Mitigation of climate change effects on salmon broodstock: effects of estrogen therapy

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Salmon Enterprises of Tasmania

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OBJECTIVES:

1. Development of a protocol for treating maiden fish held at 22^{o}C in autumn with silastic implants containing estrogen to artificially raise plasma estrogen levels.
2. Assessment of the effect of elevated plasma estrogen levels on estrogen-dependent gene expression, plasma vitellogenin levels, egg size fertility and survival.
3. On the basis of the outcomes from objectives 1 and 2, refinement of the treatment protocol for use in larger scale industry settings as a second tier tool for the mitigation of thermal stress in broodstock.

NON TECHNICAL SUMMARY:

OUTCOMES ACHIEVED TO DATE

Successful development of a treatment protocol for artificially elevating blood levels of the steroid hormone estrogen in large, maturing Atlantic salmon broodstock.

Increased understanding of the effects of estrogen on regulating egg growth, development and viability; and the protective effects that it provides against the damaging effects of elevated temperature.

Confirmation of the critical need to protect Atlantic salmon broodstock from higher than normal temperatures during summer and autumn in order to maintain industry egg production in the face of climate change.

Maiden (first spawning) Atlantic salmon were held during autumn at either 14^{o}C (an optimal temperature for ovarian and egg growth and development) or 22^{o}C (a temperature at which ovarian growth and development is inhibited). A third group was maintained at 22^{o}C but given pellet implants containing the steroid hormone estrogen. One of the known effects of exposure of fish to elevated temperature is the suppression of the synthesis of estrogen. Estrogen in turn regulates a number of key steps in egg growth and development. The objective of this study was to determine if the inhibitory effects of high temperature could be offset by artificially maintaining levels of estrogen, with the wider view of developing hormone therapy as an additional tool for the protection of salmon broodstock from the effects of high temperatures during autumn. This is the first attempt to manipulate estrogen levels in maturing adult Atlantic salmon broodstock.

As in a series of previously reported experiments, fish held at 14^{o}C matured normally and produced eggs with high fertility and survival, whereas oocyte growth was inhibited at 22^{o}C with correspondingly reduced fertility and survival. Treatment with slow-release implants containing estrogen was successful at maintaining estrogen levels at 22^{o}C when fish without implants showed the expected fall in estrogen levels. Estrogen therapy also maintained or enhanced ovarian and oocyte growth at 22^{o}C but did not prevent the associated fall in fertility and survival. This shows that while estrogens are essential for oocyte growth, it appears that additional factors also affected by high temperature, are also necessary for maintenance of egg viability.

Studies on Atlantic salmon and other salmon species have shown that an important component of egg viability rests with correct synthesis and assembly of a family of egg shell proteins collectively termed ‘ZP’. ZP synthesis is induced by estrogen and the expectation in the present study was that artificially elevated estrogen would maintain ZP synthesis at high temperature. Measurement of genes coding for the synthesis of ZP in the present study showed that while ZP activation still occurred at 22^{o}C, it was at a lower level that at 14^{o}C and this effect was not offset by estrogen treatment. Estrogen treatment was; however, successful at

inducing gene activity associated with egg yolk formation (and subsequent oocyte growth). The implication is that estrogen treatment alone is not sufficient to protect maturing female salmon from the damaging effects of exposure to high temperature during late summer and autumn. This is an unexpected but important finding and adds to our understanding of the processes involved in the thermal inhibition of reproduction. Further, it emphasizes the need for protection of broodstock from the damaging effects of exposure to high temperature, especially during early autumn, but still leaves us short of a reliable hormone therapy for managing the effects of climate change.

KEYWORDS: Atlantic salmon, thermal stress, estrogen, oocyte growth, egg fertility.

Acknowledgements

This study was supported by a Fisheries Research and Development Corporation TRF Grant with cash and in-kind contributions from Salmon Enterprises of Tasmania, and in-kind contributions from the University of the Sunshine Coast and Griffith University. Thanks are extended Pheroze Jungalwalla and the Salmon Aquaculture Subprogram for continuing advice and support for the research program, to Saltas hatchery staff for assistance with animal husbandry and management, and to the Tasmanian Aquaculture and Fisheries Institute Mount Pleasant Laboratories (Launceston, Tasmania) for preparation of histological material.

Background

Following an extensive series of studies culminating in FRDC project 2008/217 (The effect of temperature on reproductive development in maiden and repeat spawning Atlantic salmon: understanding the basis for improved egg quality and survival) it is now extremely clear that the effect of elevated summer and autumn temperature in inhibiting reproduction in Tasmanian Atlantic salmon broodstock is consistent and potentially profound. Maidens and repeats are both affected but there is evidence that repeats are a little more robust in terms of their capacity to cope at higher temperature. This is offset to an extent by maidens being a slightly better proposition than repeats in terms of egg quality and survival but only provided that they can be kept cool during summer and autumn. The results of these studies are reviewed and reported in Pankhurst and King (2010), and Pankhurst *et al*. (2010a,b).

The inhibitory effects of temperature on reproduction are mediated through the endocrine (hormonal system) system (Pankhurst and King, 2010). All parts of the endocrine chain appear to be potentially affected but a highly sensitive stage is the

aromatase-mediated production of the steroid 17β-estradiol (E_2), by the ovary. E_2 production is a key step in the maturation process of the developing ovarian follicle. E_2 is transported in the bloodstream to the liver where it binds to estrogen receptors (ER) in the hepatocyte cytoplasm. The E_2-ER complex in turn acts as a promoter for expression of the gene or genes coding for synthesis of the phospholipoprotein yolk precursor vitellogenin (Vtg) (reviewed in Watts *et al*., 2003), which is then sequestered into the developing oocyte through a process of receptor-mediated endocytosis (Tyler *et al*., 2000). The second important effect of E_2 is to stimulate hepatic synthesis of precursors of three structural proteins (collectively termed ZP) that will form the *zona pellucida* of the developing oocyte, and subsequently the chorion of the mature egg (Tyler *et al*., 2000). The genes coding for ZP are highly sensitive to stimulation by E_2 and ZP appear rapidly in the plasma soon after hepatic exposure to estrogens (Celius *et al*., 2000; Berg *et al*., 2004; Fujita *et al*., 2004). Disruption of E_2 synthesis and subsequent E_2-ER binding in the Tasmanian stock of Atlantic salmon is accompanied by chorionic abnormality, poor fertility and reduced embryonic survival (Pankhurst and King, 2010), suggesting that part of the effect may result from disruption of the expression of important E_2-inducible genes.

Management options for dealing with thermal inhibition of reproduction in Atlantic salmon are currently limited to controlling the holding temperature of broodstock with the caveat that repeats might need less intensive management than maiden fish (or management for a shorter period). This approach is achievable, provided that the temperature-controlled holding capacity is large enough (or never fails). It does not offer a solution for locations where large scale temperature management is not an option. There is still a strong need for a hormone therapy option as an additional management strategy. Our preliminary experiments with juveniles (Anderson, King, Pankhurst, Pankhurst, Ruff and Elizur; unpublished) have shown that juvenile fish remain responsive to estrogen at high temperature (i.e. it is the inhibition of estrogen production rather than the tissue sensitivity to estrogen stimulation that appears to be the main problem). This means that treatment of adults with exogenous (external) estrogen at critical

periods during thermal stress remains a strong candidate for a successful hormone therapy to offset the harmful effects of exposure to high temperature during late summer and autumn.

Our previous experiments have shown that treatment with synthetic gonadotropin releasing hormone (GnRHa) (designed to stimulate the endocrine cascade upstream of the production of E_2), is ineffective at providing protection against thermal insult during vitellogenesis (Pankhurst *et al*. 2010a). GnRHa stimulates production of the animal's own pituitary gonadotropins which in turn stimulate the ovarian production of steroid hormones, including E_2. E_2 is formed at the end of a series of stepwise, enzyme-mediated reactions and as noted earlier, its formation is dependent on the activity of the aromatase enzyme complex (reviewed in Pankhurst, 2008). One interpretation of the lack of effect of GnRHa is that it is effective at stimulating steroid production upstream of E_2 production, but if thermal stress still causes impairment of aromatase activity, there will still be no, or limited augmentation of E_2 production. In this situation, the only remaining option in terms of hormone therapy is the direct administration of exogenous E_2. Steroids are rapidly metablised under normal conditions with plasma half lives as short as 30 minutes, meaning that treatment regimes that require extended elevations of plasma estrogens as here, require the delivery of steroids through sustained-release implants. We have chosen to use the administration of crystalline E_2 in a silastic pellet matrix based on earlier studies on time-release profiles for a range of delivery methods (Pankhurst *et al*., 1986), and our more recent unpublished work showing that the same technique is effective for juvenile salmon. The current study is the first to investigate the use of the technique on full sized, reproductively maturing adult Atlantic salmon and is only possible due to the access provided to significant numbers of maturing broodstock by the industry partner Saltas.

