

Temperature and salmonid reproduction: Implications for aquaculture

N.W. PANKHURST* AND H.R. KING**

*Australian Rivers Institute, Griffith University, Gold Coast, Queensland 4212, Australia and **Salmon Enterprises of Tasmania Ltd., P.O. Box 1, Wayatinah, Tasmania 7140, Australia*

*Author to whom correspondence should be addressed: Tel.: +61 7 55528033; fax: +61 7 55528220; email: n.pankhurst@griffith.edu.au

Running Title: Temperature and reproduction in salmonids

Fish reproduction is likely to be affected by increasing water temperatures arising from climate change. Normal changes in environmental temperature have the capacity to affect endocrine function and either advance or retard gametogenesis and maturation, but above-normal temperatures have deleterious effects on reproductive processes. In Atlantic salmon (*Salmo salar*, L.) exposure to elevated temperature during gametogenesis impairs both gonadal steroid synthesis and hepatic vitellogenin production, alters hepatic estrogen receptor dynamics and ultimately results in reduced maternal investment and gamete viability. Exposure to high temperature during the maturational phase impairs gonadal steroidogenesis, delaying or inhibiting the preovulatory shift from androgen to maturation-inducing steroid production. There are also deleterious effects on reproductive development of female broodstock of rainbow trout (*Oncorhynchus mykiss* (Walbaum)) and Arctic charr (*Salvelinus alpinus* L.) when they are exposed to elevated temperature. Less is known about temperature effects on male fish but inhibition of spermiation has been observed in Atlantic salmon and rainbow trout. Among wild stocks, the response to elevated temperature will involve behavioural thermoregulation with consequent change in geographical ranges and the possibility of local extinctions in some regions. For domesticated stocks, containment in the culture environment precludes behavioural thermoregulation and aquaculturists will be required to develop adaptive strategies in order to maintain productivity. The most direct strategy is to manage the thermal environment using one or more of a range of developing aquaculture technologies. Alternatively, there is potential to mitigate the effects of elevated temperature on reproductive processes through endocrine therapies designed to augment or restore natural endocrine function. Studies largely on Atlantic salmon have demonstrated the capacity for synthetic luteinising hormone releasing hormone to offset the inhibitory effects of elevated temperature on maturational events in both sexes, but the potential for hormone therapy to provide protection during gametogenesis is still largely unexplored.

Key words: climate change; temperature; salmonids; steroidogenesis; ovulation; egg viability.

INTRODUCTION

Most temperate teleosts show seasonality of reproduction and this is usually more sharply defined at higher latitudes where there is greater amplitude of seasonal variation, and consequently greater requirement for seasonal synchronisation of reproduction (Pankhurst & Porter, 2003). Among temperate species there is extensive evidence for photoperiod being the proximate driver of the broad seasonal phasing of reproduction, consistent with its capacity to provide an unambiguous 'date' signal (reviewed in Bromage *et al.*, 2001). Temperature has a modifying role, particularly in cueing the precise timing of gamete maturation and spawning, providing the capacity for reproductive cycles to be locally 'tuned' to shorter term and less predictable variations in thermal conditions (Van Der Kraak & Pankhurst, 1997; Pankhurst & Porter, 2003). Salmonids show a strong dependence on photoperiod for entrainment of the reproductive cycle, and in the absence of other variables photoperiod can regulate complete reproductive development (Bromage *et al.*, 2001). However, there is also now good evidence for the role of falling autumn temperature in synchronising final maturation and ovulation in salmonids (Gillet, 1991; Taranger & Hansen, 1993;

Taranger *et al.*, 2000, 2003; King & Pankhurst 2000, 2007), and increasing spring temperatures also increase the rate of a number of processes in gametogenesis (reviewed in King *et al.*, 2003). Studies on salmonids also show that photoperiod and temperature interact in cueing final maturation and ovulation (Davies & Bromage, 2002; Taranger *et al.*, 2003; King & Pankhurst, 2007) confirming the cautionary views of Bromage *et al.* (2001) and Pankhurst & Porter (2003) that consideration of both variables needs to be made in any investigation of environmental regulation of reproduction.

The consequence of response to thermal variation being a part of the normal regulatory process for reproduction in salmonids and other species is that there is also the capacity for variations in temperature arising from existing climate anomalies (eg. Nakano *et al.*, 2004) or climate change occasioned by increasing atmospheric CO₂ (IPCC, 2007) to have an impact on reproduction. Increasing temperatures will at some level have adverse effects on reproduction in fish (Van Der Kraak & Pankhurst, 1997) but these effects are likely to be expressed in different ways for natural, and cultured stocks of salmonids.

Distribution of natural stocks, particularly those with ocean ranges for significant parts of their life cycle, is strongly driven by thermal preference and this preference range sits well inside the envelope of thermal tolerance. For example, Atlantic salmon *Salmo salar* L. have an approximate upper limit of thermal tolerance of 22-24 °C (Barton, 1996), but studies of ocean distribution in the North Atlantic show that fish typically stay between the 4 and 10 °C isotherms (Reddin *et al.*, 2000). Similar data have been reported for sockeye salmon *Oncorhynchus nerka* (Walbaum) and steelhead trout *O. mykiss* (Walbaum), with both species showing preference for quite narrow temperature ranges (Welch *et al.*, 1998a,b). This is supported by experimental approaches that show that Arctic charr *Salvelinus alpinus* L. have a thermal preference ~4 °C lower than the optimum temperature for growth (Larsson, 2005). This suggests that under conditions of ocean warming, scenarios for anadromous populations range from complete retreat from the North Atlantic and Pacific basins, to northward shifts in range but with periods of riverine migration where briefer exposure to elevated temperature (and the thermal tolerance, rather than thermal preference envelope) will occur. In lacustrine populations of species such as Arctic charr or whitefish *Coregonus lavaretus* (Mitchill) the consequences may be more immediate if there is limited scope for movement to deeper, cooler water (Graham & Harrod, 2009), or if stratification due to warming results in oxygen depletion at depth (Wahl & Löffler, 2009). The capacity of populations to persist under these conditions will depend initially on the ability of fish to complete maturation and spawning at higher than normal temperatures, and then the subsequent effects of high temperature on larval and juvenile survival and growth. The precise nature of many of these effects is still not known but it is already clear that elevated temperature has the capacity to impact on all stages of the salmonid life cycle (reviewed by Graham & Harrod, 2009). Some of the likely effects on reproductive processes in natural populations can be extrapolated from the effects of increased temperatures on reproduction in cultured stocks. Cultured stocks in turn present a different problem in that they are constrained in their capacity to behaviourally avoid elevated temperatures and their response is dictated by the thermal tolerance envelope. The temperature range over which these effects will apply will vary with species, with for example Arctic charr having optimal range and upper threshold of 5-16 °C and 22-24 °C respectively, whereas those of rainbow trout *Oncorhynchus mykiss* (Walbaum) are 10-22 °C and 26.5 °C respectively (Barton, 1996).

The focus of this review is to examine the state of knowledge on the effects of abnormally elevated temperatures on reproduction in salmonids, consider priorities for further research, and identify opportunities for climate change adaptation strategies for salmonid aquaculture.

THE ROLE OF TEMPERATURE IN NORMAL REPRODUCTIVE DEVELOPMENT

Salmonids in their natural range are typically autumn and winter spawners with gonadal development (or recrudescence in iteroparous species) commencing in the previous spring and continuing through summer to culminate in ovulation and spermiation in late autumn or early winter. Variations in this pattern occur, most notably in high latitude stocks where spawning may take place in spring or early summer (reviewed in Scott, 1990). Stocks transferred to the Southern Hemisphere maintain this pattern and spawn during austral autumn and winter, 6 months out-of-phase with northern conspecifics. For example, females from Tasmanian stocks of rainbow trout and Atlantic salmon initiate vitellogenesis in late austral spring (November), undergo the bulk of follicular growth through late summer and autumn (February-April) and ovulation occurs in May and June for Atlantic salmon and rainbow trout respectively (Pankhurst *et al.*, 1996; King & Pankhurst, 2000, 2003). Above a certain temperature minimum, a thermal stimulus does not appear to be necessary for the initiation and progression of gametogenesis, but vitellogenesis does proceed more quickly as temperature increases (Korsgaard *et al.*, 1986; Tyler *et al.*, 1987; Olin & Von Der Decken, 1989; MacKay & Lazier, 1993). These effects were recorded across temperature shifts of 3-10 °C, 0-25 °C, 8-16 °C, and 9-15 °C respectively, suggesting that increasing temperature can have a permissive effect on the rate of oocyte growth and development, provided that the temperature shift sits within the normal thermal range of the species concerned. There is less information on the potential for temperature to affect gametogenesis in male salmonids. Studies on rainbow trout do show that plasma levels of the gonadal steroids testosterone (T) and 11-ketotestosterone (11KT) were higher in fish held at 12 °C rather than 6 °C (Manning & Kime 1985), suggesting that temperature also has the capacity to act as a permissive factor in male gametogenesis.

