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Testing the synergistic effects of GnRH and testosterone on the reproductive physiology of pre-adult pink salmon *Oncorhynchus gorbuscha*

G. T. Crossin*†, S. G. Hinch*, S. J. Cooke‡, D. A. Patterson§, A. G. Lotto*, G. Van Der Kraak‖, Y. Zohar¶, U. Klenke¶ and A. P. Farrell**

**Centre for Applied Conservation Research and Department of Forest Sciences, University of British Columbia, Vancouver, BC, V6T 1Z4 Canada, ‡Fish Ecology and Conservation Physiology Laboratory, Department of Biology, Carleton University, Ottawa, ON, K1S 5B6 Canada, §Fisheries and Oceans Canada, Cooperative Resource Management Institute, Simon Fraser University, Burnaby, BC, V5A 1S6 Canada, ‖Department of Integrative Biology, University of Guelph, Guelph, ON, N1G 2 W1 Canada, ¶Center of Marine Biotechnology, University of Maryland Biotechnology Institute, 701 E. Pratt, Baltimore, MD 21202, U.S.A. and **Faculty of Agriculture, University of British Columbia, Vancouver, BC, V6T 1Z4 Canada*

To test the hypothesis that the hypothalmic gonadotropin-releasing hormone (GnRH) and testosterone (T) co-treatment stimulates both the hypothalmo–pituitary–gonadal (HPG) and hypothalmo–pituitary–interrenal axes, the reproductive and osmoregulatory responses of pre-adult pink salmon *Oncorhynchus gorbuscha* were compared after GnRH and T administration either alone or in combination. Relative to controls, neither GnRH nor T treatment resulted in significantly greater ovarian or testicular growth, but co-treatment significantly increased ovarian growth after 5 months. Interestingly, the stimulation was undetectable after 3 months. However, once daily photoperiod began shortening after the summer solstice, *c*. 2 months before the natural spawning date, GnRH+T-treated females were stimulated to produce larger ovaries. Final fish body length and the size of individual eggs did not differ among treatment groups. GnRH+T eggs, however, showed signs of advanced vitellogenesis relative to GnRH-treated and control eggs, whereas T-treated eggs became atretic. Testis size increased significantly from initial values and most males were spermiating, but this growth and development were independent of hormone treatments. Final plasma ion, metabolite and cortisol concentrations did not differ among treatment groups. It is concluded that GnRH+T co-treatment was effective in stimulating female but not male maturation. GnRH and T treatment, however, presumably had little effect on the hypothalmo–pituitary–interrenal axis as observed by ionoregulatory status.

Key words: cortisol; gonado-somatic index; maturation; ovarian development; photoperiod; reproductive hormones.

†Author to whom correspondence should be addressed. Tel.: +1 604 822 1969; fax: +1 604 822 9102; email: crossin@interchange.ubc.ca

INTRODUCTION

Across animal taxa, the initiation of reproductive migrations is often correlated with changes in environmental photoperiods, an increased secretion of hypothalamic gonadotropin-releasing hormone (GnRH), and the onset of gonadal development Dingle, 1996; birds: Dawson *et al*., 2001; Pacific salmon *Oncorhynchus* spp. (Ueda & Yamauchi, 1995; Hinch *et al*., 2006). Although the precise physiologic pathways that regulate the production and release of GnRH and subsequent activation of the hypothalamo–pituitary–gonadal (HPG) axis have yet to be fully elucidated, evidence points to the presence of photo-sensitive circannual oscillators located in the pineal gland, other brain regions and in retinal cells, which entrain photoperiodic cues to regulate the nocturnal secretions of melatonin (Lincoln *et al*., 2003; Falcón *et al*., 2007). By so doing, the relative concentrations of melatonin secreted at different times of year provide animals with an endogenous, hormonal means with which to measure daylight length, or alternatively night length. Migrations therefore usually occur in spring when daily photoperiods are increasing towards a peak at the summer solstice. As a result, the nocturnal secretion of melatonin declines, thus stimulating GnRH cell bodies into reproductive modes, and a cascade of hormonal messengers spill through the HPG axis leading to the production of testosterone (T) and its derivatives, and ultimately to the production of gametes. Steroidal feedback to the central nervous system also occurs, however, eliciting pleitrophic effects on behaviour, which can motivate animals into migratory modes (Dingle, 1996).

Through the adaptive co-ordination of environmental and endogenous systems, animals can modulate their physiology and behaviour in response to seasonally changing selective pressures. In birds, seasonal increases in photoperiod have been correlated with increased secretion of T and the onset of *zugenrhue*, the state of nocturnal restlessness that precedes vernal migration and facilitates premigratory fattening and activity (Dingle, 1996; Wingfield *et al*., 1999). Testosterone has also been correlated with other seasonal behaviours related peripherally to reproduction-like aggression and song (Wikelski *et al*., 1999; Wingfield & Sapolsky, 2003). Although not as well studied, fishes also modulate their physiology and behaviour in response to the change of seasons. Descriptive studies with homing sockeye salmon *Oncorhynchus nerka* (Walbaum) document an effect of increasing seasonal T concentrations in the regulation of migration timing into fresh water (Cooke *et al*., 2006*a*, 2008; Crossin *et al*., 2007, 2009*a*). Furthermore, an experimental test of the effects of GnRH and T on migratory behaviour in lacustrine kokanee salmon (*i.e.* the potamodromous sub-species of anadromous *O. nerka*) led to increases in circulating T concentrations and a premature migration from an open lake environment to a natal inlet stream for spawning (Sato *et al*., 1997; Kitahashi *et al*., 1998*a*, *b*).