Need

Strategic R & D Plan and Priority -Salmon Aquaculture Subprogram:

As a result of FRDC project 2008/217 (The effect of temperature on reproductive development in maiden and repeat spawning Atlantic salmon: understanding the basis for improved egg quality and survival) and a series of earlier studies we know that:

- The effect of elevated summer and autumn temperature in inhibiting reproduction in Tasmanian Atlantic salmon broodstock is consistent and potentially profound.
- Maidens (first spawning fish) and repeats are both affected but repeats are more robust in terms of their capacity to cope.
- Maidens are a slightly better proposition than repeats in terms of egg quality and survival but only provided that they can be kept cool during summer and autumn.

The inhibitory effects of temperature on reproduction are mediated through the endocrine (hormonal) system. All parts of the endocrine chain appear to be potentially affected but by far the most sensitive stage is the aromatase-mediated production of estrogen by the ovary. Our experiments have shown that management of aromatase inhibition is a key step in offsetting the effects of high temperature. Management options are currently limited to managing temperature of broodstock. This approach assumes that the temperature-controlled holding capacity is large enough (or never fails). There is still a strong need for a hormone therapy option as an additional management strategy. Preliminary experiments with juveniles have shown that the fish stay responsive to external estrogen at high temperature. This means that treatment of adults with estrogen at critical periods is a strong candidate for hormone therapy.

Objectives

1. Development of a protocol for treating maiden fish held at 22^{o}C in autumn with silastic implants containing estrogen to artificially raise plasma estrogen levels.
Objective achieved.
2. Assessment of the effect of elevated plasma estrogen levels on estrogen-dependent gene expression, plasma vitellogenin levels, egg size, fertility and survival.
Objective achieved.
3. On the basis of the outcomes from objectives 1 and 2, refinement of the treatment protocol for use in larger scale industry settings as a second-tier tool for the mitigation of thermal stress in broodstock.
Objective partially achieved. Experiments were completed as planned and estrogen therapy was effective at maintaining oocyte growth at high temperature. However, fertility and survival from estrogen treated fish was low indicating that optimal treatment regimes have yet to be achieved. This is further complicated by the observation that all fish were unintentionally exposed to higher than normal temperatures earlier in the summer before introduction to experimental regimes.

Methods

Fish Husbandry and treatment groups

Three groups of maiden spawning Atlantic salmon broodstock were set up at Saltas Wayatinah on the 17th February 2010 in the following three treatment groups.

Treatment groups:

1. Fish held at 14^{o}C throughout
2. Fish held at 22^{o}C throughout
3. Fish held at 22^{o}C but implanted twice with an estradiol (E_2) pellet.

The initial intention was to set up fish at the beginning of February but set-up had to be delayed by approximately 10 days due to the close approach of a bushfire to both Saltas hatcheries during the first week of February. This delay had no overall impact on the conduct of the project but resulted in unintentional exposure of all experimental fish to a period of higher-than-normal temperatures prior to introduction into the experimental regimes.

Pellets containing E_2 were manufactured in the laboratory by combining 35 mg of crystalline E_2 with 500µl of unpolymerised silastic elastomer (Dow Corning), adding accelerant and then spinning down the silastic mix in 1.5-ml Eppendorf tubes to provide solid cured pellets. Each fish (approximate weight 3.5 kg) received one pellet at each treatment to give a notional dose of 10 $mg.kg^{-1}$, which previous experiments (Pankhurst *et al*., 1986; and authors' unpublished data) have shown elevated E_2 levels to plasma values expected of fish undergoing normal vitellogenesis.

Fish were sampled (n=7) as a baseline sample at the time of set up (17th February; Sample 1), then on 5th March (sample 2), 19th March (Sample 3) and on 9th April (Sample 4); with 7 remaining fish in each group being left to mature through to ovulation and stripping in May-June. Fish in Group 3 were given E_2 pellets at Samples 1 and 2. For implant, fish were anaesthetised in Aqui-S™, a small ventral incision was made with a scalpel, the pellet was inserted using forceps and the wound sealed with medical adhesive (Stoma Powder). The temperature for all fish was ramped down to the pre-spawning, maturational temperature of 8°C, approximately one week before sample 4. Temperature profiles for the treatment groups are shown in Fig. 1.

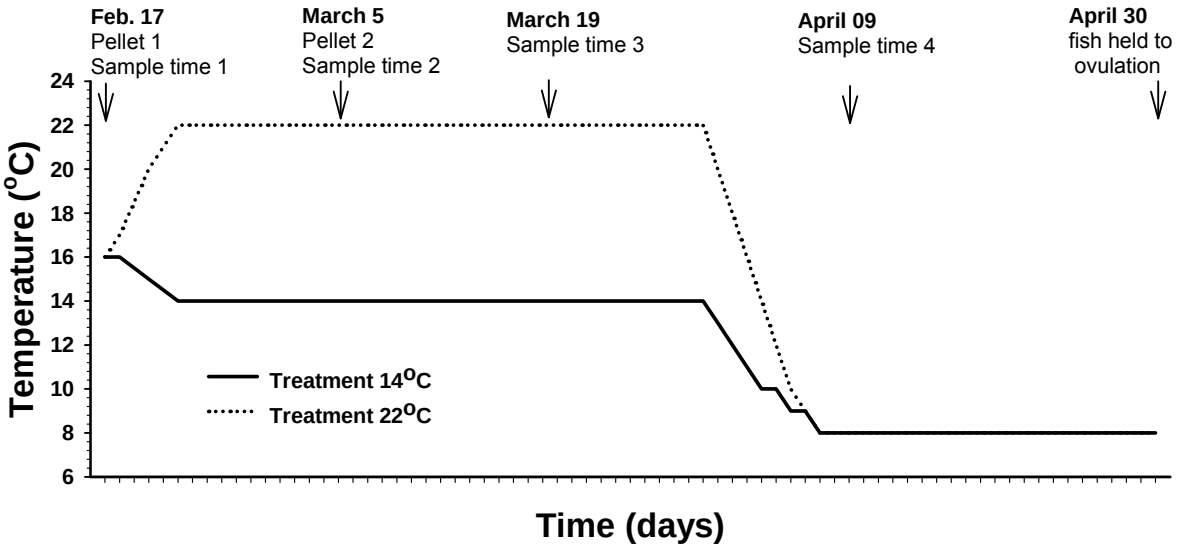

Figure 1. Thermal treatment regimes, hormone pellet implant dates and sampling times for Atlantic salmon spawners held at 14°C and 22°C during the latter stages of ovarian follicle development in the autumn of 2010.

Sampling Protocol

At each sampling time, fish were terminally anaesthetised, weighed and measured, ovaries weighed and samples of ovary and liver were stabilised in RNA Later™ for later analysis of gene expression using real-time RT PCR. A blood sample was taken by caudal puncture and the plasma separated for storage and later analysis of the steroids E_2, testosterone (T) and cortisol by radioimmunoassay, and vitellogenin (Vtg) by enzyme-linked immunosorbent assay. A segment of ovarian tissue was also fixed in 10% neutral buffered formalin for later histological examination, and a weighed sub-sample of ovarian tissue was used for estimation of ovarian fecundity, and also to measure oocyte size.

Gonadosomatic indices (GSI) were calculated as (gonad weight/total body weight) x 100, and condition factor (CF) as (body weight/length^3) x 100. Fecundity was determined by dispersing all ovarian follicles from an ovarian segment of ~5g in teleost saline using 22G needles, and counting all vitellogenic (opaque) follicles

present in the sample. Total fecundity was determined by correction for total ovarian weight and expressed as relative fecundity.kg^{-1} body weight. Mean follicle size was determined by measuring the horizontal diameter of 20 follicles from each fish using an eye piece micrometer fitted to a dissecting microscope. Fixed tissue for histology was dehydrated in an ethanol series, embedded in paraffin wax, sectioned at 5 μm and stained with hematoxylin and eosin.