Under normal conditions, maturing salmonids experience both declining photoperiod and temperature coinciding with the completion of vitellogenesis and the resumption of oocyte meiosis. Changes in temperature advance or delay maturation. Both Northern (Taranger & Hansen, 1993; Vikingstad *et al.*, 2008) and Southern Hemisphere stocks of Atlantic salmon (King & Pankhurst, 2000) show earliest progression to ovulation at temperatures of 5-8 °C, intermediate times to ovulation at 8-11 °C, and delayed or no ovulation at temperatures above 13-15 °C. Subsequent exposure of fish held at higher temperature to a reduction in temperature to ~8 °C results in resumption of maturation and subsequent ovulation, but with a reduced proportion of fish maturing (Taranger & Hansen, 1993; King & Pankhurst, 2000; Watts *et al.*, 2004). Similar effects were observed in Arctic charr but over the lower temperature range of 5-11 °C (Gillet, 1991). Brook trout *Salvelinus fontinalis* (Mitchill) show the same inhibition of ovulation at high temperature but with a higher upper threshold of 16 °C (Hokanson *et al.*, 1973). The effect is also present in non-salmonids with the high latitude wolfish *Anarhichas lupus* L. showing a delay in progression to ovulation, and subsequent reductions in egg fertility and survival at 12 °C (Tveiten *et al.*, 2001). Time-series data for a natural population of whitefish

Coregonus lavaretus L. show later autumn spawning in warmer years (Wahl & Löffler, 2009) indicating that the phenomenon is also present in natural populations. Earlier studies on a Lake Erie population of coho salmon *Oncorhynchus kisutch* (Walbaum) showed reduced plasma levels of T, the maturational steroid 17,20 β -dihydroxy-4-pregnen-3-one (17,20 β P) and fertility in fish exposed to temperatures 2-4 °C higher than other lake stocks, during the two month prespawning migratory period (Flett *et al.*, 1996).

Studies combining the effects of both shortening photoperiod and decreasing temperature indicate that both advance the timing of oocyte maturation and ovulation, and also that the effects are essentially additive. Taranger *et al.* (2003) reported a further 2-3 week advance in spawning of Atlantic salmon over a 5 week advance occasioned by short photoperiod alone, and King & Pankhurst (2007) observed a 4 week advance under shortened photoperiod, and a further 3 week advance when this was combined with an earlier autumn reduction in temperature. Davies & Bromage (2002) confirmed the dominant role of photoperiod in advancing spawning times in rainbow trout but also reported an additional advance of 3-4 weeks in fish exposed to cool borehole water at 7-9 °C compared with river water fluctuating over a summer range of 6-14 °C. Collectively these studies show that temperature differences associated with normal seasonal change have the capacity to either advance or retard gametogenic and maturational events in the reproductive cycle.

EFFECTS OF EXPOSURE TO ABNORMALLY ELEVATED TEMPERATURE

Many Southern Hemisphere stocks of salmonids experience temperature profiles in both the natural and culture environment that are towards the upper end of their thermal tolerance ranges (King & Pankhurst, 2000, 2007; Pankhurst *et al.*, 1996), and this can have some beneficial effects for aquaculture in terms of increased growth rates and shorter production times (King & Pankhurst, 2007). There is; however, a relatively small thermal window between these temperatures and the higher temperatures at which detrimental effects on reproduction, growth and disease susceptibility begin to appear (Battaglene *et al.*, 2008). It appears that these effects are now increasingly also being reported for northern hemispheres stocks (McClure *et al.*, 2007; Steinum *et al.*, 2008). The need to quantify those effects with respect to reproduction in aquaculture has stimulated a series of studies mainly focussed on female salmonids.

VITELLOGENESIS AND OOCYTE DEVELOPMENT

Exposure of a Tasmanian stock of Atlantic salmon to temperatures of 14, 18 or 22 °C (corresponding to cool, normal and warm austral summer water temperatures respectively) from midsummer until late autumn resulted in reduced egg size at 22 °C and corresponding reductions in egg fertility and survival (King *et al.*, 2003). This was correlated with reduced plasma 17 β -estradiol (E₂) and to a lesser extent, T levels at most sample times at 22° C. Plasma vitellogenin (Vtg) levels were consistently lower in fish at 22 than 14° C through autumn, then showed a marked increase in April (Fig. 1). It was concluded that reduced egg size, fertility and survival was at least partly a result of impaired E₂ secretion, and subsequent hepatic Vtg synthesis and sequestration during critical stages of vitellogenesis. Similar effects have been reported for brown trout *Salmo trutta* L., where reductions in plasma Vtg resulting

from stress were associated with smaller eggs and markedly reduced egg survival (Campbell *et al.*, 1994). E₂ is also a key regulatory factor in chorion assembly through its role in stimulating the hepatic synthesis of zona radiata proteins (ZP) (Oppen-Bernsten *et al.*, 1994; Hyllner & Haux, 1995; Celius & Walther, 1998). There was indirect evidence for a possible effect of elevated temperature on chorion assembly in the study by King *et al.* (2003) in the form of higher incidence of structural abnormalities in the chorions of ovulated eggs at higher temperatures.

Earlier experiments conducted on a Tasmanian stock of rainbow trout showed that exposure to autumn temperatures of 9-15° C resulted in high frequency of ovulation and high egg fertility and survival, whereas survival was considerably reduced at 18 and 21 °C. There was no consistent effect of elevated temperature on plasma levels of T, E₂ or luteinising hormone (LH) (Pankhurst *et al.*, 1996). Chmylevsky (2000) also reported arrest of gonadal development in rainbow trout following exposure to 22-23 °C. This suggests that the observed effects on ovulation and fertility probably arise from effects on maturational events (see next section) and that if there is thermal inhibition of E₂ production of the type seen in Atlantic salmon, then it occurs at temperatures above the ranges tested in these studies.

A subsequent study on Atlantic salmon exposed fish to 14 or 22 °C throughout austral autumn, or 22 °C for 6 weeks followed by 14 °C for the remaining period. Extended exposure to 22 °C resulted in reduced plasma levels of E₂, T and Vtg early in the exposure period (King *et al.*, 2007). Exposure to 22 °C for the first half of autumn only resulted in depressed E₂ then recovery from March onwards but with no reduction in Vtg. Egg size was reduced in both groups exposed to 22 °C but there was a stepwise decrease in fertility and survival (14 °C > 22-14 °C > 22 °C) indicating that there was some scope for recovery if the exposure to high temperature was not maintained. In a following experiment, fish were exposed to 22 °C as before but only for a single month over mid January - mid February, mid February - mid March, or mid March - mid April. Plasma E₂ levels were depressed in all groups exposed to 22 °C but there was some evidence of recovery at later sample times (Fig. 2). In contrast, plasma Vtg levels were depressed in all groups at 22 °C but only during the month of exposure. There was a significant reduction in fertility and survival only in fish exposed to 22° C from mid February - mid March, showing that there was a window of heightened thermal sensitivity coinciding with the period when the main increase in egg volume normally occurs (King & Pankhurst, 2003). This suggests that the principle effect of thermal stress is to reduce the capacity for Vtg to be sequestered into growing oocytes. The limited data available for non-salmonid species indicate that the effects of exposure to elevated temperature are quite consistent across taxonomic groups. Wolffish exposed to 12 °C during ovarian recrudescence had reduced plasma T and E₂ levels (Tveiten & Johnsen, 2001), and the temperature freshwater pejerrey *Odontesthes bonariensis* (Valenciennes) also shows reduced plasma E₂ in females and T in males in response to short term exposure to elevated temperature (Soria *et al.*, 2008).

The mechanisms of thermal inhibition of vitellogenesis and egg growth appear to be associated with impairment of E₂ secretion. Steroidogenic capacity was assessed in ovarian follicles harvested from Atlantic salmon maintained at 14, 18 or 22 °C for up to 3 months during austral autumn, and where the previously reported declines in plasma E₂ Vtg, egg size, fertility and survival were again recorded at 22 °C (Watts *et al.*, 2004). Follicles from fish held at 22 °C showed low capacity to produce E₂ in response to steroid precursors and gonadotropin (hCG), whereas all treatments stimulated E₂ production at 14 and 18 °C. In contrast, follicles from fish at 22 °C

showed high capacity to produce T from both steroid precursors and hCG (Fig. 3). The effect was most marked in follicles from fish sampled in February indicating that a major effect of thermal stress, especially in early to mid vitellogenesis is inhibition of P₄₅₀ aromatase (arom)-mediated conversion of T to E₂. High temperature inhibition of arom has been reported elsewhere, particularly in relation to brain-derived arom activity during sex differentiation in juvenile teleosts (reviewed in Baroiller & D’Cotta, 2001; Devlin & Nagahama, 2002) but there is limited research on the effects of elevated temperatures on gonadal arom in adults. Exposure to temperatures of 20° C or more for 4-8 weeks was found to suppress arom mRNA levels in adult red sea bream *Pagrus major* (Temminck & Schlegel) (Lim *et al.*, 2003).