Migrations, however, are inherently risky and mortality is a natural outcome. With few exceptions, the HPG axis is inhibited by severe stress (Sapolsky, 2002). Stress activates the hypothalamo–pituitary–adrenal (HPA) axis [or in fishes, the homologous hypothalamo–pituitary–interrenal (HPI) axis], leading to corticosteroid secretion, which mobilizes stored energy to facilitate the appropriate responses to stress but at the same time exerting strong anti-gonadotrophic effects. Thus, the stress response, within limits, is perhaps adaptive by bringing costly anabolic processes such as gamete production to a temporary halt and directing resources to

glycolytic, lipolytic and gluconeogenic metabolism to support immediate survival responses (*i.e.* locomotion). Semelparity (single lifetime opportunities to reproduce) may be an important exception to this paradigm. For example, in *Oncorhynchus* spp. spawning dates are fixed and death follows shortly after, so delayed reproductive development may result in zero fitness. Indeed in *Oncorhynchus* spp., it is well documented that plasma cortisol levels, the principle stress hormone, are secreted in massive quantities during spawning migrations (upwards of *c*. 650 ng ml^{-1}: Robertson *et al*., 1961; Idler & Truscott, 1963; Carruth *et al*., 2000; Cooke *et al*., 2006*a*, *b*; Hinch *et al*., 2006). For most animals, these corticosteroid levels would be more than sufficient to completely inhibit the HPG axis (Sapolsky, 2002). Yet homing *Oncorhynchus* spp. develop gametes in an almost linear trajectory during migration, and cortisol does not generally inhibit reproductive development. In fact, cortisol secretion seems an integral part of both reproductive and osmoregulatory development (Makino *et al*., 2007), and plays a critical role in the programmed death that occurs after spawning, which so characterizes their life history (Pankhurst & Van Der Kraak, 2000; Wingfield & Sapolsky, 2003).

Recent studies with *Oncorhynchus* spp. have suggested that an HPG and HPI cross-axis stimulation occurs during reproductive development through sustained cortisol release, which, in conjunction with prolactin and thyroid hormones, enables a suite of osmoregulatory changes that are necessary for the transition from salt to fresh water during migration (Norris & Hobbs, 2006). Cortisol can provide positive feedback to the HPG axis by enhancing the sensitivity of gonadal receptors to gonadotropin binding (Hirano *et al*., 1990; Seidelin *et al*., 1997; Uchida *et al*., 1997). Cortisol also affects the expression and activity of gill Na^+, K^+-ATPase, the primary osmoregulatory enzyme that is downregulated when transitioning from salt to fresh water (Shrimpton *et al*., 2005; Norris & Hobbs, 2006). The resultant changes in gill membrane permeability and ion-uptake capacity that allow *Oncorhynchus* spp. to retain ions and counter the diluting effects of freshwater entry therefore occur in parallel with reproductive development. Thus, rather than working in opposition, the two axes appear to work collectively in *Oncorhynchus* spp. to ensure an effective entry into fresh water and successful reproductive development for spawning. Cortisol still plays a role in the stress response during migration, but the responsiveness of the HPA axis is probably shifted to a higher set-point to minimize the negative feedback effects of cortisol secretion (Pottinger *et al*., 1995; Carruth *et al*., 2000; Wingfield & Sapolsky, 2003). Again, this may be adaptive in semelparous animals, especially those making long migrations and changing their osmotic environment. Only at times of acute stress does cortisol appears to be anti-gonadotrophic in salmonids (Pankhurst & Van Der Kraak, 2000). For example, the stress associated with swimming upstream through a stretch of exceedingly turbulent rapids (aptly named Hell's Gate) in the Fraser River canyon of British Columbia temporarily depresses circulating concentrations of T and 17β oestradiol (E_2) in adult *O. nerka* (Hinch *et al*., 2006).

Previous studies examining the physiology and behaviour of homing *O. nerka* have uncovered links between reproductive maturity, osmoregulatory capacity and migratory success (Cooke *et al*., 2008; Crossin *et al*., 2009*a*). These studies indicate the activation of HPG and HPI functions. In order to test hypotheses about hormonal regulation of migratory behaviour, homing *O. nerka* were implanted with GnRH either alone or in combination with T and marine travel times compared with

acoustic telemetry through the North Pacific Ocean to the mouth of the Fraser River over 800 km away (Crossin *et al*., 2009*b*). Because fish could not be sampled after hormonal treatment and subsequent migration, it was impossible to say whether observed migratory behaviours (*i.e.* swim speeds) were influenced by HPA cross-axis stimulation in response to GnRH implantation. Thus, the present experiment was conducted to evaluate the temporal pattern of reproductive and osmoregulatory physiology in maturing *Oncorhynchus* spp. held in salt water, in response to GnRH and T implantation. More generally, few studies have investigated the functional interaction of GnRH and sex steroids on reproductive and osmoregulatory physiology (Ando *et al*., 2004), despite numerous studies investigating their individual effects on either or both processes (Sato *et al*., 1997; Dickey & Swanson, 1998; Kitahashi *et al*., 1998*a*, *b*, 2001; Amano *et al*, 1999; Bhandari *et al*., 2003; Taranger *et al*., 2003; Abe & Urano, 2005; Onuma *et al*., 2005; Makino *et al*., 2007). A possible interaction between GnRH and sex steroids can be inferred from studies showing a link between the responsiveness of gonadotropins to GnRH and circulating steroid levels (Ando *et al*., 2004). GnRH and steroidal interactions should thus be examined to fully understand the neuroendocrine control of reproductive development and other processes. Particular attention was paid to the stage of maturity at which pink salmon *Oncorhynchus gorbuscha* (Walbaum) were implanted, as the response to hormonal treatment depends very much on how close to normal spawning dates implantation occurred (Dickey & Swanson, 1998; Antonopoulou *et al*., 1999).

