10-fold as sampling progressed. King & Pankhurst (2003) reported that plasma E₂ and T in unmanipulated female Atlantic salmon increased from ~ 2-5 ng.ml⁻¹ to 20-25 ng.ml⁻¹ over a similar timeframe. Furthermore, the generally lower E₂ production in fish held at 22°C is consistent with the observations of King & Pankhurst (2000) of an apparent impairment of ovarian steroidogenesis during the latter stages of vitellogenesis in salmon maintained at 16°C. This supports the contention that exposure to elevated temperatures may influence vitellogenic as well as maturational processes in Atlantic salmon (King & Pankhurst, 2000) but contrasts with the conclusions of Pankhurst *et al.* (1996) and Pankhurst & Thomas (1998) who reported inconsistent effects of elevated temperatures on ovarian steroidogenesis during vitellogenesis in female rainbow trout.

The present reduction in E₂ and elevation in T observed during the earlier stages of sampling (January to March) in fish held at 22°C is suggestive of impaired E₂ synthesis. As conversion of T to E₂ is dependent on the activity of the aromatase cytochrome P450

(P450_{arom}) enzyme system (reviewed by Nagahama *et al.*, 1993), the present observations suggest that the impairment of ovarian steroidogenesis during the early stages of sampling was largely restricted to inhibition of P450_{arom} activity. In contrast, the observation of significant reductions in both E₂ and T in fish from the 18 and 22°C treatments at the final sample point indicates that impairment of ovarian steroidogenesis involved enzymes higher in the synthetic pathway at that stage.

At the commencement of sampling, Vtg levels in fish held at 14 and 18°C were consistent with those reported in rainbow trout at a similar stage of development (~ 10-15 mg.ml⁻¹, Tyler *et al.*, 1990). However, the absence of a subsequent increase in plasma Vtg, concomitant with the observed increases in plasma E₂ and T, is unexpected and the cause unclear. In contrast, in spite of a later, rapid elevation, initial Vtg levels at 22°C were low (~ 5 mg.ml⁻¹), implying impairment of vitellogenesis at 22°C during January and February. The majority of the salmonid literature tends to indicate that increased temperature actually favours rather than impairs Vtg production. For example, Olin & Von Der Decken (1989) observed an increase in serum Vtg from ~ 10 mg.ml⁻¹ to ~ 28 mg.ml⁻¹ in Atlantic salmon smolts in association with an increase in temperature from 8 to 16°C and calculated a Q₁₀ temperature coefficient for Vtg production of 3.71. Korsgaard *et al.* (1986), using serum levels of alkali-labile phosphorous and calcium as indicators of circulating Vtg, observed a failure to produce Vtg following E₂ treatment in Atlantic salmon post-smolts held at 3°C. In contrast, E₂-treated fish held at 10°C or transferred to 10°C from 3°C exhibited increased serum levels of alkali-labile phosphorous (~ 12 µg.ml⁻¹) and calcium (~ 4-5 mM) relative to controls (~ 0.7 µg.ml⁻¹ and ~ 2.8 mM respectively). Similarly, rainbow trout held at 15°C exhibited measurable Vtg production within 24h of E₂ injection, with plasma Vtg peaking at ~ 70 mg.ml⁻¹ within 10 days (MacKay and Lazier, 1993) whereas, in fish held at 9°C plasma Vtg

appeared later (~ 72h) and reached a level of only 8.9 mg.ml⁻¹ after 10 days. Furthermore, production of Vtg mRNA was detected within 8h in fish held at 15°C but took ~ 24 and ~ 48h at 9 and 4°C respectively. As all the above studies were conducted at temperatures near or below the lowest temperature used in the present study this implies that increasing temperature is stimulatory in terms of Vtg production up to a certain point, beyond which it becomes inhibitory. In Tasmanian populations of Atlantic salmon this effect becomes apparent at temperatures above 18°C.