Thermal effects on estrogen-mediated processes downstream of E₂ synthesis and secretion also occur. Assessment of binding characteristics of hepatic estrogen receptors (ER) in Atlantic salmon using ³H-E₂ saturation binding analysis of hepatic cytosols from fish held at 14, 18 or 22 °C from February to April showed the appearance of a large low-affinity binding component at 22 °C (Watts *et al.*, 2005). This corresponded with the peak period of sensitivity to exposure to 22° C in terms of subsequent egg fertility and survival (King *et al.*, 2007), suggesting that part of the effect arises from reduced ER-ligand binding. Experiments currently in progress show that hepatic expression of genes encoding VtgA and ZPB isoforms is reduced in fish held at 22 °C relative to 14 °C (K. Anderson, A. Elizur [University of the Sunshine Coast, Maroochydore, Queensland], N.Pankhurst & H. King, unpublished data). It is not clear whether the reduction in E₂-dependent gene expression is a direct result of thermal challenge, or results from reduced E₂ synthesis and subsequent ER binding. Plasma T levels were also periodically depressed during vitellogenesis in Atlantic salmon held at 22 °C (King *et al.*, 2003; 2007), indicating that processes other than arom activity are probably also inhibited at high temperature. Studies on non-salmonids show that exposure to elevated temperature can reduce expression of genes coding for gonadotropin-releasing hormone and its pituitary receptor and the LH β-subunit (LHβ) in red sea bream (Okuzawa *et al.*, 2003), and LHβ and the ovarian receptor for follicle stimulating hormone (FSH) in pejerrey (Soria *et al.*, 2008).

FINAL MATURATION AND OVULATION

Early studies showed that exposure to high temperature during autumn delayed or inhibited ovulation. In Arctic charr, the delaying effect was present at temperatures above 8° C, with complete inhibition of ovulation at 11° C (Gillet, 1991). Jobling *et al.* (1995) also found maintenance of Arctic charr at 12 or 16° C for 3 months from mid summer to delay ovulation by 3-4 weeks relative to fish held at 4° C, although here ovulation was not inhibited at the higher temperatures. This may relate to the fact that all fish were exposed to reducing temperature towards the end of the experiment. Delay in ovulation also occurred in brook trout and Atlantic salmon exposed to high temperatures in autumn, but at the higher temperatures of 16, and 13-14 °C respectively (Hokanson *et al.*, 1973; Taranger & Hansen, 1993). In all species, ovulation at higher temperature was accompanied by reduced egg fertility and subsequent survival.

Maintenance of females from a Tasmanian stock of Atlantic salmon at 16 °C from early April (at the completion of vitellogenesis) resulted in complete inhibition of ovulation (King & Pankhurst, 2000). A proportion of the fish held at 16 °C subsequently ovulated within 10 days of the temperature being reduced to 8 °C (Fig. 4). Maintenance at 16 °C was accompanied by lower fertility, egg survival and plasma

E₂ levels, and a delayed peri-ovulatory decline in T and rise in 17,20βP (Fig.4). This indicates that a major effect of exposure to elevated temperature was to delay or inhibit the preovulatory steroidogenic shift from androgen to maturational steroid production, either by inhibiting synthesis, release or signal transduction of LH, or suppression of 20β-hydroxysteroid dehydrogenase (20βHSD) activity, preventing the conversion of precursors to 17,20βP. Similar results are reported for Northern Hemisphere stocks of Atlantic salmon where exposure of post-vitellogenic fish to 14-16 °C completely inhibited ovulation in females, and also spermiation in males (Taranger *et al.*, 2003). Plasma 17,20βP levels showed preovulatory peaks in females held at cooler temperatures, but remained at low levels in fish maintained at 14-16 °C. In contrast, 17,20βP levels in males were only slightly higher in lower temperature groups than at high temperatures (Taranger *et al.*, 2003) suggesting that events other than 17,20βP suppression were having an impact on reduced spermiation at higher temperature. Inhibitory effects on males have also been reported in rainbow trout where exposure to 18 °C for up to 3 months resulted in reduced milt volumes (Billard & Breton, 1977).

A study on female rainbow trout (Pankhurst & Thomas, 1998) showed similar effects to those described for Atlantic salmon. At 12 °C, treatment of post-vitellogenic fish approximately one month in advance of the normal time of ovulation with an injection of luteinising hormone releasing hormone analogue (LHRHa) resulted in ovulation of all fish in advance of controls. LHRHa was ineffective at 18 °C but did stimulate ovulation when a second injection was given 23 days after the first. Fish treated with LHRHa at 12 °C showed the expected decline in plasma E₂, transitory peak in T and pre-ovulatory surge in 17,20βP. This response was absent at 18 °C after the first LHRHa injection but present after the second injection. The effect of exposure to elevated temperature was interpreted as being due to either initial lack of pituitary responsiveness to LHRHa, or ovarian incapacity to synthesize 17,20βP. In contrast to Atlantic salmon, the effect of exposure to elevated temperature was to delay rather than inhibit maturational capacity and this may relate to the higher upper limits for thermal tolerance in rainbow trout.

MANAGEMENT OF THERMAL STRESS IN AQUACULTURE

Options for management of broodstock in the face of potential thermal stress fall into several (non-exclusive) groups. The first is complete isolation of broodstock from thermal stress either in sea cages where conditions permit relocation to higher latitudes or greater depth, or maintenance in land based systems where temperature control is possible. Sea cage management will be constrained by reliable access to cooler water and will probably only be possible in the medium term. Nevertheless, such a management response may represent an opportunity to apply technologies such as submersible cages or floating closed containment systems. There is already a precedent for the use of submersible cages for management of the thermal environment for salmonids with a significant driver for their development being the need to avoid winter ice in regions such as Atlantic Canada (reviewed by Beveridge, 2004). The alternative of floating closed-containment technology is as yet largely unproven as a grow-out technology but could provides additional opportunities for environmental control such as drawing cooler influent water from depth (DFO, 2008). Land-based options are more capital intensive but ultimately offer the maximum level of thermal security. Owing to the high value of the brood stock concerned, this is the

approach currently being pursued by the operators of the Tasmanian Atlantic salmon farming industry's selective breeding program (H.King – pers.comm.). However, scale and site constraints mean that this option may not be available to a significant proportion of the propagation sector of the global salmonid production industry.

A second option involves thermal protection but only for critical periods of the reproductive cycle. The identification of a critical window of sensitivity for Tasmanian stocks of Atlantic salmon from mid-February to mid-March (King *et al.*, 2007) offers the possibility of thermal protection only being necessary for a short period. There is some risk associated with this approach in that it assumes that there will be no shift in the window of sensitivity in the face of thermal variations leading up to the period of protection. The limited data available for other stocks and species in relation to possible variation in timing of periods of high thermal sensitivity mean that the capacity to exploit this effect more widely as a management option remains largely unexplored.