In the present study, the working hypothesis was that GnRH and T would collectively elicit a reproductive and osmoregulatory response. Using pre-adult *O. gorbuscha* held in salt water during the 5 months preceding natural spawning dates, it was predicted that: (1) both males and females co-treated with GnRH and T would have higher plasma T concentrations relative to controls at the end of the experiment, (2) GnRH+T females would have higher E_2 concentrations relative to controls, (3) larger livers and ovaries as a result of E_2-mediated vitellogenesis and finally (4) GnRH+T treatment would stimulate plasma cortisol secretion due not from stress, but from a programmed cross-axis stimulation of the HPI axis as fish matured and prepared for entry to fresh water, an essential component of the maturation process. It was further predicted that other osmoregulatory indicators such as plasma ions or osmolality would not differ between hormonally treated and control fish as all *O. gorbuscha* were expected to maintain ionic homeostasis irrespective of treatment.

MATERIALS AND METHODS

STUDY ANIMALS

The model species for this study was *O. gorbuscha*, an anadromous, semelparous species whose 2 year life history begins when adults spawn in freshwater streams in autumn, then fertilized eggs incubate in redds overwinter and fry emerge in spring at which time they migrate immediately to the ocean. It takes *c*. 6 months from egg fertilization to smolt emigration. Juveniles spend the next 16–18 months rearing and maturing at sea, then adults return to natal streams to spawn (Heard, 1991). In November 2004, fertilized eggs were collected at the Quinsam Hatchery on Vancouver Island, located near Campbell River, British Columbia, and transferred to the Department of Fisheries and Oceans Canada, Pacific Biological Station in Nanaimo, British Columbia. The eggs were incubated in freshwater Heath trays

through to hatching, after which smolts were transferred to large tanks containing ambient sea water pumped from the adjacent Strait of Georgia. Salinity (*c*. 26–32) and temperature (6·7–18·3° C) varied seasonally. Although housed indoors, the facility had windows to allow natural photoperiod (49° 11′ N).

At 15 months, 236 *O. gorbuscha* were transferred to the Centre for Aquaculture and Environmental Research, West Vancouver, British Columbia, for the present study. Fish were divided randomly among four, 3 m diameter, outdoor tanks that were supplied with continuous, ambient sea water (from Burrard Inlet, salinity *c*. 26–32, 8·9–12·3° C). The tanks were large enough to provide room for swimming, and the circular flow allowed fish to hold station at 1·5 body lengths (BL) s^{-1} at the periphery, or less if fish moved towards the centre standpipe. Tanks were covered with netting to prevent avian predation and were partially shaded to reduce the intensity of direct sunlight, but they were effectively exposed to full, natural photoperiod. Fish were, and had been previously, fed daily to satiety with a commercial fish feed (Skretting Canada; www.skretting.ca), usually between 1000 and 1400 hours. Transport permits and veterinary inspections were obtained from the Department of Fisheries and Oceans Canada.

EXPERIMENTAL DESIGN AND HORMONAL DELIVERY SYSTEM

Oncorhynchus gorbuscha were acclimated in the outdoor facility for 3 months (February to April 2006), then the experiment began and conducted for 5 months. Thus, the fish were *c*. 23 months old at the end of the experiment and were within a few weeks of their natural spawning dates. At the start of the experiment on 28 April, fish were individually and randomly removed from tanks by dip-net and transferred to a bucket with aerated, ambient sea water (salinity *c*. 27, 9° C), and anaesthetized in a dilute bath (20 mg l^{-1}) of tricane methane sulphinate (MS-222). Fish then had a passive integrated transponder (PIT) tag implanted in their intraperitoneal cavity. Nose-to-fork length (L_F), post-orbital-to-hypural length (L_{POH}) and body depth (D_B) were measured to the nearest mm, and body mass (M) was recorded to the nearest 0·1 g. To provide a sustained, 8 week delivery of hormones to fish, biodegradable microspheres were prepared with (D-Ala 6, Pro 9 Net) GnRH analogue and T according to a modified solvent-evaporation method (Mylonas *et al*., 1995; Mylonas & Zohar, 2001, Taranger *et al*., 2003). Individual fish received a mass-specific injection of one of four hormonal treatments: (1) 150 μg kg^{-1} GnRHa, (2) 150 μg kg^{-1} GnRHa and 4 mg kg^{-1} T, (3) 4 mg kg^{-1} T or (4) sham injection (saline). Each treatment group was housed in a separate, identical tank to prevent treatment-related social interactions that could exert a disproportionate influence on GnRH regulation in subordinate individuals (Soma *et al*., 1996).

TIME-SERIES BIOLOGICAL SAMPLING

At each sampling date, a minimum of 10 fish per treatment were removed, killed by concussion, their individual identities were determined with a PIT-tag reader and body morphometrics were re-measured. In addition, a 1·5 ml blood sample was then taken from the caudal vein using a 38·1 mm, 23 gauge heparinized (lithium) vacutainer syringe (Houston, 1990). Sampling began at 1000 hours and was usually finished by 1500 hours. Blood samples were immediately centrifuged for 10 min at 6048 *g* to separate plasma, which was pipetted into separate, numbered cryo-vials. In addition, six to eight gill filament tips (*c*. 0·03 g) were clipped from the first gill arch and placed in numbered cryo-vials. Gill and plasma samples were then stored on dry ice for several hours until transfer to a −80° C freezer. Ovaries (M_O) and testes (M_T) were then dissected from the carcass and weighed to the nearest 0·1 g. The sex of each fish was recorded at this time. Dissected ovaries were fixed in Bouin's fixative for 6 h and then transferred to 70% ethanol for at least a week. Livers were also dissected and weighed (M_I) (0·1 g). The gonado-somatic (I_G) and hepato-somatic (I_H) indices were calculated as: $I_G = 100\ M_O\ M^{-1}$ or $100\ M_T\ M^{-1}$ and $I_H = 100\ M_I\ M^{-1}$. Condition factor (K) was calculated for each fish as $K = M\ 100\ L_F^{-3}$. Concentrations of plasma T and E_2 were measured by radioimmunoassay (McMaster *et al*., 1992). Plasma ions (Na^+, Cl^-), glucose, lactate, osmolality and cortisol were quantified by procedures described in Farrell *et al*.