At ovulation, ova were stripped and fertilised as described in King *et al.* (2003) for measurement of egg size, fertility and survival to the eyed stage at 250 degree-days of incubation. All animal experiments were conducted in accordance with Australian law under ethical approval ENV/01/10/AEC issued by the Griffith University Animal Ethics Committee.

Plasma Steroid and Vitellogenin Measurement

Plasma levels of E_2, T and cortisol were measured by radioimmunoassay in ethyl acetate-extracted plasma using the protocol and reagents for E_2 and T as described in Pankhurst and Carragher (1992), and for cortisol as in Pankhurst *et al.* (2008). Briefly, 100 μl of plasma were extracted with 1 ml ethyl acetate, 100 μl of the extract transferred to assay tubes and dried down before addition of assay buffer, radio-labelled steroid and antibody. Extraction efficiency was determined by recovery of ^{3}H-labelled steroid from replicates of a plasma pool and was 89, 92 and 84% for E_2, T and cortisol, respectively. Assay values were corrected accordingly for extraction losses. Interassay variability was determined by repeat measurement of a pooled internal standard and was (%CV) 4.4, 4.1 and 20 (n=2) for E_2, T and cortisol, respectively. Plasma Vtg levels were measured by enzyme linked immunosorbent assay using the reagents and protocol described in Watts *et al.* (2003). Plasma samples were diluted at 1:1000 in assay buffer for measurement. Interassay variability was assessed by repeat measurement of a Vtg standard from the central part of the assay curve and was (%CV) 8.9% (n=3). Pooled internal standards were not used here due to the tendency of Vtg to denature following repeated freeze-thaw cycles.

Measurement of hepatic gene expression

Methods for measurement of gene expression were described previously in Pankhurst *et al*. (2010a) but are presented here again for reference.

RNA isolation and cDNA synthesis

Total RNA was isolated from hepatic tissue using the Illustra RNAspin Mini kit (GE Healthcare, United Kingdom) according to the manufacturer's protocol, except no more than 15mg of tissue was used. Four hundred nanograms of liver-derived RNA were used to synthesize cDNA for use in polymerase chain reactions (PCRs), and real-time/quantitative PCR (qPCR) using the QuantiTect® reverse transcription kit (Qiagen, Germany). This kit includes a DNA elimination step to remove potential contamination of PCRs by genomic DNA. Following synthesis, cDNA was diluted 5-fold with diethylprocarbonate treated water (DEPC) and stored at -20°C until use.

Partial isolation of ZPC genes

To amplify a fragment of the zona pellucida C (ZPC, equivalent to ZP3, ZPγ and choriogenin L) gene from *S. salar*, the ZPC mRNA nucleotide sequences from rainbow trout (*Oncorhynchus mykiss*, GenBank accession number AF231708), masou salmon (*Oncorhynchus masou*, EU042126) and Java medaka (*Oryzias javanicus*, AY913760) were aligned using the ClustalW2 sequence alignment tool (http://www.ebi.ac.uk/) and degenerate primers were designed from conserved regions (ZPC F1 and ZPCR1, Table 1). The 25 µl PCR reaction contained 17.9 µl PCR grade water, 2.5 µl 10x PCR buffer, 0.5 µl 10mM dNTPs, 2 µl 25mM $MgCl_2$, 0.5 µl 10 µm forward primer, 0.5 µl 10 µm reverse primer, 0.1 µl TAQ (Fisher Biotec, Australia) and 1 µl liver derived cDNA template. Thermal cycling consisted of an initial denaturation step at 94^{o}C for 1 min; followed by 34 cycles of 94^{o}C for 30 seconds (s), 49^{o}C for 30 s and 72^{o}C for 4 min; with a final 10 min extension at 72^{o}C. The PCR product was purified using the QIAquick® PCR purification kit (Qiagen) and cloned using the pGEM®-T easy vector system (Promega, Madison,

WI) according to the manufacturer's instructions. Clones were selected via blue/white screening and successful transformation was confirmed via colony PCRs using M13 primers designed by Promega. PCRs which yielded a product of the expected size were purified (as above) then sequenced by the Australian Genome Research Facility before being submitted to GenBank (accession number GU075906, http://www.ncbi.nlm.nih.gov/Genbank/).

Table 1. Degenerate and gene specific PCR primers

Gene name	Primer name	Sequence (5'→3')	Product size
ZPC	ZPCF1	ACT CCC TSR TCT ACA YCT TCA C	491bp
	ZPCR1	CTG GAA CCT GAA RGC YTC CA	

bp = base pairs. Degenerate base codes: S = GC, R = AG, Y = CT and K = GT

qPCR primer design

Gene specific primers (GSPs) for vitellogenin A (Vtg), zona pellucida B (ZPB, designed to detect all known ZPB genes), ZPC, estrogen receptor α (ERα), and the potential qPCR reference genes: hypoxanthine phosphoribosyltransferase 1 (HPRT1), TATA box binding protein (TBP), elongation factor 1 alpha (EF1α) and beta tubulin (β-tubulin) were designed from species-specific mRNA sequences using Primer3 software (http://frodo.wi.mit.edu/primer3/, Table 2). All primers were designed to have an optimum annealing temperature of 60°C and were supplied by GeneWorks (Australia).

Table 2. qPCR primers

Gene name	Primer name	Sequence (5'→3')	Prod. size	E*	Source sequence
Vtg	VtgF4	AAC TTT GCC CCT GAA TTT GC	95bp	0.984	DQ834857
	VtgR4	GCT CTA GCC AGA CCC TCC TC			
ZPB	ZPBF1	GTTT CCA GGG ATG CCA CTC T	113bp	0.937	AJ000664, AJ000665
	ZPBR1	TGG TAG ATG GCA AAG GCA GA			
ZPC	ZPCF5	GTC CCC CTG CGT ATC TTT GT	121bp	0.969	GU075906
	ZPCR4	AAC CTG TCA CTT TGG CAT CG			
	GPR54R10	GGC AGC CAG GTT AGC TAT GTA			
ERα	ERαF1	AAG CAT GCC GCC TCA GAA AG	150BP	1.003	X89959
	ERαR2	TCC TGT GCT CCA GGT CAC CA			
HPRT1	HPRT1F1	GAT GAT GAG CAG GGA TAT GAC	165bp	0.963	BT043501
	HPRT1R1	GCA GAG AGC CAC GAT ATG G			
TBP	TBPF1	TCC CCA ACC TGT GAC GAA CA	117bp	1.037	BT059217
	TBPR1	GTC TGT CCT GAG CCC CCT GA			
EF1α	EF1αF2	GCA CCA CGA GAC CCT GGA AT	94bp	0.969	AF321836
	EF1αR2	CAC GTT GCC ACG ACG GAT AT			
β-tubulin	βTubF1	CCG TGC TTG TCG ACT TGG AG	144bp	0.975	DQ367888
	βTubR2	CAG CGC CCT CTG TGT AGT GG			

E = efficiency, bp = base pairs.

qPCR validations

qPCRs were conducted on a Rotor-gene 6000 series thermal cycler (Qiagen) using Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen) as the fluorescent label according to the manufacturer's instructions. All primers were tested for specificity via melt curve analysis which consisted of a 90s preconditioning step at 72°C, followed by a temperature gradient up to 95°C at 1°C

per 5s. Following amplification, the size of all qPCR products was determined by running 4 µl of the products on a 2% agarose gel; gene identity was then confirmed through sequencing. Validation curves were carried out in triplicate using serially diluted cDNA as the template. In each qPCR run, negative reverse transcription and no-template controls were analysed to ensure the absence of genomic DNA and other contamination. Reaction efficiencies were automatically calculated by Rotor-gene software version 1.7.87 using the equation: $E = [10^{(-1/M)}] - 1$, where E is equal to efficiency and M is equal to slope. The reaction efficiencies for optimised qPCRs can be found in Table 2.

General qPCR protocol

For determination of the relative expression of target genes, the qPCR reaction generally contained 5 µl SYBR, 0.2 µl 10 µM forward primer, 0.2 µl 10 µM reverse primer, 1.6 µl PCR grade water and 3 µl of diluted cDNA template; although, the amount of template and water was occasionally adjusted to optimise detection. Cycling parameters were as follows:

50°C for 2 min;
95°C for 2 min;
40 cycles of 95°C for 15s;
60°C for 15s, and
72°C for 20s (acquiring).