A third option involves the uses of exogenous hormone treatment to offset the inhibitory effects of thermal stress on endocrine processes, either alone or in combination with temperature manipulation. There is equivocal evidence for how effective this strategy might be. Treatment of pre-ovulatory Atlantic salmon maintained at 6, 11 or 18 °C with the des-Gly¹⁰ [D-Ala⁶] ethylamide analogue of LHRH (LHRHa) at 25 µg kg⁻¹ body weight resulted in rapid synchronised ovulation at 6 and 11 °C, but no response at 18 °C. This was associated with rapid rises in 17,20βP levels at 6 and 11 °C but no plasma changes at 18 °C (King & Pankhurst 2004a). In a later experiment, fish were exposed to either 11 or 16 °C for a month prior to the normal timing of ovulation, then injected three days apart with LHRHa, 17-hydroxyprogesterone (17P), or a combination of the two. Some fish held at 16 °C were also exposed to a ramp down to 11 °C over the injection period (King & Pankhurst, 2004b). Fish held at 16 °C throughout were unresponsive to any treatment but in fish exposed to temperature ramp down there was advanced ovulation in response to LHRHa and to a lesser extent, 17P (Fig. 5). Fish exposed to a combination of ramp down temperature and LHRHa also had fertility that was as high as fish in all groups at 11 °C, indicating that LHRHa did have the capacity to restore fertility in fish previously exposed to high temperature. Increased plasma levels of 17,20βP in response to both LHRHa and 17P indicated that this effect was associated with both pituitary responsiveness to LHRHa and the 20βHSD mediated conversion of steroid precursors to 17,20βP. Similarly, improved protection was reported for a Norwegian stock of Atlantic salmon held at cold (6.9 °C), normal (10.6 °C) or high temperature (14.3 °C) during autumn and treated with an injection of microspheres of the D-Ala⁶, Pro⁹ NEt analogue of LHRH at a dose of 50 µg kg⁻¹ (Vikingstad *et al.*, 2008). In this experiment, treatment with LHRHa was effective at synchronising and advancing ovulation in fish from all temperature groups, suggesting that LHRHa therapy can offset the inhibitory effects of high temperature on maturational events, if the dose is relatively high, and delivery is sustained. However, the deleterious effects of temperature were not completely overcome with reduced survival to the eyed egg stage in the warm-LHRHa group compared to most other groups. Collectively these studies suggest that LHRHa can have protective effects on maturational events in thermally stressed fish, but also that the dose, choice of analogue and time of exposure to LHRHa are likely to be important, and that the protective effect is likely to be higher if combined with a reduction in temperature.

There has been less attention to the potential for endocrine manipulation earlier in the reproductive cycle. If as available data suggest, one of the primary effects of

exposure to elevated temperature during vitellogenesis is to inhibit the production of E₂, then there may be scope to manipulate E₂ levels for critical periods. This may be achievable by direct administration of E₂ by implant or in food, or by treatment with doses of LHRHa below the threshold to trigger maturational events. LHRHa is primarily assumed to increase plasma levels of LH. For example, Breton *et al.* (1998) found that rainbow trout treated with 40 µg kg⁻¹ D-Arg⁶ LHRHa showed no effect in terms of changes in plasma levels of FSH, but predictable and marked increases in plasma LH, with the largest effect being seen in fish at the end of vitellogenesis. However, *in vitro* studies by Dickey & Swanson (2000) showed that LHRH can stimulate both the expression of FSHβ subunit mRNA and the release of FSH into the medium in perfused pituitary preparations from coho salmon. The means that fish at early stages of maturity may have the capacity to respond to stimulation with LHRHa with an increase in both the synthesis and release of FSH. Under these conditions, follicles will respond with increased production of E₂ and subsequent increased vitellogenin and egg shell protein synthesis by the liver, provided that aromatase activity is not compromised. LHRHa may also stimulate E₂ production even without activation of FSH synthesis and release. In both cyprinids (Van Der Kraak *et al.*, 1992) and salmonids (Swanson, 1991), FSH and LH are approximately equipotent in the stimulation of the synthesis of estrogen and androgen synthesis *in vitro*, and LHRHa does generate initial increases in plasma E₂ *in vivo* in Atlantic salmon prior to subsequent increases in T and then 17,20βP (King & Pankhurst, 2007). Crim *et al.* (1986) reported a similar effect of LHRHa in advancing the vitellogenic state of a Canadian stock of Atlantic salmon. As noted earlier, the scope for the use of LHRHa to ameliorate thermal inhibition of vitellogenesis in salmonids is essentially unknown. Studies on non-salmonids do show that treatment with LHRHa can have a protective effect on the maintenance of vitellogenic follicles in captive fish stocks where stress effects would otherwise generate gonadal regression (Hodson & Sullivan, 1993; Mylonas & Zohar, 2001; Corriero *et al.*, 2007), and in some species at least, LHRHa is capable of stimulating full gonadal maturation and ovulation in fish that were previtellogenic at the time of treatment (Morehead *et al.*, 1998). This suggests that investigation of the protective effects of LHRHa in thermally stressed salmonids at earlier stages of reproductive development is a sensible line of enquiry.

In summary, there is now increasing understanding of the inhibitory effects of elevated temperature on both gametogenesis and maturational events in female salmonids. In contrast, there is very little knowledge of the effects of elevated temperature on male reproductive development and fitness, and still considerable uncertainty about the level(s) at which inhibitory mechanisms operate in both sexes. The capacity of the aquaculture industry and managers of natural stocks to respond to challenges posed by increasing water temperatures will depend in large part on a more complete understanding of those mechanisms and effects.

References

- Baroiller, J-F. & D’Cotta, H. (2001). Environment and sex determination in farmed fish. *Comparative Biochemistry and Physiology* **130C**, 399-409.
- Barton, B.A. (1996). General biology of salmonids. In *Principles of Salmonid Aquaculture* (Pennell, W. & Barton, B.A., eds), pp.29-95. Amsterdam: Elsevier.
- Battaglione, S., Carter, C., Hobday, A.J., Lyne, V. & Nowak, B. (2008). *Scoping study into adaptation of the Tasmanian salmonid aquaculture industry to potential*

- impacts of climate change*. National Agriculture and Climate Change Action Plan: Implementation Programme Report, 84p, Hobart: Tasmanian Aquaculture and Fisheries Institute.
- Beveridge, M.C.M. (2004). *Cage Aquaculture, 3rd Edition*. Blackwell Publishing Ltd. Oxford, UK. 368pp.
- Billard, R., & Breton, B. (1977). Sensibilité à la température des différentes étapes de la reproduction chez la truite arc-en-ciel. *Cahiers Laboratory Montereau* **5**, 5-24.
- Breton, B., Govoroun, M. & Mikolajczyk, T. (1998). GTH I and GTH II secretion profiles during the reproductive cycle in female rainbow trout: relationship with pituitary responsiveness to GnRH-A stimulation. *General and Comparative Endocrinology* **111**, 38-50.
- Bromage, N., Porter, M. & Randall, C. (2001). The environmental regulation of maturation in farmed finfish with special reference to the role of photoperiod and melatonin. *Aquaculture* **197**, 63-98.
- Campbell, P.M., Pottinger, T.G. & Sumpter, J.P. (1994). Preliminary evidence that chronic confinement stress reduces the quality of gametes produced by brown and rainbow trout. *Aquaculture* **120**, 151-169.
- Celius, T. & Walther, B.T. (1998). Oogenesis in Atlantic salmon (*Salmo salar* L.) occurs by zonagenesis preceding vitellogenesis *in vivo* and *in vitro*. *Journal of Endocrinology* **158**, 259-266.
- Chmielevsky, D.A. (2000). Effects of extreme temperature on oogenesis in tilapia and rainbow trout. In *Proceedings of the 6th International Symposium on the Reproductive Physiology of Fish* (Norberg, B., Kjesbu, O.S., Taranger, G.L., Andersson, E., & Stefansson, S.O., eds), p. 344. Bergen: John Grieg A/S.
- Corriero, A., Medina, A., Mylonas, C.C., Abascal, F.J., Deflorio, M., Aragón, L., Bridges, C.R., Santamaria, N., Heinisch, G., Vassallo-Agius, R., Belmonte, A., Fauvel, C., Garcia, A., Gordin, H. & De Metrio, G. (2007). Histological study of the effects of treatment with gonadotropin-releasing hormone agonist (GnRHa) on the reproductive maturation of captive-reared Atlantic bluefin tuna (*Thunnus thynnus* L.). *Aquaculture* **272**, 675-686.
- Crim, L.W., Glebe, B.D. & Scott, A.P. (1986). The influence of LHRH analog on oocyte development and spawning in female Atlantic salmon, *Salmo salar*. *Aquaculture* **56**, 139-149.
- Davies, B. & Bromage, N. (2002). The effects of fluctuating seasonal and constant water temperatures on the photoperiodic advancement of reproduction in female rainbow trout, *Oncorhynchus mykiss*. *Aquaculture* **205**, 183-200.
- Devlin, R.H. & Nagahama, Y. (2002). Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. *Aquaculture* **208**, 191-364.
- DFO. (2008). Potential technologies for closed-containment saltwater salmon aquaculture. *DFO Canadian Science Advisory Secretariat Science Advisory Report 2008/001*, 17 pp.
- Dickey, J.T. & Swanson, P. (2000). Effects of salmon gonadotropin-releasing hormone on follicle stimulating hormone secretion and subunit gene expression in coho salmon (*Oncorhynchus kisutch*). *General and Comparative Endocrinology* **118**, 436-449.