(2001). Gill tissue Na^+, K^+-ATPase activity was not determined because gill samples were damaged by accidental thawing. Ovary samples were embedded in wax, sectioned (3 μm) and stained with haematoxylin and eosin stain. Ovarian morphology was examined with a dissecting scope, and classification of the developing oocytes followed the methods outlined in Tyler & Sumpter (1996).

STATISTICAL ANALYSES

All biological data were examined for normality, and variables were transformed when necessary to reduce heteroscedasticity. MANCOVA was used to explore physiological differences between the sexes while accounting for allometric variation related to size (L_{POH}). Variables examined were: plasma [Na^+] and [Cl^-], plasma osmolality, plasma lactate and glucose. Sex-specific variables were excluded from this analysis: [T] and [E_2] (females only). If the sexes did not differ, variables were pooled in subsequent analyses. Multivariate differences in [Na^+] and [Cl^-], osmolality, lactate and glucose were then examined by treatment group. Because L_{POH}, [T] and [E_2] were expected, *a priori,* to vary by sex, these variables were left out of the model, and treatment differences by sex were explored with univariate models.

All analyses were conducted using JMP 4.0 (SAS Institute; www.sas.com). Because of multiple comparisons, Bonferroni corrections were made to minimize the potential for type II errors. Statistical significance was designated at $\alpha = 0{\cdot}05$. Due to the high conservatism of Bonferroni corrections (Cabin & Mitchell, 2000), however, significance was indicated at $\alpha = 0{\cdot}05$ and $\alpha = 0{\cdot}01$, thus allowing readers to define for themselves the levels that are most biologically meaningful (Cabin & Mitchell, 2000).

RESULTS

SURVIVAL DURING EXPERIMENT

Of the 236 experimental fish, 46% were males and 54% were females. Fish survival over the course of the experiment was 97%. When mortality did occur, it was due to fish jumping out of the tanks ($n = 5$ males and 2 females), with no apparent treatment bias (one GnRH fish, two GnRH+T fish, two control fish and no T fish). Visual examinations did not reveal any overt signs of infection or disease.

SOMATIC GROWTH

After accounting for allometric differences in L_F, male and female *O. gorbuscha* grew at similar rates independent of treatment (K: two-way ANCOVA, sex $P > 0{\cdot}05$, treatment $P > 0{\cdot}05$; L_{POH} covariate $P < 0{\cdot}001$, $n = 227$) (Fig. 1). Growth was slow between April and June, and accelerated between July and September such that final K values were significantly higher in both sexes relative to baseline values (ANCOVA, both sexes $P < 0{\cdot}001$).

PHYSIOLOGICAL AND REPRODUCTIVE RESPONSES TO HORMONAL TREATMENT

Relative to baseline samples, plasma [T], [E_2], I_H and I_G varied little in the GnRH-treated and control females during the 5 month long experiment (final September pooled means ± S.E., [T] = $135{\cdot}2 \pm 46{\cdot}4$ ng ml^{-1}, [E_2] = $13{\cdot}1 \pm 46{\cdot}4$ ng ml^{-1}, $I_H = 1{\cdot}25 \pm 0{\cdot}09$ and $I_G = 2{\cdot}07 \pm 1{\cdot}14$) [Fig. 2(a)–(d)]. In contrast, GnRH+T-treated

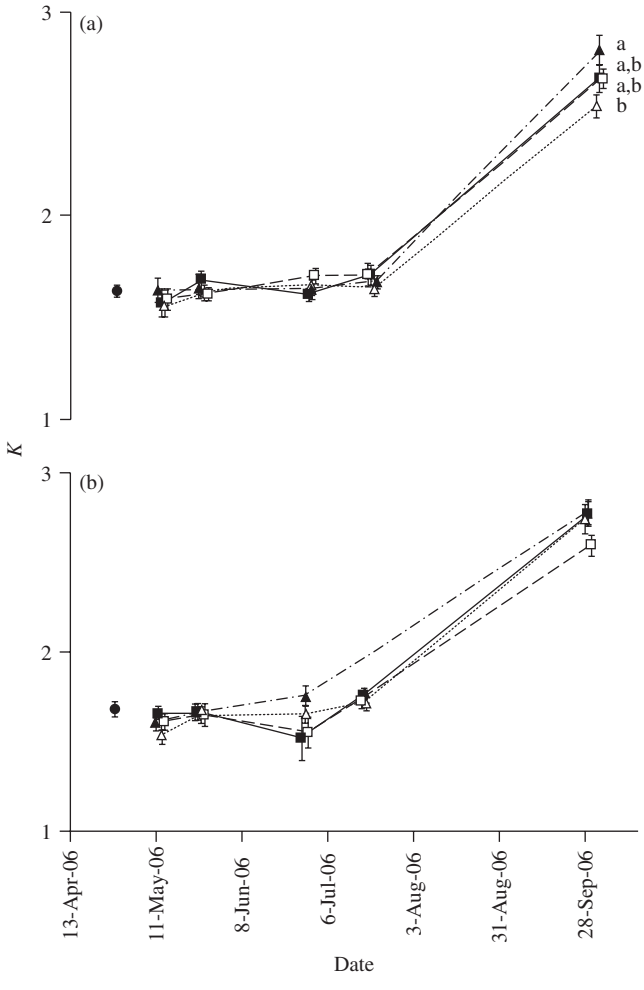

FIG. 1. Temporal response of condition factor (K, calculated from $K = 100\ \mathrm{W}\ L_{\mathrm{F}}^{-1}$, where W is mass and L_{F} is fork length) in (a) female and (b) male *Oncorhynchus gorbuscha* to experimental hormonal manipulation: pre-treatment baseline values (●), controls (■), hypothalmic gonadotropin-releasing hormone (GnRHa) (△), GnRHa + testosterone (T) (▲) and T (□). Values are least-squares means ± S.E. ($n = 5$–8 for each point). Different lowercase letters indicate a statistically significant contrast at $\alpha = 0{\cdot}05$.

and T-treated females showed significant increases in [T] from April to June, but T-treated females did not significantly increase in [E_2], I_{H} or I_{G} at the end of the experiment in September [Fig. 2(a)–(d)]. Despite the similar patterns of I_{H} and I_{G} among all treatment groups to late June, the GnRH+T-treated females showed a significant, positive departure from other groups to an I_{H} of 2·10 and a final I_{G} of 9·88 by late September (ANCOVA, treatment $P < 0{\cdot}001$) [Fig. 2(c), (d)].