At the end of cycle 40, a melt curve analysis was always performed. In each qPCR run and for every gene analysed, negative no-template controls and a calibrator sample were included to detect possible contamination, and control for – between-run variability, respectively. The reference gene selected for expression normalisation was TBP as it showed high stability under the experimental conditions. The software Rest 2008, V2.0.7 (http://gene-quantification.com/rest.html) was used to calculate expression of key genes relative to TBP expression for each sample. This software also normalises the data for between-run variance based on variability in calibrator expression.

Statistical analysis

Comparison of means of morphometric and plasma hormone data was made using one-way ANOVA with subsequent comparison of means by Tukeys-b using the SPSS (version 17.0) statistical package. Differences in relative gene expression levels were detected non-parametrically using the Kruskal-Wallis test coupled with Bonferronis Correction to reduce the risk of type 1 error. The P value for significance was set at 0.05 for all analyses.

Results and Discussion

Morphometric Data

There was no significant difference in length or weight of fish in any treatment group (Fig. 2) indicating that experimental outcomes were unaffected by any size bias within groups. Similarly, there was no difference in condition factor among groups but there was a significant increase in gonad mass in fish held at 22^{o}C and treated with an E_2 pellet, at Sample time 3 (Fig. 2). Relative gonad mass (expressed as gonadosomatic index) was elevated in E_2-treated fish at Sample 3, and higher in fish at 14^{o}C and E_2-treated fish at 22^{o}C, than untreated fish at 22^{o}C at Sample 4 (Fig. 3). The same pattern was seen in changes in follicle diameter with an identical pattern of significant difference to that found for GSI (Fig. 3).

There were no significant differences in absolute or relative fecundity between groups at any time (Fig. 4). However, fish from all groups, at all times showed the presence of atretic follicles in the ovary (Fig. 4), including quite high levels in fish held at 14^{o}C at Sample times 1, 2 and 3. This is an unusual finding and has not been observed by us previously, with fish held at 14^{o}C typically showing no, or at least very low incidence of ovarian atresia (Pankhurst *et al*., 2010b). At Sample 3, there were higher levels of atresia in fish held at 22^{o}C and treated with E_2, than in the other 2 groups, whereas at sample 4 there were high levels of atresia, and

high variance between fish in both groups at 22°C. Due to high variances, only the 22°C plus E_2 pellet group was significantly different from 14°C fish. In contrast, fish held at 14°C showed strong evidence of recovery by Sample 4, with only a small proportion of follicles displaying atresia (Fig. 4). Histological characteristics of healthy, and atretic follicles are shown in Figs. 5 and 6, and Fig. 7, respectively.

Examination of hatchery temperature records prior to set up (Saltas temperature data) showed that all fish had been exposed to temperatures ranging from 18-22°C during the early part of February, compared with pre-set up temperatures in previous years and experiments which were not above 17°C. This resulted from both the later set up for the current experiment (partly occasioned by the early February bushfire at Wayatinah) and the generally warmer water temperatures over the 2009/2010 summer. This coincided with the period of vitellogenesis which previous studies have shown to be highly temperature sensitive (King *et al*., 2007) and in consequence, it appears that all fish had experienced some level of thermal insult at the time of establishment of the experimental groups. Fish held at 14°C appear to have undergone significant recovery, whereas in terms of levels of ovarian atresia, those at 22°C did not, irrespective of whether there was the presence of exogenous E_2 or not. This occurred in the face of the apparent growth promoting effects of E_2 on the gonad mass and follicle diameter of fish held at 22°C and treated with E_2 pellets.

Fish held at 14°C began ovulating in early June and the group had completed ovulation three weeks later (Fig. 8). The initiation of ovulation among fish held at 22°C was delayed by one week compared to 14°C, and only 70% of fish at 22°C had ovulated by the 22nd June. The pattern was similar in fish held at 22°C and treated with E_2 pellets, with the additional effect that only 50% of the fish ovulated (Fig. 8). For fish that did ovulate, there were no differences between groups in absolute or relative fecundity (based on measured egg production) (Fig. 9). Egg diameters and volumes were larger in fish held at 22°C plus E_2 pellet than in both other groups, and eggs of fish held at 14°C were in turn larger than those at 22°C (Fig. 9). In contrast, the reverse pattern was found for both egg fertility and survival

to the eyed stage (Fig. 9) with fertility and survival highest at 14°C, reduced at 22°C, and further reduced in the 22°C plus E_2 pellet group. This confirms that the protecting effects of E_2 administration in maintaining oocyte growth at 22°C, did not extend to egg quality, fertility and survival. The implication here is that either more than maintenance of E_2 levels alone is required to maintain healthy ovarian function at high temperature, or the E_2 profiles generated by pellet implant were not adequate or appropriate. These considerations are discussed further in relation to endocrine and gene expression data in the following sections.

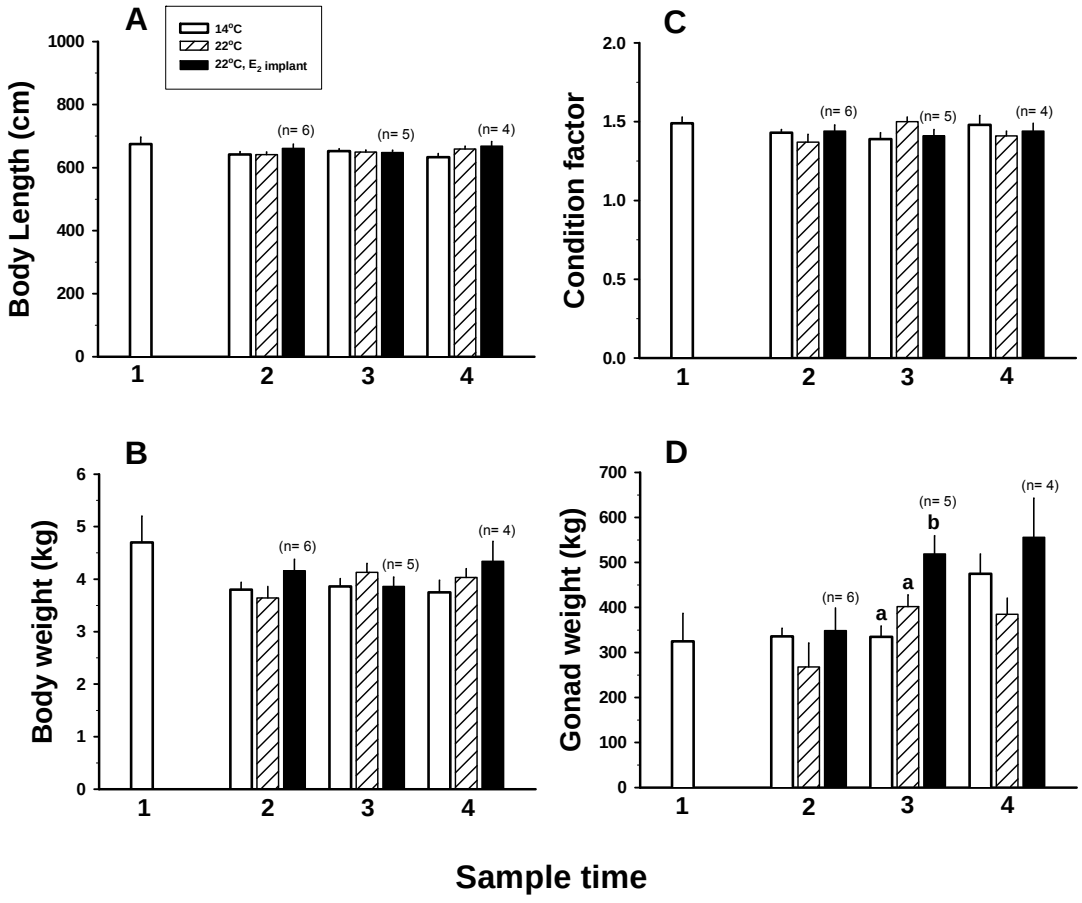

Figure 2. Body lengths, weights, condition factors and gonad weights of maiden Atlantic salmon spawners without hormone pellet implants held at 14°C (open bars) or 22°C (cross-hatched bars), and fish with E_2 pellet implants held at 22°C (black bars). Values are means + SE (n=7 unless stated otherwise). Different alphabetical superscripts at each sample time denote significant differences ($P<0.05$).