- Flett, P.A., Munkittrick, K.R., Van Der Kraak, G. & Leatherland, J.F. (1996). Over-ripening as the cause of low survival to hatch in Lake Erie coho salmon (*Oncorhynchus kisutch*) embryos. *Canadian Journal of Zoology* **74**, 851-857.
- Gillet, C. (1991). Egg production in an Arctic charr (*Salvelinus alpinus* L.) brood stock: Effects of temperature on the timing of spawning and the quality of eggs. *Aquatic Living Resources* **4**, 109-116.
- Graham, C.T. & Harrod, C. (2009). Implications of climate change for the fishes of the British Isles. *Journal of Fish Biology* **74**, 1143-1205.
- Hodson, R. & Sullivan, C.V. (1993). Induced maturation and spawning of domestic and wild striped bass, *Morone saxatilis* (Walbaum) broodstock with implanted GnRH analogue and injected hCG. *Aquaculture and Fisheries Management* **24**, 895-903.
- Hokanson, K.E.F., McCormick, J.H., Jones, B.R. & Tucker, J.H. (1973). Thermal requirements for maturation, spawning, and embryo survival of the brook trout, *Salvelinus fontinalis*. *Journal of the Fisheries Research Board of Canada* **30**, 975-984.
- Hyllner, S.J. & Haux, C. (1995). Vitelline envelope proteins in teleost fish. In *Reproductive Physiology of Fish 1995*, (Goetz, F.W. & Thomas, P., eds), pp. 331-335. Austin: Fish Symposium 95.
- IPCC. (2007). Summary for policymakers. In *Climate change 2007: the physical science basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change*, pp. 1-18. Cambridge: Cambridge University Press.
- Jobling, M., Johnsen, H.K., Pettersen, G.W. & Henderson, R.J. (1995). Effect of temperature on reproductive development in Arctic charr, *Salvelinus alpinus* (L.). *Journal of Thermal Biology* **20**, 157-165.
- King, H.R. & Pankhurst, N.W. (2000). Ovulation of Tasmanian Atlantic salmon maintained at elevated temperatures: implications of climate change for sustainable industry development. In *Proceedings of the 6th International Symposium on the Reproductive Physiology of Fish*, (Norberg, B., Kjesbu, O.S., Taranger, G.L. Andersson, E. & Stefansson, S.O., eds), pp. 396-398. Bergen: John Grieg A/S.
- King, H.R. & Pankhurst, N.W. (2003). Ovarian growth and plasma sex steroid and vitellogenin profiles during vitellogenesis in Tasmanian female Atlantic salmon (*Salmo salar*). *Aquaculture* **219**, 797-813.
- King, H.R. & Pankhurst, N.W. (2004a). Effect of maintenance at elevated temperatures on ovulation and luteinizing hormone releasing hormone analogue responsiveness of female Atlantic salmon (*Salmo salar*) in Tasmania. *Aquaculture* **233**, 583-597.
- King, H.R. & Pankhurst, N.W. (2004b). Effect of short-term temperature reduction on ovulation and LHRHa responsiveness in female Atlantic salmon (*Salmo salar*) maintained at elevated water temperatures. *Aquaculture* **238**, 421-436.
- King, H.R. & Pankhurst, N.W. (2007). Additive effects of advanced temperature and photoperiod regimes and LHRHa injection on ovulation in Atlantic salmon (*Salmo salar*). *Aquaculture* **273**, 729-738.
- King, H.R., Pankhurst, N.W., Watts, M. & Pankhurst, P.M. (2003). Effect of elevated summer temperatures on gonadal steroid production, vitellogenesis and egg quality in female Atlantic salmon. *Journal of Fish Biology* **63**, 153-167.

- King, H.R., Pankhurst, N.W. & Watts, M. (2007). Reproductive sensitivity to elevated water temperatures in female Atlantic salmon is heightened at certain stages of vitellogenesis. *Journal of Fish Biology* **70**, 190-205.
- Korsgaard, B., Mommsen, T.P. & Saunders, R.L. (1986). The effect of temperature on the vitellogenic response in Atlantic salmon post-smolts (*Salmo salar*). *General and Comparative Endocrinology* **62**, 191-201.
- Larsson, S. (2005). Thermal preference of Arctic charr, *Salvelinus alpinus*, and brown trout, *Salmo trutta*: implications for their niche segregation. *Environmental Biology of Fishes* **73**, 89-96.
- Lim, B-S., Kagawa, H., Gen, K. & Okuzawa, K. (2003). Effects of water temperature on the gonadal development and expression of steroidogenic enzymes in the gonad of juvenile red seabream, *Pagrus major*. *Fish Physiology and Biochemistry* **28**, 161-162.
- MacKay, M.E. & Lazier, C.B. (1993). Estrogen responsiveness of vitellogenin gene expression in rainbow trout (*Oncorhynchus mykiss*) kept at different temperatures. *General and Comparative Endocrinology* **89**, 255-266.
- Manning, N.J. & Kime, D.E. (1985). The effect of temperature on testicular steroid production in the rainbow trout, *Salmo gairdneri*, *in vivo* and *in vitro*. *General and Comparative Endocrinology* **57**, 377-382.
- McClure, C.A., Hammell, K.L., Moore, M., Dohoo, I.R. & Burnley, H. (2007). Risk factors for early sexual maturation in Atlantic salmon in seawater farms in New Brunswick and Nova Scotia, Canada. *Aquaculture* **272**, 370-379.
- Morehead, D.T., Pankhurst, N.W. & Ritar, A.J. (1998). Effect of treatment with LHRH analogue on oocyte maturation, plasma sex steroid levels and egg production in female striped trumpeter *Latris lineata* (Latrididae). *Aquaculture* **169**, 315-331.
- Mylonas, C.C. & Zohar, Y. (2001). Endocrine regulation and artificial induction of oocyte maturation and spermiation in basses of the genus *Morone*. *Aquaculture* **202**, 205-220.
- Nakano, K., Takemura, A., Nakamura, S., Nakano, Y. & Iwama, G.K. (2004). Changes in the cellular and organismal stress responses of the subtropical fish, the Indo-pacific sergeant, *Abudefduf vaigiensis*, due to the 1997-1998 El Niño/Southern Oscillation. *Environmental Biology of Fishes* **70**, 321-329.
- Okuzawa, K., Kumakura, N., Gen, K., Yamaguchi, S., Lim, B-S. & Kagawa, H. (2003). Effect of high water temperature on brain-pituitary-gonad axis of the red seabream during its spawning season. *Fish Physiology and Biochemistry* **28**, 439-440.
- Olin, T. & Von Der Decken, A. (1989). Vitellogenin synthesis in Atlantic salmon (*Salmo salar*) at different acclimation temperatures. *Aquaculture* **79**, 397-402.
- Oppen-Berntsen, D.O., Olsen, S.O., Rong, C.J., Taranger, G.L., Swanson, P. & Walther, B.T. (1994). Plasma levels of eggshell zr-proteins, estradiol-17 β , and gonadotropins during an annual reproductive cycle of Atlantic salmon (*Salmo salar*). *Journal of Experimental Zoology* **268**, 59-70.
- Pankhurst, N.W. & Porter, M.J.R. (2003). Cold and dark or warm and light: variations on the theme of environmental control of reproduction. *Fish Physiology and Biochemistry* **28**, 385-389.
- Pankhurst, N.W. & Thomas, P.M. (1998). Maintenance at elevated temperature delays the steroidogenic and ovulatory responsiveness of rainbow trout *Oncorhynchus*