In males, [T] increased from baseline values in the GnRH+T-treated and T-treated groups between April and early June [Fig. 2(e)], and both groups were again significantly higher than baseline values in September (ANCOVA, $P < 0{\cdot}05$), but with GnRH+T-treated fish having significantly higher concentrations than the

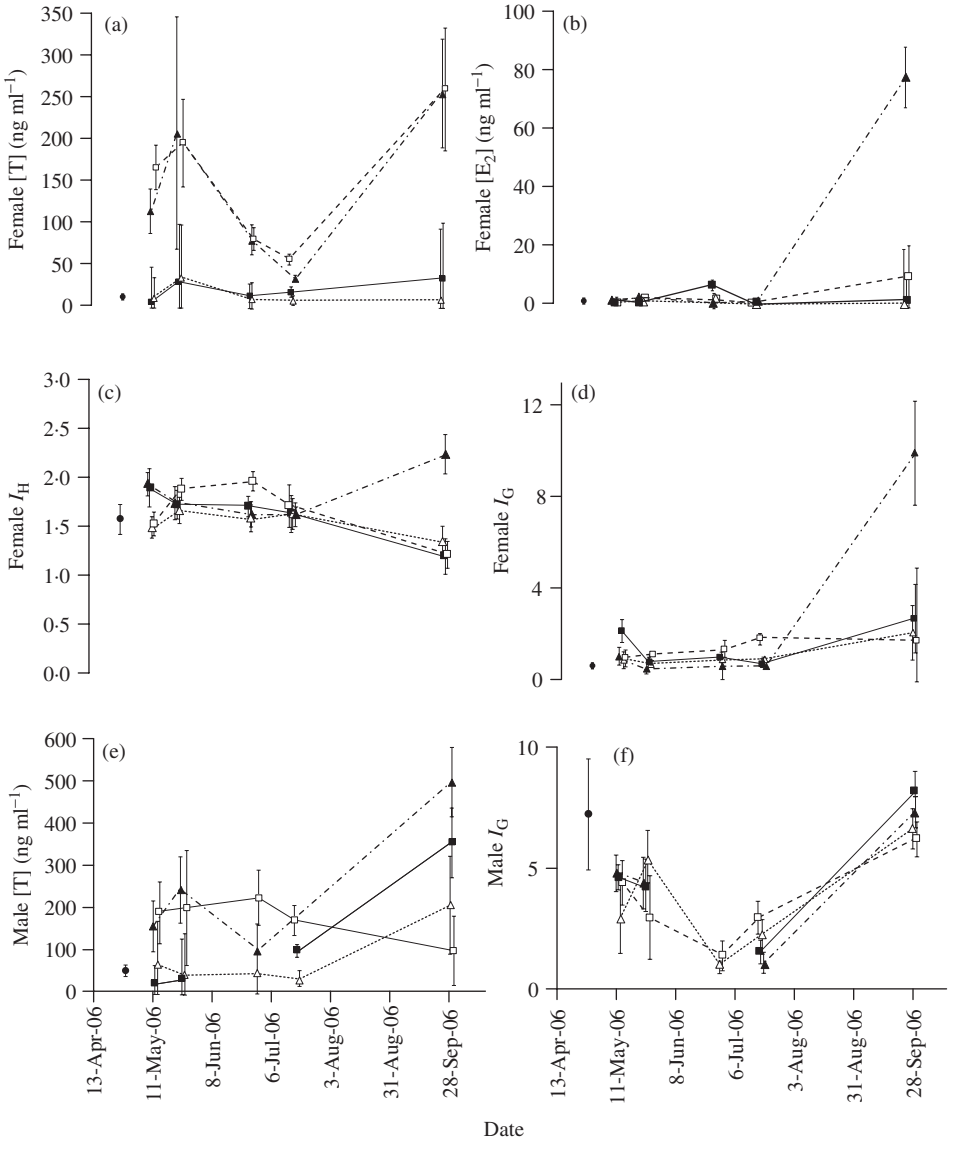

FIG. 2. Temporal response of female *Oncorhynchus gorbuscha* to: (a) testosterone (T), (b) 17β oestradiol (E_2), (c) hepato-somatic index (I_H), (d) gonado-somatic index (I_G) and of male (e) T and (f) I_G to experimental hormonal manipulations [●, pre-treatment baseline values; ■, controls; △, hypothalmic gonadotropin-releasing hormone (GnRHa); ▲, GnRHa+T; (□), T]. Values are least-squares means ± S.E. ($n = 5$–8 for each point).