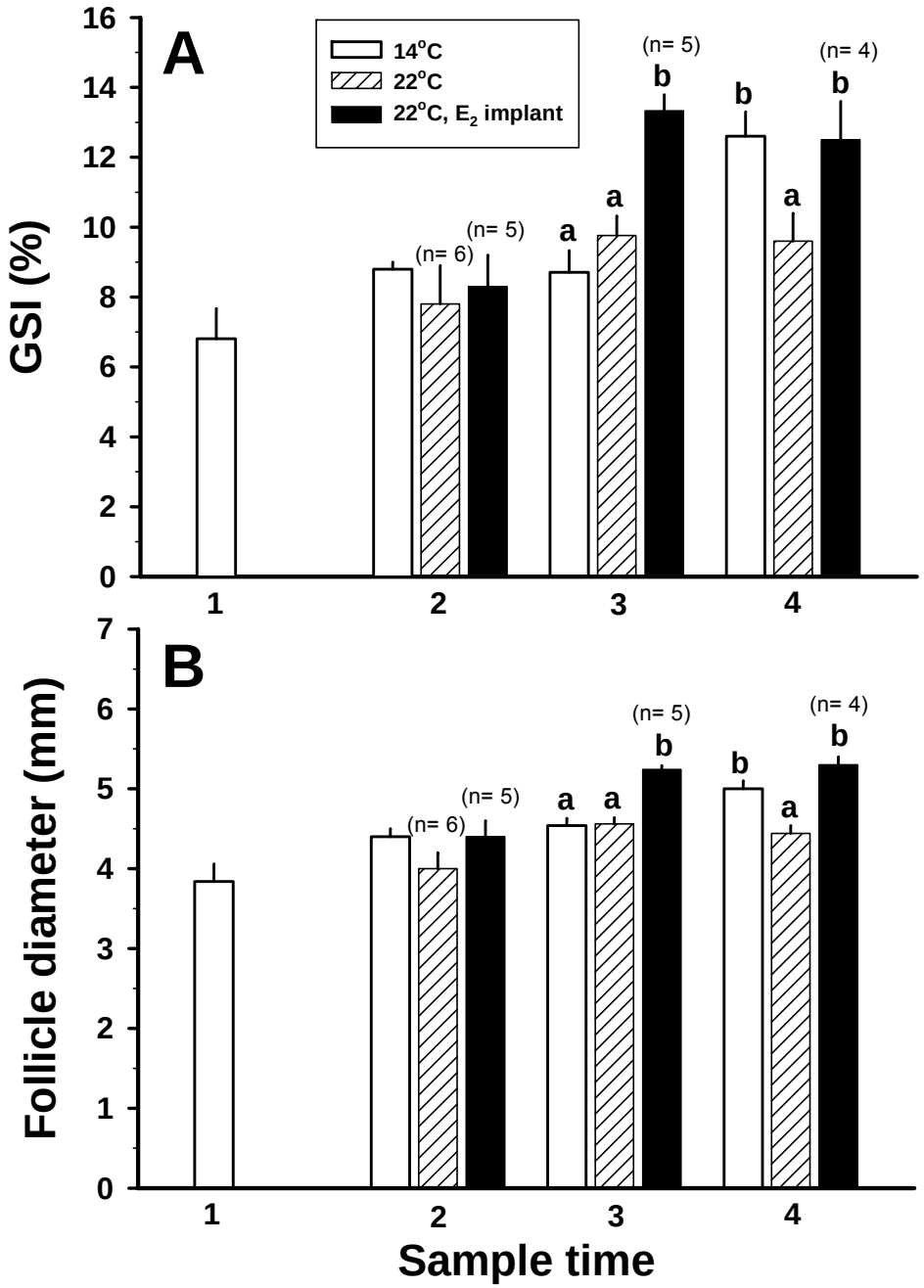

Figure 3. Gonadosomatic index (GSI) and follicle diameters among maiden Atlantic salmon spawners without hormone pellet implants held at 14°C (open bars), or 22°C (cross-hatched bars), and fish with E_2 pellet implants held at 22°C (black bars). Values are means + SE (n=7 unless stated otherwise). Different alphabetical superscripts at each sample time denote significant differences ($P<0.05$).

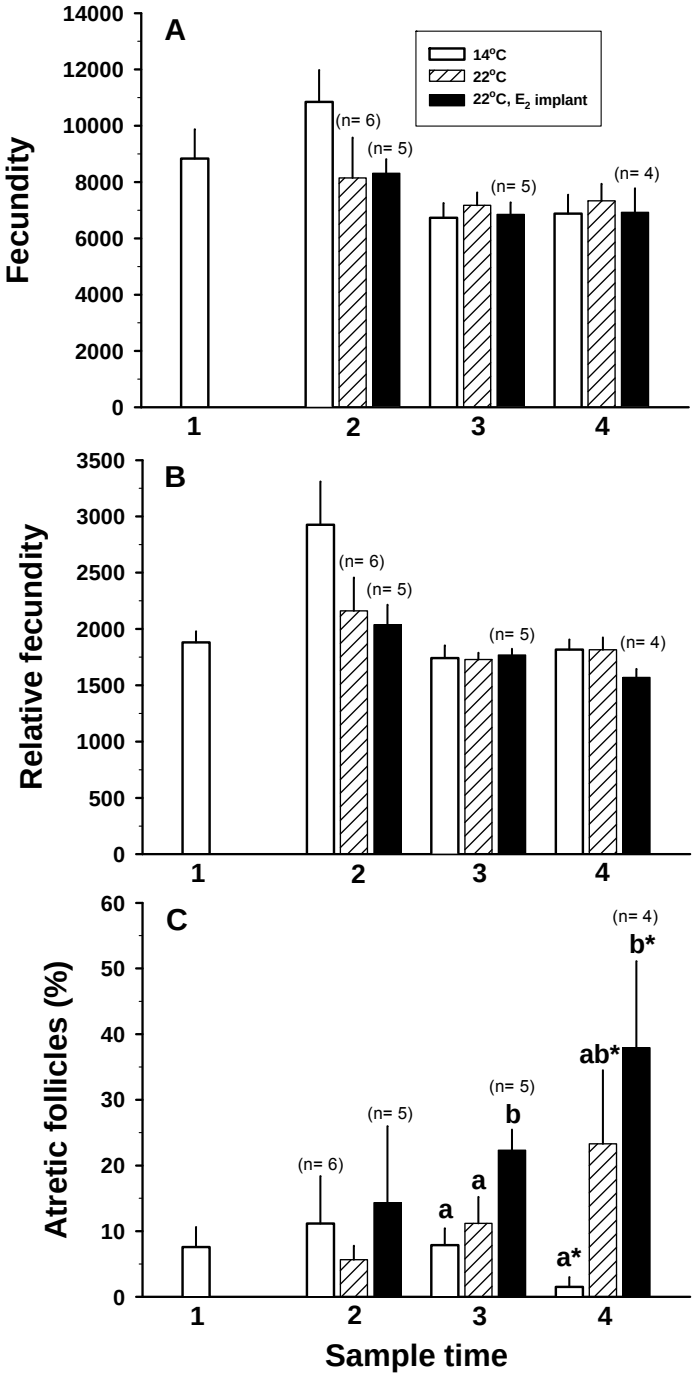

Figure 4. Absolute and relative fecundity and the proportions of atretic follicles in the ovaries of maiden Atlantic salmon spawners without hormone pellet implants held at 14°C (open bars), or 22°C (cross-hatched bars), and fish with E_2 pellet implants held at 22°C (black bars). Values are means + SE (n=7 unless stated otherwise). Different alphabetical superscripts at each sample time denote significant differences ($P<0.05$) except for % atresia at Sample time 4 (*$P<0.057$; $F=3.486$).