- mykiss* to luteinizing hormone releasing hormone analogue. *Aquaculture* **166**, 163-177.
- Pankhurst, N.W., Purser, G.J., Van Der Kraak, G., Thomas, P.M. & Forteach, G.N.R. (1996). Effect of holding temperature on ovulation, egg fertility, plasma levels of reproductive hormones and *in vitro* ovarian steroidogenesis in the rainbow trout *Oncorhynchus mykiss*. *Aquaculture* **146**, 277-290.
- Reddin, D.G., Helbig, J., Thomas, A., Whitehouse, B.G. & Friedland, K.D. (2000). Survival of Atlantic salmon (*Salmo salar* L.) related to marine climate. In *The Ocean Life of Atlantic Salmon* (Mills D. ed), pp. 88-91. Oxford: Blackwell Science.
- Scott, A.P. (1990). Salmonids. In *Reproductive Seasonality in Teleosts: Environmental Influences*, (Munro, A.D., Scott, A.P. & Lam, T.J., eds), pp. 33-51. Boca Raton, Florida: CRC Press.
- Soria, F.N., Strüssman, C.A. & Miranda, L.A. (2008). High water temperatures impair the reproductive ability of the pejerrey fish *Odontesthes bonariensis*: Effects on the hypophyseal-gonadal axis. *Physiological and Biochemical Zoology* **81**, 898-905.
- Steinum, T., Kvellestad, A., Rønneberg, L.B., Nilsen, H., Asheim, A., Fjell, K., Nygård, S.M.R., Olsen, A.B. & Dale, A.B. (2008). First cases of amoebic gill disease (AGD) in Norwegian seawater farmed Atlantic salmon, *Salmo salar* L., and phylogeny of the causative amoeba using 18s cDNA sequences. *Journal of Fish Diseases* **31**, 205-214.
- Swanson, P. (1991). Salmon gonadotropins: Reconciling old and new ideas. In *Reproductive Physiology of Fish 1991*, (Scott, A.P., Sumpter, J.P., Kime, D.E. & Rolfe, M.S., eds), pp. 2-7. Sheffield: FishSymp 91,
- Taranger, G.L. & Hansen, T. (1993). Ovulation and egg survival following exposure of Atlantic salmon, *Salmo salar* L., broodstock to different water temperatures. *Aquaculture and Fisheries Management* **24**, 151-156.
- Taranger, G.L., Stefansson, S.O., Oppedal, F., Andersson, E., Hansen, T. & Norberg, B., 2000. Photoperiod and temperature affect spawning time in Atlantic salmon (*Salmo salar* L.). In *Proceedings of the 6th International Symposium on the Reproductive Physiology of Fish*, (Norberg, B., Kjesbu, O.S., Taranger, G.L. Andersson, E. & Stefansson, S.O., eds), p. 345. Bergen: John Grieg A/S.
- Taranger, G.L., Vikingstad, E., Klenke, U., Mayer, I., Stefansson, S.O., Norberg, B., Hansen, T., Zohar, Y. & Andersson, E. (2003). Effects of photoperiod, temperature and GnRH α treatment on the reproductive physiology of Atlantic salmon (*Salmo salar* L.) broodstock. *Fish Physiology and Biochemistry* **28**, 403-406.
- Tveiten, H. & Johnsen, H.K. (2001). Thermal influences on temporal changes in plasma testosterone and oestradiol-17 β concentrations during gonadal recrudescence in female common wolfish. *Journal of Fish Biology* **59**, 175-178.
- Tveiten, H., Solevåg, S.E. & Johnsen, H.K. (2001). Holding temperature during the breeding season influences final maturation and egg quality in common wolfish. *Journal of Fish Biology* **58**, 374-385.
- Tyler, C., Sumpter, J. & Bromage, N. (1987). Uptake of vitellogenin into cultured ovarian follicles of rainbow trout. In *Reproductive Physiology of Fish 1987*, (Idler, D.R., Crim, L.W. & Walsh, J.M., eds), p. 221. St. Johns: Memorial University of Newfoundland.

- Van Der Kraak, G. & Pankhurst, N.W. (1997). Temperature effects on the reproductive performance of fish. In *Global Warming: Implications for Freshwater and Marine Fish*, (Wood, C.M. & McDonald, D.G., eds), *Society for Experimental Biology Seminar Series* **61**, 159-176. Cambridge: Cambridge University Press.
- Van Der Kraak, G., Suzuki, K., Peter, R.E., Itoh, H. & Kawauchi, H. (1992). Properties of carp gonadotropin I and gonadotropin II. *General and Comparative Endocrinology* **85**, 217-229.
- Vikingstad, E., Andersson, E., Norberg, B., Mayer, I., Klenke, U., Zohar, Y., Stefansson, S.O. & Taranger, G.L. (2008). The combined effects of temperature and GnRHa treatment on the final stages of sexual maturation in Atlantic salmon (*Salmo salar* L.) females. *Fish Physiology and Biochemistry* **34**, 289-298.
- Wahl, B. & Löffler, H. (2009). Influences on the natural reproduction of whitefish (*Coregonus lavaretus*) in Lake Constance. *Canadian Journal of Fisheries and Aquatic Sciences* **66**, 547-556.
- Watts, M., Pankhurst, N.W. & King, H.R. (2004). Maintenance of Atlantic salmon (*Salmo salar*) at elevated temperature inhibits cytochrome P₄₅₀ aromatase activity in isolated ovarian follicles. *General and Comparative Endocrinology* **135**, 381-390.
- Watts, M., Pankhurst, N.W., King, H.R. & Geraghty, D.P. (2005). Differential effects of temperature and maturity stage on hepatic estrogen receptor characteristics of Atlantic salmon. *Comparative Biochemistry and Physiology* **140A**, 377-383.
- Welch, D.W., Ishida, Y., & Nagasawa, K. (1998a). Thermal limits and ocean migrations of sockeye salmon (*Oncorhynchus nerka*): long-term consequences of global warming. *Canadian Journal of Fisheries and Aquatic Sciences* **55**, 937-948.
- Welch, D.W., Ishida, Y., Nagasawa, K. & Eveson, P. (1998b). Thermal limits on the ocean distribution of steelhead trout (*Oncorhynchus mykiss*). *North Pacific Anadromous Fish Committee Bulletin* **1**, 396-404.

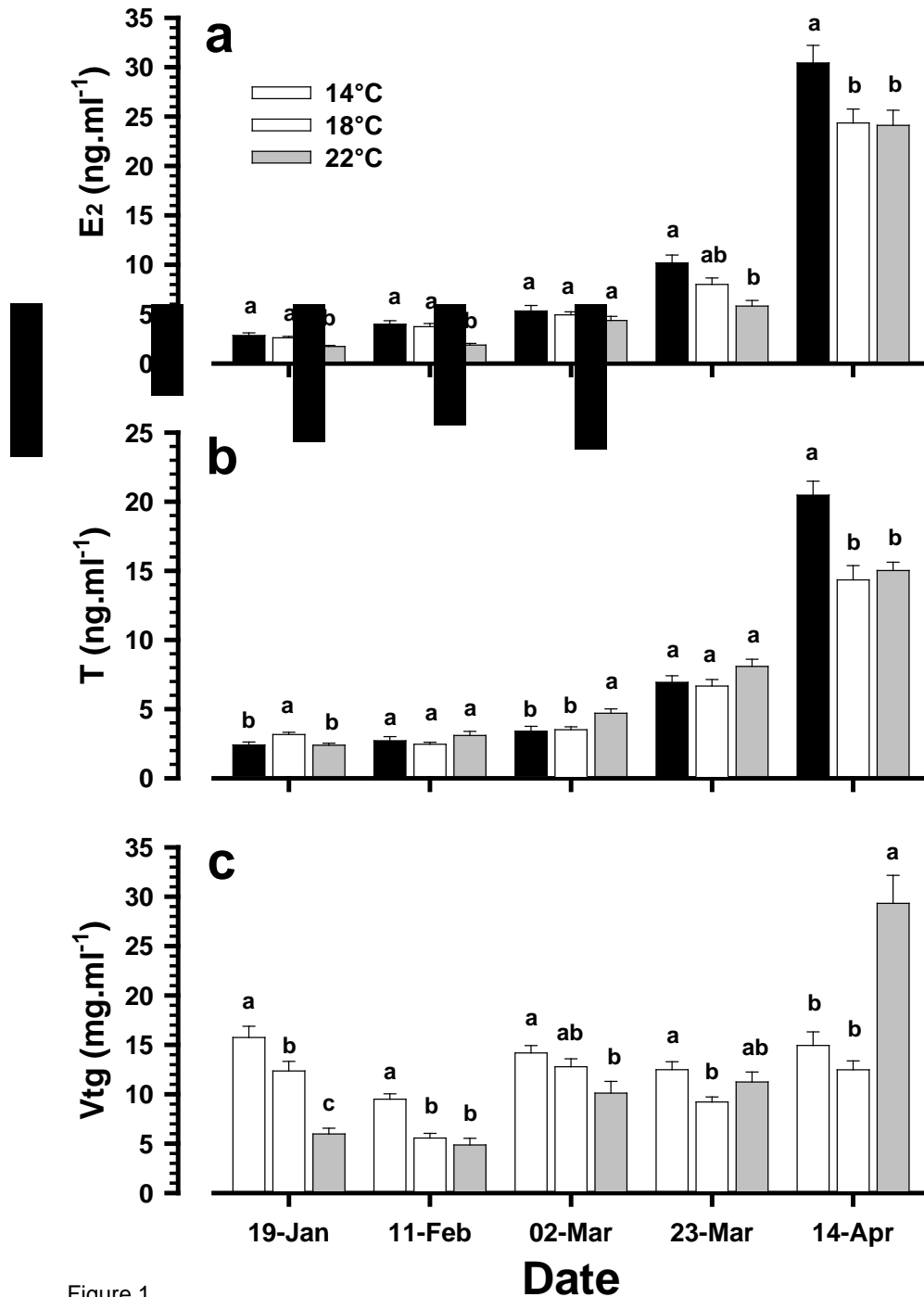


Figure 1

FIG. 1. Plasma levels of E₂, T and Vtg in a Tasmanian stock of Atlantic salmon held at 14, 18 or 22°C for ~3 months prior to the normal time of ovulation. Values at each sample time with common superscripts are not significantly different (P > 0.05). Redrawn from data in King *et al.*, 2003.