T-treated fish (ANCOVA, $P < 0{\cdot}001$). Final [T] (mean ± S.E.) among all treatment groups was: GnRH+T = 496·6 ± 82·2 ng ml^{-1}, control = 352·3 ± 82·2 ng ml^{-1}, GnRH = 204·7 ± 116·2 ng ml^{-1} and T = 95·6 ± 82·0 ng ml^{-1}) [Fig. 2(e)]. Male I_G varied little among groups between April (12·0 ± 4·2 g) and mid-July [Fig. 2(f)]. By September, M_T had increased significantly from baseline levels in all groups

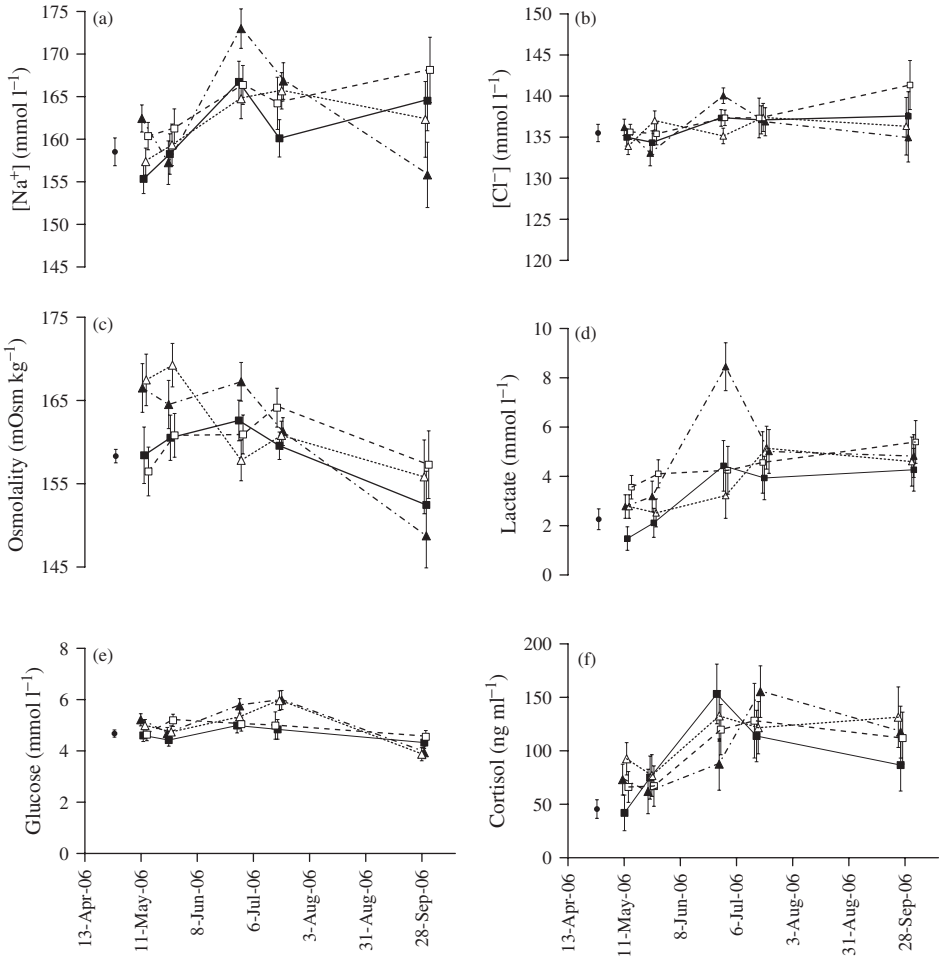

FIG. 3. Temporal response of plasma (a) sodium (Na), (b) chloride (Cl), (c) glucose, (d) lactate, (e) osmolality and (f) cortisol to hormonal treatment in maturing *Oncorhynchus gorbuscha*. Sexes were pooled [●, pre-treatment baseline values; ■, controls; △, hypothalmic gonadotrophin-releasing hormone (GnRHa); ▲, GnRHa + testosterone (T); □, T]. Values are least squares means ± S.E. ($n = 5$–8 for each point).

($P < 0{\cdot}001$), irrespective of treatment (ANCOVA, $P > 0{\cdot}05$, pooled mean ± S.E. $= 28{\cdot}6 \pm 2{\cdot}4$ g), but male I_G had not increased significantly [Fig. 2(f)].

OSMOREGULATORY AND STRESS PHYSIOLOGY

Initial and final plasma [Na^+], [Cl^-] and osmolality values did not differ among treatment groups or sexes (all $P > 0{\cdot}05$) [Fig. 3(a)–(c)]. Final pooled treatment mean ± S.E. values were: [Na^+] $= 162{\cdot}8 \pm 2{\cdot}0$ mmol l^{-1}, $n = 35$; [Cl^-] $= 137{\cdot}6 \pm 1{\cdot}5$ mmol l^{-1}, $n = 35$; osmolality $= 320{\cdot}8 \pm 5{\cdot}0$ mOsm kg^{-1}, $n = 35$).

Plasma lactate, glucose and cortisol also did not differ among treatment groups and sexes at the end of the experiment (all $P > 0{\cdot}05$; pooled mean ± S.E. values