Almost all of the contents which determine the quality of a fish egg must be incorporated into the oocyte during ovarian growth (reviewed by Brooks *et al.*, 1997; Tyler *et al.*, 2000). Accordingly, since uptake of Vtg can account for more than 90% of final oocyte volume in salmonids (reviewed by Tyler, 1991; Tyler *et al.*, 2000) the significance of impaired vitellogenic growth for gamete quality and subsequent embryo viability is likely to be high. In rainbow trout, for example, treatments which resulted in a ~ 46% reduction in plasma Vtg levels also resulted in reductions in egg weight and egg volume of ~ 13% and ~ 17% respectively, and were associated with reduced survival of eggs to the eyed stage of development (~ 54% c.f. ~ 96% in controls) (Campbell *et al.*, 1994). During the present study, the size, fertility and survival of ova from fish held at 22°C were also significantly reduced. In addition, significantly reduced levels of Vtg were observed at each of the first three sample points in fish held at 22°C relative to those at 14°C. The observation of weak but statistically significant correlations between plasma Vtg levels and plasma E₂ levels at the first and second samples are consistent with the known role of E₂ in stimulating vitellogenesis (reviewed by Mommsen & Walsh, 1988; Tyler, 1991; Specker & Sullivan, 1994) and suggest that the reduced performance of ova from fish held at 22°C was associated with the significant reductions in E₂ observed during January and February.

Reductions in circulating E_2 and Vtg in fish held at high temperatures may represent a stress effect. Carragher *et al.* (1989) reported a depression of plasma E_2 and Vtg levels in female rainbow trout following artificial elevation of plasma cortisol levels. In later experiments, plasma Vtg levels in rainbow trout were halved following 2 weeks of confinement stress, while implantation of female brown trout (*Salmo trutta* L.) with a slow-release cortisol pellet resulted in reduced plasma E_2 (2 c.f. 7 ng.ml⁻¹) and Vtg (6 c.f. 24 mg.ml⁻¹) (Pottinger *et al.*, 1991). During the present study, plasma cortisol levels were relatively high at all temperatures (~ 30-100 ng.ml⁻¹) and no doubt primarily reflected an acute response to the stress of sampling. Nonetheless, plasma cortisol levels were generally higher at elevated temperatures and there was a weak but significant negative correlation between plasma cortisol and Vtg levels in early samples, suggesting a temperature treatment-related stress effect on vitellogenesis. However, there was no significant relationship between plasma cortisol and plasma E_2 . This is not unexpected since the role of cortisol as a direct mediator of stress on reproduction and, in particular, ovarian steroidogenesis has been difficult to demonstrate unequivocally (eg. Pankhurst *et al.*, 1995; Pankhurst, 1998, Leatherland, 1999) and it has only recently become apparent that cortisol-mediated inhibition of reproduction may result from effects at the level of GtH signal-transduction (Pankhurst & Van Der Kraak, 2000).

E_2 is also responsible for stimulating hepatic synthesis of egg-shell proteins in teleost fish (zonagenesis, Hyllner & Haux, 1995). In Atlantic salmon, plasma levels of egg shell or zona radiata (ZR) proteins closely correlate with plasma levels of E_2 throughout the annual reproductive cycle (Oppen-Berntsen *et al.*, 1994), and *in vitro* ZR protein synthesis has been shown to be more responsive than Vtg synthesis to E_2 (Celius & Walther, 1998). This suggests that the temperature-related differences in E_2 and Vtg observed during the present study might also be reflected in the reduced production of

ZR proteins. The ZR assists in protecting the developing embryo against mechanical damage, desiccation and fluctuating environmental conditions while also exhibiting bactericidal and fungicidal properties (reviewed by Hyllner & Haux, 1995). In consequence, impairment of ZR protein synthesis or ZR assembly is likely to be reflected in lowered egg survival. In the present study, weak but significant correlations were observed between survival and the incidence of egg shell damage (at least in ova from fish held at 14 and 18°C) and the fact that the damage was already present at the point of release from the female genital pore confirms the maternal origin of the phenomenon. However, the possible role of holding temperature as a causal factor is less clear. Here, the limitations of the ova sampling should be acknowledged. Only 10-20 ova were sampled from each female and only one quarter of each ovum was examined, while the numbers and sizes of holes were not evaluated and only their presence or absence recorded. Nonetheless, the incidence of shell damage was lowest and embryo survival highest in ova from fish held at 14°C.