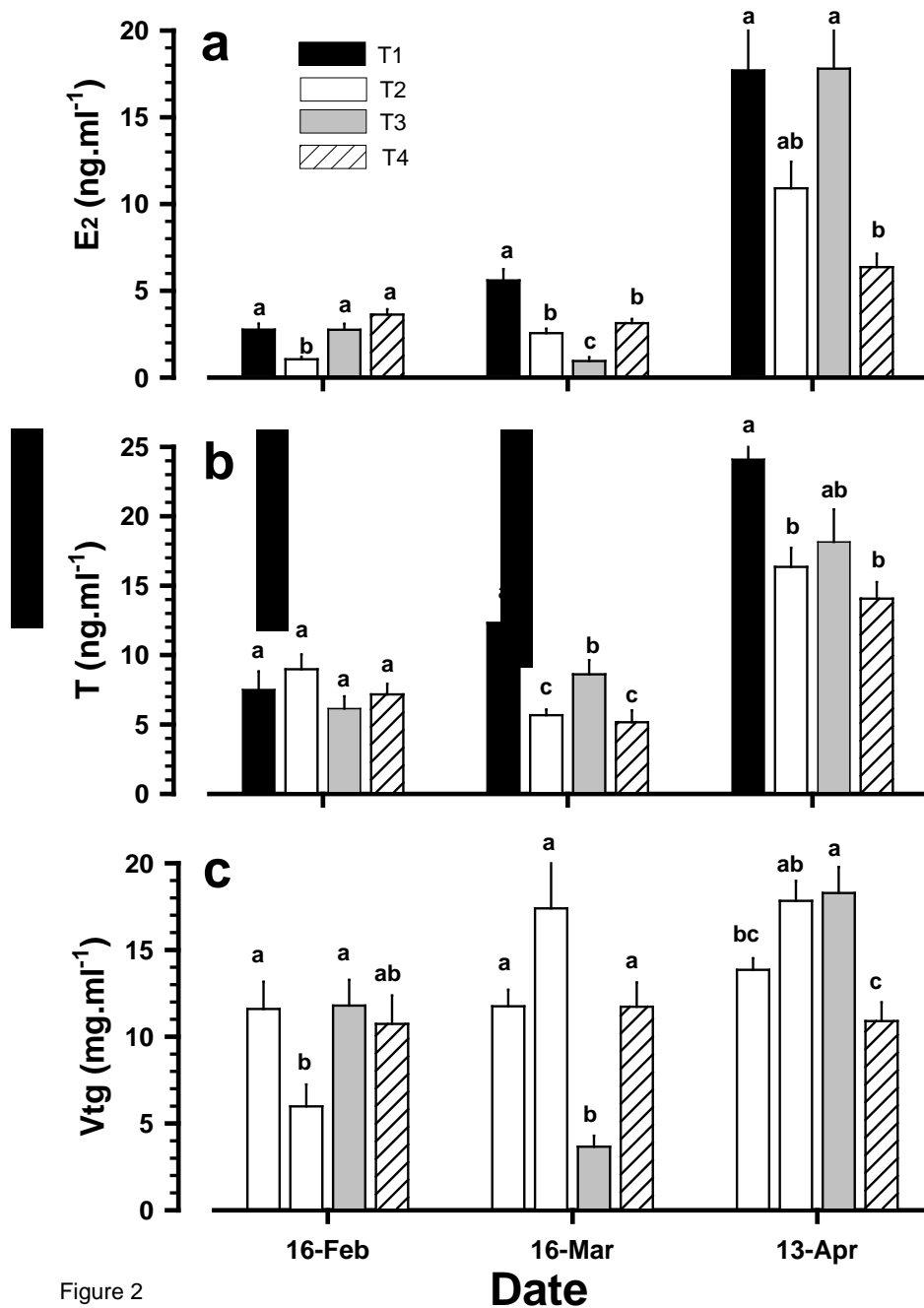


Figure 2

FIG. 2. Plasma levels of E₂, T and Vtg in a Tasmanian stock of Atlantic salmon maintained throughout austral autumn at 14° C (T1), or with 28 days of exposure to 22° C during mid January to mid February (T2), mid February to mid March (T3) or mid March to mid April (T4). Values at each sample time with common superscripts are not significantly different (P > 0.05). From King *et al.*, 2007.

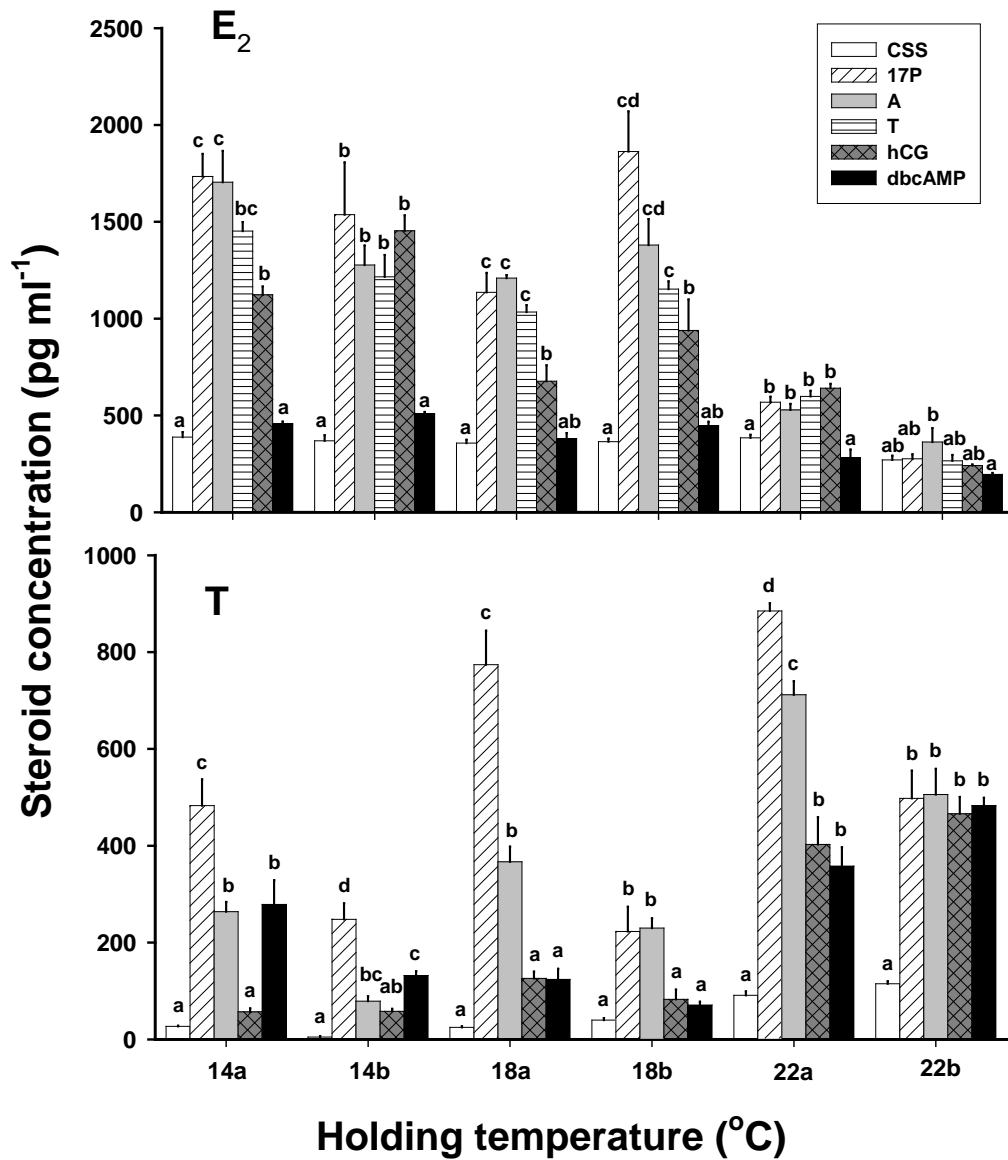


Figure 3

FIG. 3. E₂ and T production by isolated ovarian follicles from Atlantic salmon held at 14, 18 or 22° C during February, in response to incubation with Cortland salt solution (CSS) alone or containing 100 ng ml⁻¹ of 17-hydroxyprogesterone (17P), 10 ng ml⁻¹ androstenedione (A), 10 ng ml⁻¹ T, 100 U ml⁻¹ human chorionic gonadotropin (hCG) or 10 mM dibutyryl cyclic AMP (dbcAMP). Different superscripts (comparisons between treatments for each fish) indicate significantly different means (P < 0.05); ‘a’ and ‘b’ denote duplicate fish for each temperature. From Watts *et al.*, 2004.