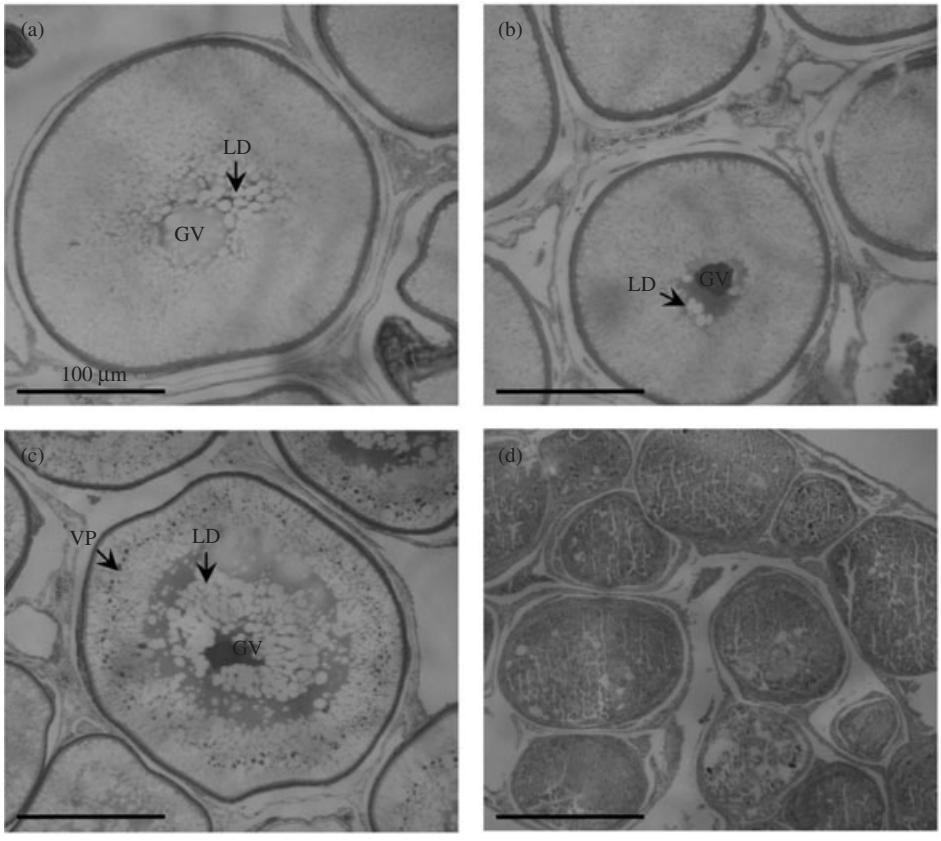

FIG. 4. Representative histological sections from the developing ovaries of maturing *Oncorhynchus gorbuscha*. Samples were collected on the same date (18 July 2006), and the responses of (a) control, (b) hypothalmic gonadotrophin-releasing hormone (GnRH)-treated, (c) GnRH + testosterone (T)-treated and (d) T-treated females are shown. As indicated by the position of the germinal vesicle (GV) and the presence of cortical alveoli (CA), control and GnRH-treated oocytes are in the early stages of vitellogenesis. The additional presence of vitelline plaques shows that (c) GnRH+T oocytes are in a more advanced stage of vitellogenesis. In contrast, (d) T-treated oocytes have been enucleated and are in advanced atresia.

were: glucose $= 4{\cdot}2 \pm 0{\cdot}12$ mmol l^{-1}, $P > 0{\cdot}05$, $n = 35$; lactate $= 4{\cdot}7 \pm 0{\cdot}43$ mmol l^{-1}, $P > 0{\cdot}05$, $n = 35$; cortisol $= 109{\cdot}9 \pm 13{\cdot}4$ ng ml^{-1}, $P > 0{\cdot}05$, $n = 35$) [Fig. 3(d)–(f)]. Final lactate and cortisol but not glucose concentrations increased significantly from baseline levels (both $P < 0{\cdot}01$) [Fig. 3(d)–(f)]. Final glucose concentrations in the GnRH-treated and GnRH+T-treated fish declined significantly from baseline levels ($P < 0{\cdot}05$), but the change was relatively small [Fig. 3(e)].

OVARIAN HISTOLOGY

Mean egg diameters did not differ among treatments at any sampling time. Mean ± S.E. diameter on 18 July for pooled treatments was $4{\cdot}40 \pm 0{\cdot}30$ mm. Ovarian histology at this date is shown in Fig. 4(a)–(d). Control and GnRH-treated oocytes were in the early stages of vitellogenesis, as indicated by the position of the germinal

vesicle and the presence of lipid drops. GnRH+T oocytes were in a more advanced stage of vitellogenesis as indicated by the additional presence of vitelline plaques. In contrast, T-treated oocytes were enucleated and in advanced atresia.

DISCUSSION

Five months after treatment, a significant, positive effect of GnRH+T co-treatment on circulating T and E_2 concentrations was observed in *O. gorbuscha*. Associated with these elevated reproductive hormones were hepatic hypertrophy and increased ovarian size and egg numbers (*i.e.* increased I_G and no change in egg size). GnRH+T co-treatment also led to a significant increase in circulating [T] in males. These results support the first three predictions. Final male I_G did not differ significantly with time or among treatment groups, possibly because male I_G throughout the experiment was at a level commonly observed in mature adult *O. gorbuscha* (McBride *et al*., 1986), and many males were spermiating on the final sample date. These observations suggest that only the GnRH+T females were spurred towards full sexual maturity, and all male groups were capable of spawning irrespective of hormonal treatment.

The hormonal delivery system used in this study was designed to provide a sustained release of hormones over an 8 week period (Mylonas & Zohar, 2001), with depletion then occurring near the summer solstice on 21 June. Ovarian responsiveness was first detectable on the first sampling date after the solstice and increased appreciably 2 months later in September. The initial increases and then decreases in July of circulating concentrations of T and E_2 in the GnRH-treated and GnRH+T-treated females are consistent with the expected delivery of hormones from the microspheres. It is likely that sometime after the solstice, when daily photoperiods began decreasing, a synergistic interaction of GnRH and T led to enhanced ovarian growth in advance of genetically programmed November spawning dates. Histological analysis of the ovaries showed qualitatively that vitellogenesis was active in the developing oocytes of GnRH+T females, suggesting that the high levels of E_2 circulation was due to a synergistic priming of follicle stimulating hormone (FSH) production and secretion. In contrast, GnRH administered alone resulted in oocytes similar to control, *i.e*. early vitellogenesis and less advanced than the GnRH+T oocytes. The distinct signs of oocyte atresia of T-treated females, however, suggest a strong, negative feedback of T at this stage of maturity even though other studies have documented positive feedback to T treatment in juvenile female fishes (Crim & Evans, 1979; Tiwary *et al*., 2002). It is possible that the observed atresia might have been due to a T overdose.