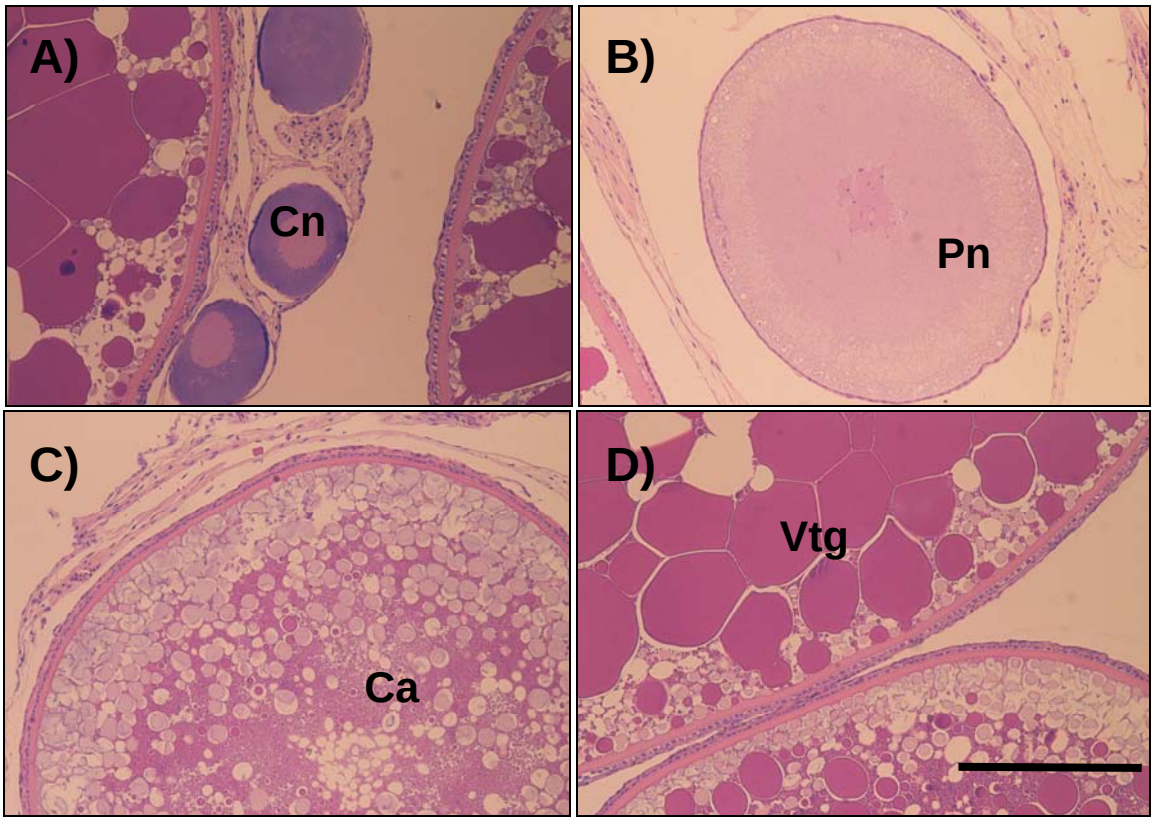

Figure 5. Photomicrographs of histological sections through a range of developmental stages of Atlantic salmon ovarian follicles showing healthy follicle structure: Cn – Chromatin nucleolus stage; Ca – Cortical alveolus stage; Pn - Perinucleolus stage; Vtg – Vitellogenic stage. Scale bar is 20 μm, for all micrographs.

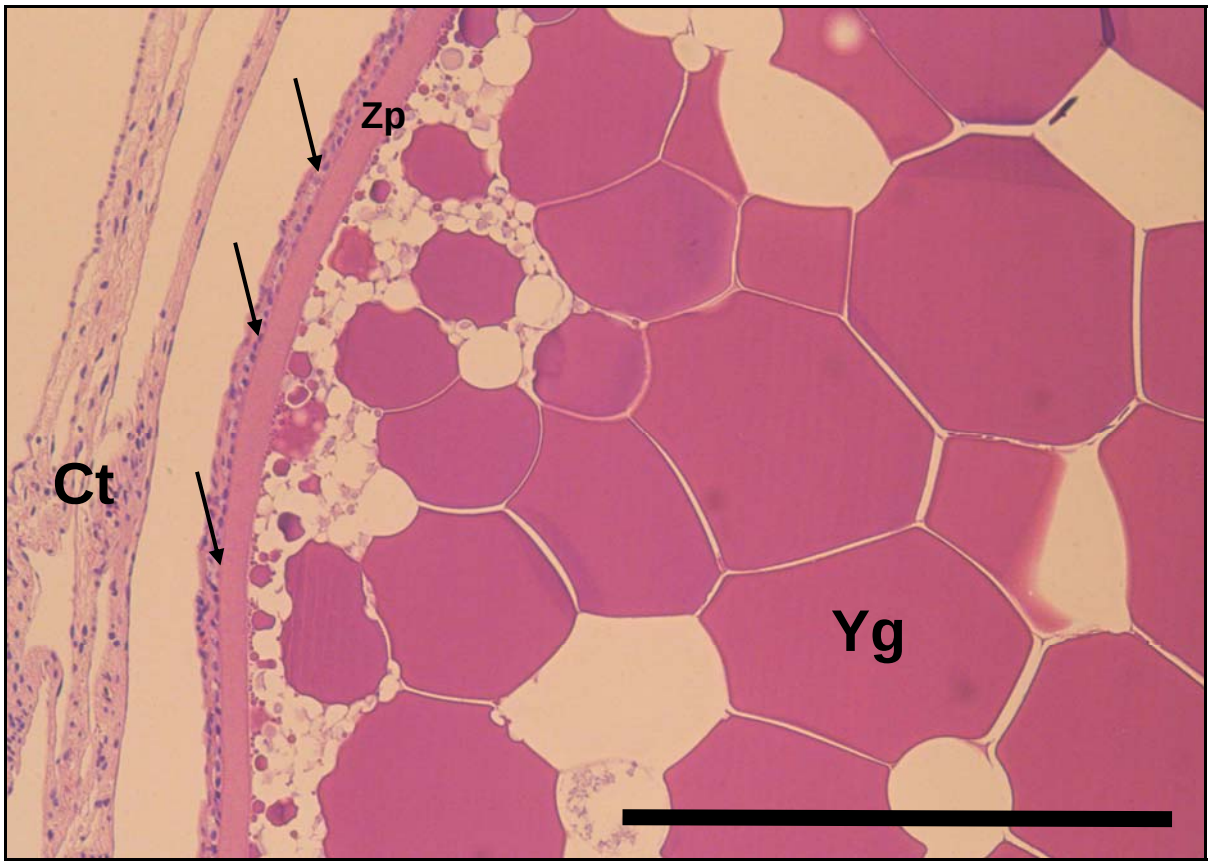

Figure 6. Photomicrograph of a histological section through a healthy late-maturation-stage vitellogenic follicle showing the developing *zona pellucida* (Zp; also known as the egg-membrane or 'chorion') which is contained within a sheath of maternal tissue, the theca (arrows). Ovarian connective tissue (Ct) lies adjacent to the theca. The follicle is filled with large yolk globules (Yg) largely composed of the yolk protein vitellogenin. Scale bar is 50 µm.