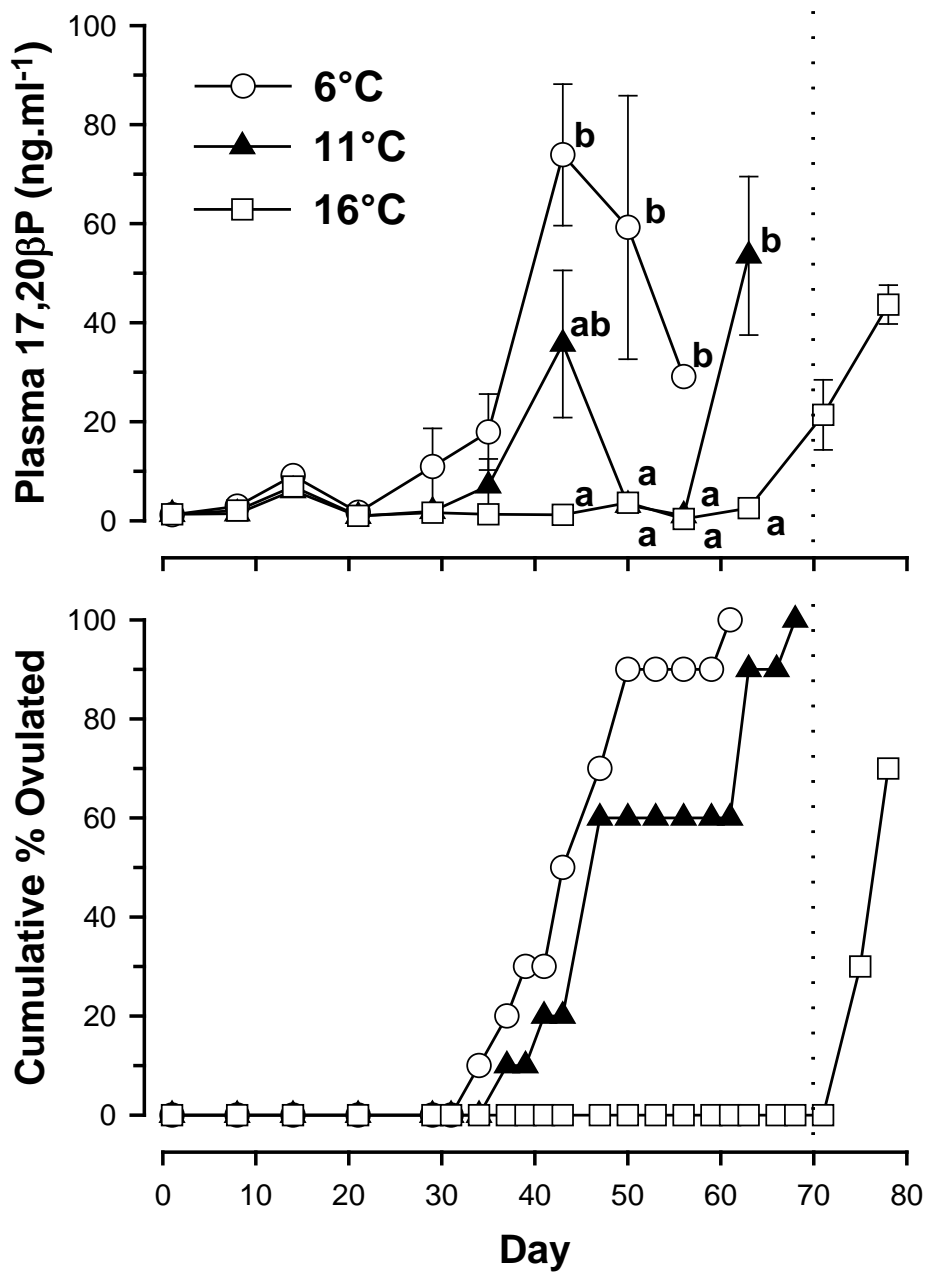


Figure 4

FIG. 4. Plasma levels of 17,20βP and cumulative percentage ovulation in females from a Tasmanian stock of Atlantic salmon held at 6, 11 or 16°C from early April onwards. 17,20βP values at each time with common superscripts are not significantly different ($P > 0.05$). Dotted line indicates reduction of temperature for 16°C group to 8°C. Redrawn from data in King & Pankhurst (2000).

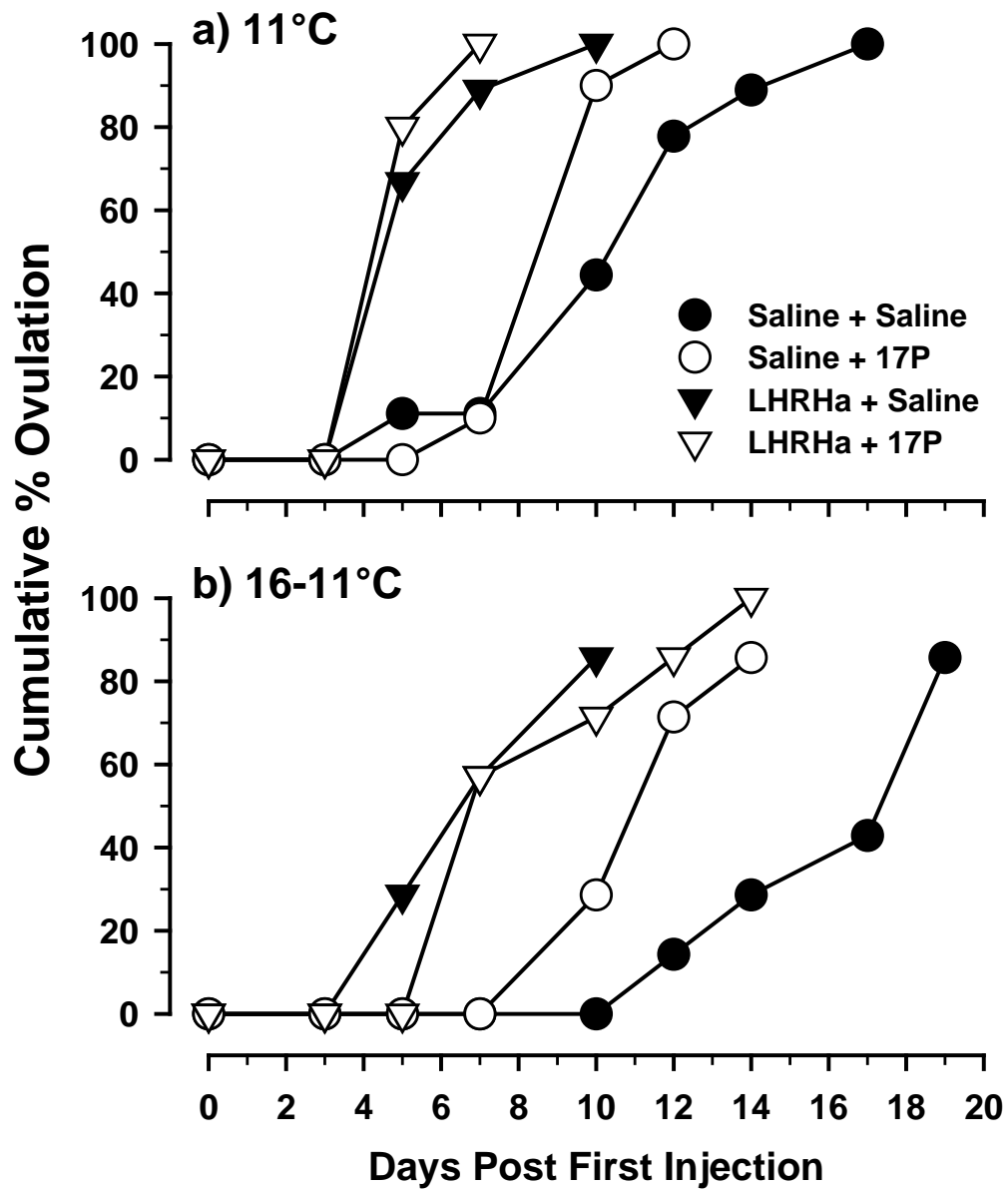


Figure 5

FIG. 5. Cumulative percentage ovulation in females from a Tasmanian stock of Atlantic salmon maintained at 11°C or a reducing temperature profile of 16 to 11°C and treated with saline, 1 mg kg⁻¹ of 17-hydroxyprogesterone (17P) or 25 µg kg⁻¹ luteinising hormone releasing hormone analogue (LHRHa). From King and Pankhurst (2004b).