The present results are consistent with a study examining GnRH+T co-treatment on juvenile and pre-vitellogenic white sturgeon *Acipenser transmontanus* Richardson in which long-term T treatment stimulated the accumulation of pituitary gonadotropins but did not affect basal GnRH-induced secretions until more than a year later (Pavlick & Moberg, 1997). Although FSH and LH were not measured in this study, the ovarian responses suggests a GnRH+T-induced accumulation of pituitary gonadotropin (GTH), but release occurred only when other cues (presumably photoperiodic) were perceived by the fish and transduced into the HPG axis. Steroidal treatment in juvenile fishes usually stimulates FSH production and accumulation in the pituitary, but not necessarily its release to the bloodstream [Atlantic salmon

Salmo salar L.; Crim & Peter, 1978: rainbow trout *Oncorhynchus mykiss* (Walbaum); Crim & Evans, 1979; Crim *et al*., 1981; Magri *et al*., 1985: European eel *Anguilla anguilla* (L.); Dufour *et al*., 1983]. But when T was administered with leutinizing hormone releasing hormone (LHRH, now known as GnRH) in juvenile *O. mykiss*, both pituitary and plasma GTH concentrations rose (Crim & Evans, 1983). A similar response has been observed in postspawning goldfish *Carassius auratus* (L.) in which GTH production and secretion occurred in response to T and E_2 co-treatment with GnRH, which in effect reversed gonadal regression that usually follows spawning (Trudeau *et al*., 1991).

Despite initial differences in female [T], [E_2], I_H and I_G in the GnRH, T and control groups, final values did not differ among groups, nor were they different from pre-treatment baselines. The lack of response in the GnRH-treated fish suggests a low receptivity to GnRH stimulation in juvenile and maturing *O. gorbuscha*, and the lack of response to T treatment alone is consistent with the known inhibitory effects of steroids on FSH secretion, despite the fact that T treatment can stimulate FSH production in some circumstance (Yaron *et al*., 2003). That no effect of individual GnRH or T treatment on M_O was detected suggests that these female fish would not have likely spawned in November, which is when the population would normally have done so in the wild. It was anticipated that *O. gorbuscha*, with their 2 year life span (Heard, 1991), would have been nearly fully mature by the completion of the experiments. There is evidence, however, that rates of maturation in captive salmonids can become de-coupled from natural cycles (Gross, 1998). Several studies have indicated that critical size and energy thresholds must be attained by specific times of year to induce maturation (Simpson, 1992; Thorpe, 1994; Hopkins & Unwin, 1997). For example, British Columbia *O. gorbuscha* that were accidentally introduced to the Great Lakes in 1956 have evolved a 3 year rather than 2 year life history. It is possible that the relatively low primary production of the lakes, coupled with the impossibility of anadromy, has protracted their life span to allow an effective size-at-maturity.

Nonetheless, GnRH+T co-treatment induced female maturation in this study, whereas independent GnRH and T treatments did not. The case is less clear for males as all groups were spermiating in September and had morphed into spawning forms and colourations despite the lack of differences in M_T among treatment groups.

Throughout the experiment, treated and control fish grew at similar rates, and K did not differ significantly until the end of the experiment, when GnRH+T-treated females, but not males, were heavier at a given length than the other treatment groups. This greater mass at length was probably due to their larger ovaries. GnRH and T treatments, either alone or in combination, had little influence on cortisol secretion, which had increased over time but did not differ among treatment groups. Plasma ions and osmolality did not differ among sexes or treatment groups at any stage of the experiment, suggesting effective homeostasis despite different hormonal treatments.

In conclusion, GnRH in combination with T induced maturation in captive female *O. gorbuscha* held in salt water, but the reproductive response occurred many months after the initial treatment. Presumably, GnRH+T lead to the accumulation of FSH and luteinizing hormone (LH) transcripts in the pituitary gland, which were released only when daily photoperiods became shorter in the weeks or months preceding

natural spawning dates, leading to hepatic hypertrophy and increased vitellogenic deposition to oocytes. Male *O. gorbuscha* did not show a clear response to hormonal treatment, but had I_G throughout the experiment (mean $\pm$ S.E. $= 7{\cdot}1 \pm 0{\cdot}8$) that were comparable with those measured in mature adults upon river entry during spawning migrations (*c*. 5·5: McBride *et al*., 1986). In nature, male salmonids are more likely to mature precociously than females (Foote & Larkin, 1988; Foote *et al*., 1997). Thus, despite tank confinement and their small stature, all were ready to spawn, whereas only the GnRH+T females were ready to do so. During the last 6 months of ocean residency, fishes make 'decisions' about whether to initiate spawning migrations *via* the integration of exogenous (photoperiod and temperature) and endogenous (gross somatic energy and circannual rhythms) cues (Hinch *et al*., 2006). That females in this study appeared to require hormonal intervention to mature, while males did not, suggests that females are more risk averse and perhaps at greater susceptibility to a confinement or tank effect. Ultimately, GnRH and T co-treatment spurred reproductive physiology in males and females, thus supporting predictions 1–3, but little support was found for the idea that GnRH provides cross-axis stimulation of HPA cortisol release (prediction 4).

The stimulation of gill Na^+, K^+-ATPase activity *via* GnRH/T treatment (prediction 5) could not be addressed due to the degradation of samples, but plasma [Na^+], [Cl^-] and osmolality did not differ among treatments, which, not surprisingly, suggests that all fish were maintaining effective homeostasis. Cortisol has been shown to increase Na^+ and Cl^- uptake from the environment and to increase the number of freshwater-type chloride cells in the gills of tilapia *Oreochromis mossambicus* (Peters) (Dang *et al*., 2000). Thus, differences in gill Na^+, K^+-ATPase activities may not have been seen had the samples been processed. Many studies have described a central role of prolactin and arginine vasotocin in freshwater adaptation, especially during the upstream migration of salmonids, hormones that are regulated in part by GnRH (Bhandari *et al*., 2003; Abe & Urano, 2005; Onuma *et al*., 2005; Makino *et al*., 2007). Neither prolactin nor arginine vasotocin were measured in this study due to cost and due to the number of other variables that were required to be measured and the small volumes of plasma available for analysis. These hormones might have allowed a more rigorous assessment of whether any cross-axis stimulation and freshwater adaptation might have occurred.

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