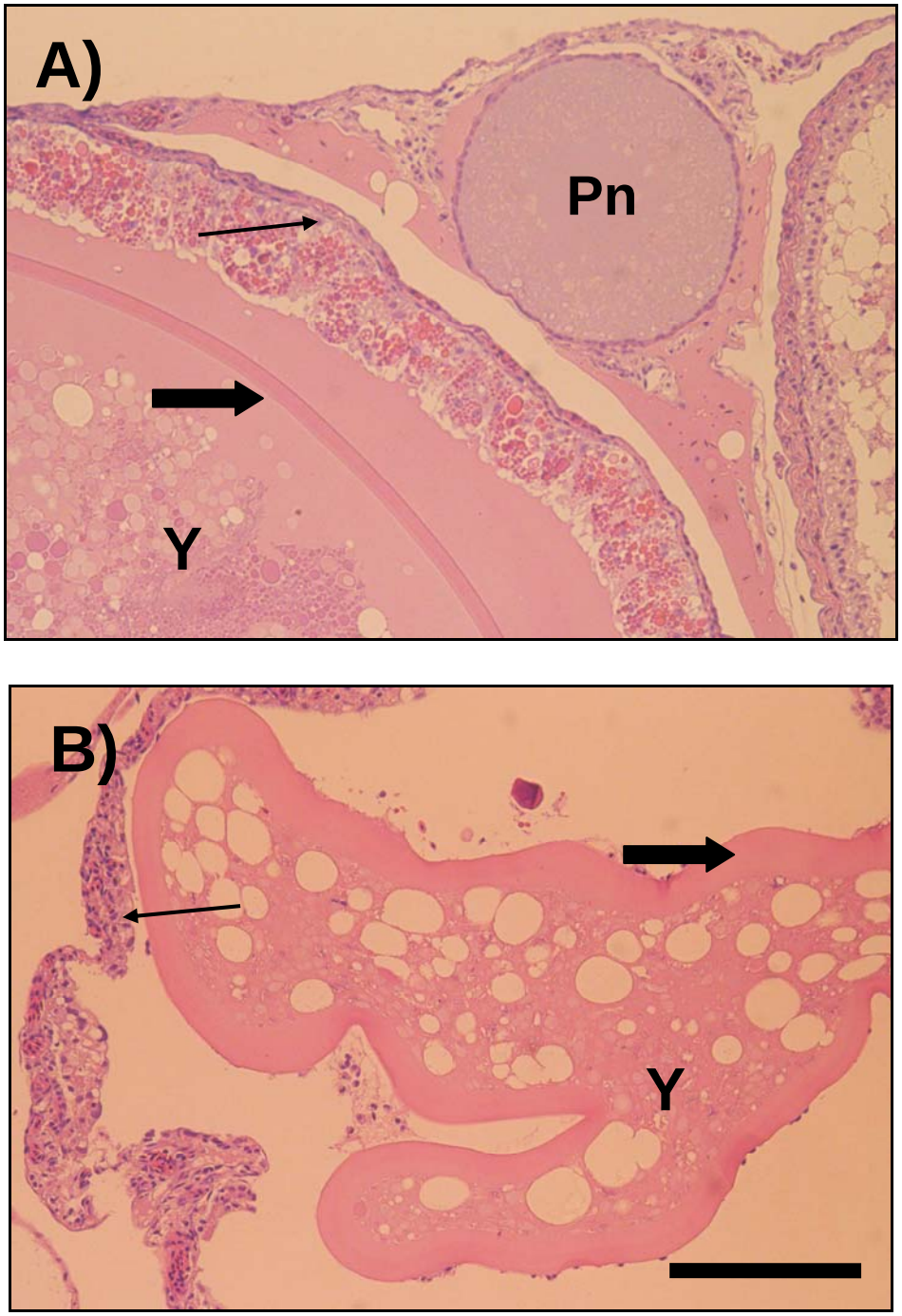

Figure 7. Photomicrographs of histological sections of atretic follicles and an adjacent perinucleolus (Pn) stage follicle. A), A vitellogenic follicle in which onset of atresia is recent; B) follicle showing advanced atresia in which most of the follicle contents have been resorbed. Thick arrows within atretic follicles show remnants of the *zona pellucida,* and thin arrows indicate the thecal tissue. Y – yolk material. Scale bar is 10 µm; same for both micrographs.

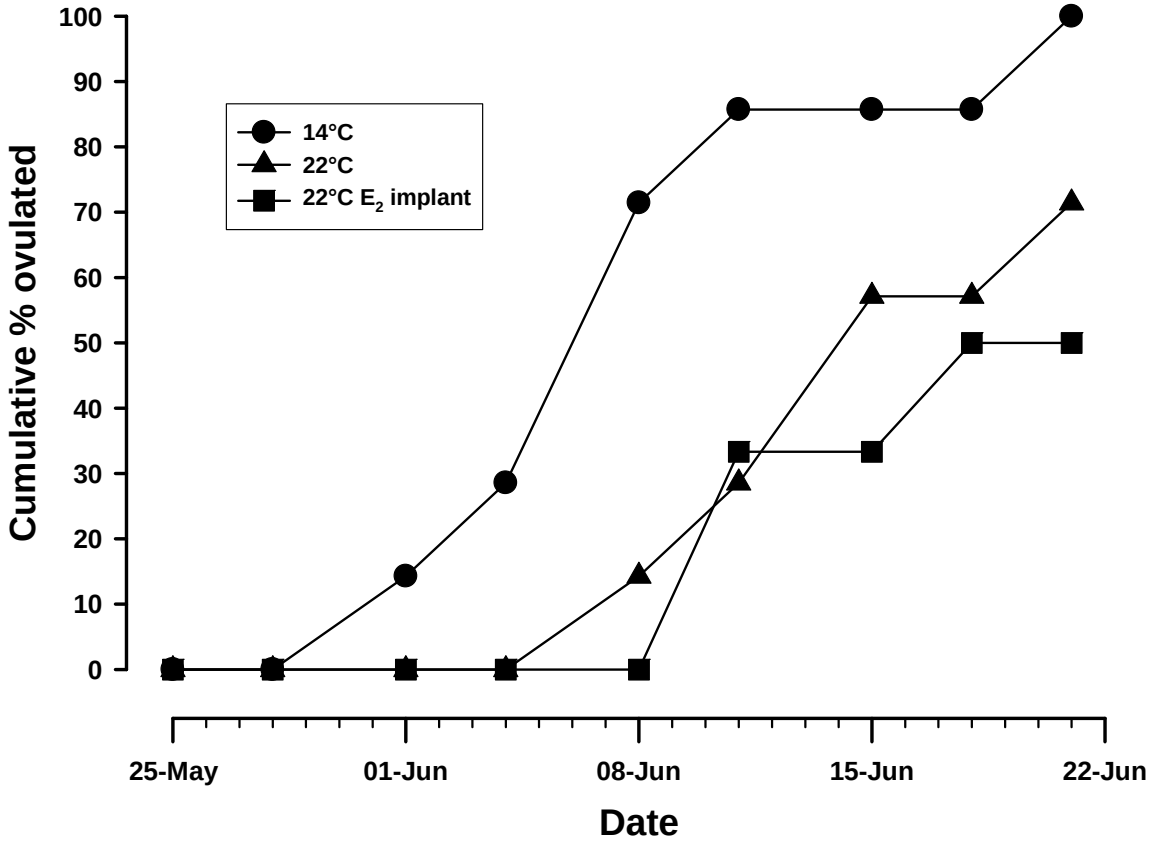

Figure 8. Cumulative ovulation in maiden Atlantic salmon spawners without hormone pellet implants held at 14°C (circles) and 22°C (triangles), and fish with E_2 pellet implants held at 22°C (squares) during autumn.

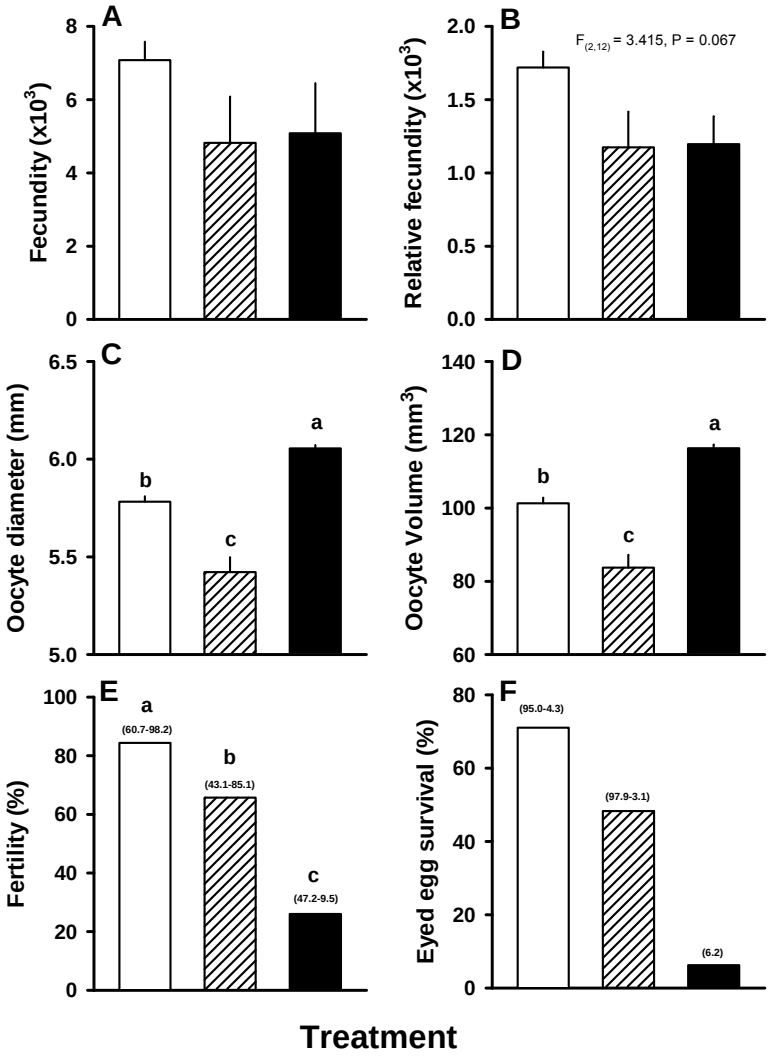

Figure 9. Post-ovulatory fecundity, fertility, oocyte diameter and volume, and survival to eyed egg stage among maiden Atlantic salmon spawners without hormone pellet implants held at 14°C (open bars) or 22°C (cross-hatched bars), and fish with E_2 pellet implants held at 22°C (black bars). Values are mean + SE (or 95% confidence limits for % data) (n=7,5 and 3 for the three groups respectively; n=1 for eyed egg survival in 22°C + pellet group). Different alphabetical superscripts among sample times denote significant differences ($P<0.05$) unless stated otherwise.