In summary, maintenance of female Atlantic salmon at a water temperature of 22°C during vitellogenesis was associated with a general reduction in plasma E₂ levels and an early reduction in plasma Vtg levels relative to those observed in fish held at 14 and 18°C. A resultant reduction in maternal investment was confirmed by a significant reduction in oocyte diameter in ova from fish held at 22°C while a concomitant increase in the incidence of previously undescribed chorion damage indicated possible impairment of zonagenesis as well as vitellogenesis. Elevated temperature may have acted directly at the level of the reproductive endocrine cascade or indirectly via the stress axis. The net effect was a significant reduction in the viability of ova from fish exposed to high temperatures during vitellogenesis.

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Table I Mean ($\pm$ SEM) diameter (mm), mean fertility (%) and mean survival to the eyed stage (%) of ova from female Atlantic salmon maintained at 14, 18, or 22°C (C.L. = confidence limit). Figures with the same superscript are not significantly different ($P > 0.05$).

	Temperature Regime (°C)		
	14	18	22
Mean Egg Diameter (mm)	5.760 ^a	5.668 ^a	5.396 ^b
SEM	± 0.056	± 0.032	± 0.058
Mean Egg Fertility (%)	93.4 ^a	86.5 ^a	69.4 ^b
Upper 95% C.L.	100.00	97.11	99.95
Lower 95% C.L.	75.38	36.40	16.69
Mean Egg Survival (%)	85.6 ^a	84.5 ^a	41.9 ^b
Upper 95% C.L.	95.74	88.90	98.30
Lower 95% C.L.	30.43	16.76	0.11

Table II Proportion of egg batches from female Atlantic salmon maintained at 14, 18, or 22°C which contained damaged ova and mean ($\pm$ SEM) incidence of damage in those egg batches. Egg batch refers to the ova from a single female. Figures with the same superscript are not significantly different ($P > 0.05$).

	Temperature Regime (°C)		
	14	18	22
Proportion of Damaged Egg Batches (%)	27.3 ^a	60.9 ^b	38.5 ^{ab}
Mean Incidence of Damaged Eggs (%)	32.6 ^a ± 5.1	31.9 ^a ± 3.0	38.4 ^a ± 7.8

FIGURE LEGENDS

- FIG. 1. Cumulative percent ovulation in female Atlantic salmon maintained at 14°C (○), 18°C (&), or 22°C (').
- FIG. 2. Mean ($\pm$ S.E.M.) plasma levels ($\text{ng}\cdot\text{ml}^{-1}$) of 17 β -estradiol (E_2) and testosterone (T) in female Atlantic salmon maintained at 14°C (○), 18°C (&), or 22°C ('). At each sample time data points with the same superscript are not significantly different ($P>0.05$), $n = 23-29$.
- FIG. 3. Mean ($\pm$ S.E.M.) plasma levels of cortisol ($\text{ng}\cdot\text{ml}^{-1}$) and vitellogenin (Vtg, $\text{mg}\cdot\text{ml}^{-1}$) in female Atlantic salmon maintained at 14°C (○), 18°C (&), or 22°C ('). At each sample time data points with the same superscript are not significantly different ($P>0.05$), $n = 23-29$.
- FIG. 4. Scanning electron micrographs of (a) and (b) the surface of a typical Atlantic salmon ovum displaying protein plugs sealing the pore channels of the zona radiata (scale bar = 40 μm and 25 μm respectively), (c) the micropyle of a typical Atlantic salmon ovum (scale bar = 15 μm), (d) a damaged Atlantic salmon ovum displaying blister-like holes in the chorion (scale bar = 150 μm) and, (e) a damaged Atlantic salmon ovum displaying the detail of a blister-like hole in the chorion (scale bar = 15 μm).