Endocrine and gene expression data

Plasma cortisol levels were low and typical of values expected from unstressed fish from this stock of salmon (Thomas *et al*., 1999) at Sample 1 and all groups of fish at Sample 2 (Fig. 10). At Sample 3, plasma cortisol levels were elevated in the group that had received E_2 pellets, and at Sample 4 all groups were showing signs of elevation in cortisol but there was no significant difference between groups (Fig. 10). This suggests that the process of surgery and pellet implant was to a degree stressful, and this may have affected outcomes, as stress can have strong inhibitory effects on reproduction which are independent of the thermal effects being examined here (reviewed by Schreck, 2010). This is difficult to assess in the absence of data from fish exposed to less invasive or less frequent hormone implant protocols, but the dual pellet implant approach chosen here (and which may have contributed to the stress effects observed) was designed to maintain E_2 levels for an extended period. The use of a single pellet implant protocol possibly warrants investigation but would still need to meet the requirement of being able to elevate plasma E_2 levels for an extended period.

Plasma E_2 levels were suppressed in fish held at 22^{o}C relative to 14^{o}C controls at Samples 2 and 3 (Fig. 10). E_2 levels were at intermediate values in the pellet group at Sample 2 but elevated over values in both the other groups at Sample 3. This indicates that the pellet treatment was effective in terms of elevating plasma E_2 levels in thermally stressed fish; however, whether there was suitable and uniform elevation of plasma E_2 for the duration of the experiment is less clear. Shorter term experiments with other species (Pankhurst *et al*., 1986) and juvenile Atlantic salmon (our unpublished data), show that single implants of silastic pellets can maintain plasma elevations of E_2 at stable levels for 7-14 days, suggesting that the pellet implants used here are likely to have maintained plasma E_2 levels at values similar to those of fish held at 14^{o}C for most of the experimental period. This appears to have been reflected in the oocyte growth promoting effects seen in the pellet-treated group in the current study. In the absence of E_2 treatment, maintenance at 22^{o}C resulted in the same depression of plasma E_2 levels and

reduction in oocyte size and volume consistently found in previous experiments with this stock of salmon (Pankhurst and King, 2010).

Testosterone (T) levels were depressed in both 22°C groups at Sample 2 and 4, and in the pellet group at Sample 3 (Fig. 10). Depression of plasma T levels at 22°C is indicative of inhibitory effects on the endocrine system in addition to the inhibition of aromatase-mediated conversion of T and other androgen substrates to E_2. This effect has been observed previously in this stock of salmon (Pankhurst and King, 2010) but is generally less consistent and less profound than the falls in plasma E_2 levels at elevated temperature. *In vitro* incubation of isolated ovarian follicles with gonadotropins, analogues of cyclic AMP, and steroid precursors has shown that ovarian steroidogenesis upstream of E_2 production continues at 22°C (Watts *et al*., 2004), suggesting that suppression of plasma T levels arises higher in the hormone pathway than at the level of gonadal steroidogenesis. Studies on other species showing that thermal inhibition can occur at the pituitary level (Okuzawa *et al*., 2003; Soria *et al.,* 2008) suggest that this may also be an effect that is present in salmon. We know that augmentation of pituitary function through the administration of GnRHa is ineffective in offsetting the inhibitory effects of elevated temperature. The same appears to be the case for treatment with E_2 alone. We have yet to examine the effects of combined E_2 and GnRHa therapy.

An interesting additional point is that plasma T levels were depressed in E_2-treated fish and this may relate to the effect that E_2 is known to have for a range of species, in up-regulating expression of the genes coding for aromatase (reviewed in Guiguen *et al.,* 2010). Aromatase mediates the conversion of T to E_2 and the low levels of T present could reflect greater rates of conversion of T to E_2, although given the suppressive effects of elevated temperature on aromatase activity, this may be unlikely. Gonadal steroids including E_2 also have feedback effects at most levels of the endocrine cascade. In maturing fish, the effects of E_2 are generally reported to be stimulatory, with increases in hypothalamic GnRH content, pituitary sensitivity to GnRH and pituitary gonadotropin content all increasing in fish treated with E_2. However, E_2 is also reported to have direct

inhibitory action on gonadal steroidogenesis by an as yet undescribed membrane receptor-mediated (non-genomic) process (reviewed in Pankhurst, 2008) and it is possible that an effect of this type was present here.

Plasma Vtg levels were relatively stable across sample times among fish held at 14^{o}C (Fig. 10). This is an unexpected finding given the very consistent increase in plasma Vtg levels typically seen across this period of vitellogenesis in previous seasons (Pankhurst and King, 2010). This may be a reflection of the lower than normal plasma E_2 levels occurring in the 14^{o}C fish (Fig. 10), in turn possibly a legacy of the period of high temperature exposure before introduction to the experimental regimes. In contrast, plasma Vtg levels were elevated in fish held at 22^{o}C and treated with an E_2 pellet relative to 22^{o}C fish at Sample 2, and both 14^{o}C and 22^{o}C groups at Samples 3 and 4. This indicates that fish in this group retained hepatic responsiveness to E_2, despite the exposure to elevated temperature. This is consistent with our studies on juveniles (unpublished data) showing that Atlantic salmon retain hepatic E_2 responsiveness even at high temperature. This is borne out by the increase in Vtg A gene expression occurring in fish at 22^{o}C with E_2 implantation, at Sample 3 (Fig. 11).

ZP gene expression data are harder to interpret, with ZPB and ZPB gene expression levels both being quite low at samples 2 and 3, and both 22^{o}C groups showing higher levels of expression of ZPC genes at Sample 3 than fish held at 14^{o}C (Fig. 11). The expression data were not strongly coupled with plasma E_2 data despite the expectation that they would be, based on the high sensitivity of ZP genes to E_2-induction (Celius *et al*., 2000; Berg *et al*., 2004; Fujita *et al*., 2004). However, at Sample 4 expression of both sets of ZP genes had increased significantly at 14^{o}C compared with both groups held at 22^{o}C, in line with increasing plasma E_2 levels (Figs. 10 and 11). ZP synthesis and assembly is critical in determining chorion integrity (Modig *et al.,* 2007), and subsequent egg viability and fertility in both Atlantic salmon (King *et al.,* 2003) and chinook salmon (*Oncorhynchus tshawytscha*) (Barnes *et al*., 2003). The increasing levels of ZP gene expression seen in fish from the 14^{o}C group are consistent with the

subsequent high fertility and survival in eggs from this group of females. It is also clear that elevated E_2 alone was not sufficient to maintain ZP gene expression in fish held at 22^{o}C, despite its capacity to induce both Vtg gene expression and subsequent Vtg synthesis, release to the plasma and (based on the measured increase in follicle size), sequestration into growing oocytes. This suggests that a key step in egg viability resides with the development of chorion integrity and that E_2 treatment alone is not sufficient to protect this process from thermal disruption. It is not clear whether this effect is generated by impaired transcription alone (as evidenced by reduced expression of both ZPB and ZPC genes), or also impacts on subsequent translation and protein assembly. ERα gene expression data (Fig. 11) do not show any evidence that there was impairment of estrogen receptor gene expression as a result of exposure to elevated temperature, suggesting that the effects may lie with impairment of activation of estrogen response elements of the relevant genes by transcription factors, rather than receptor binding and translocation.

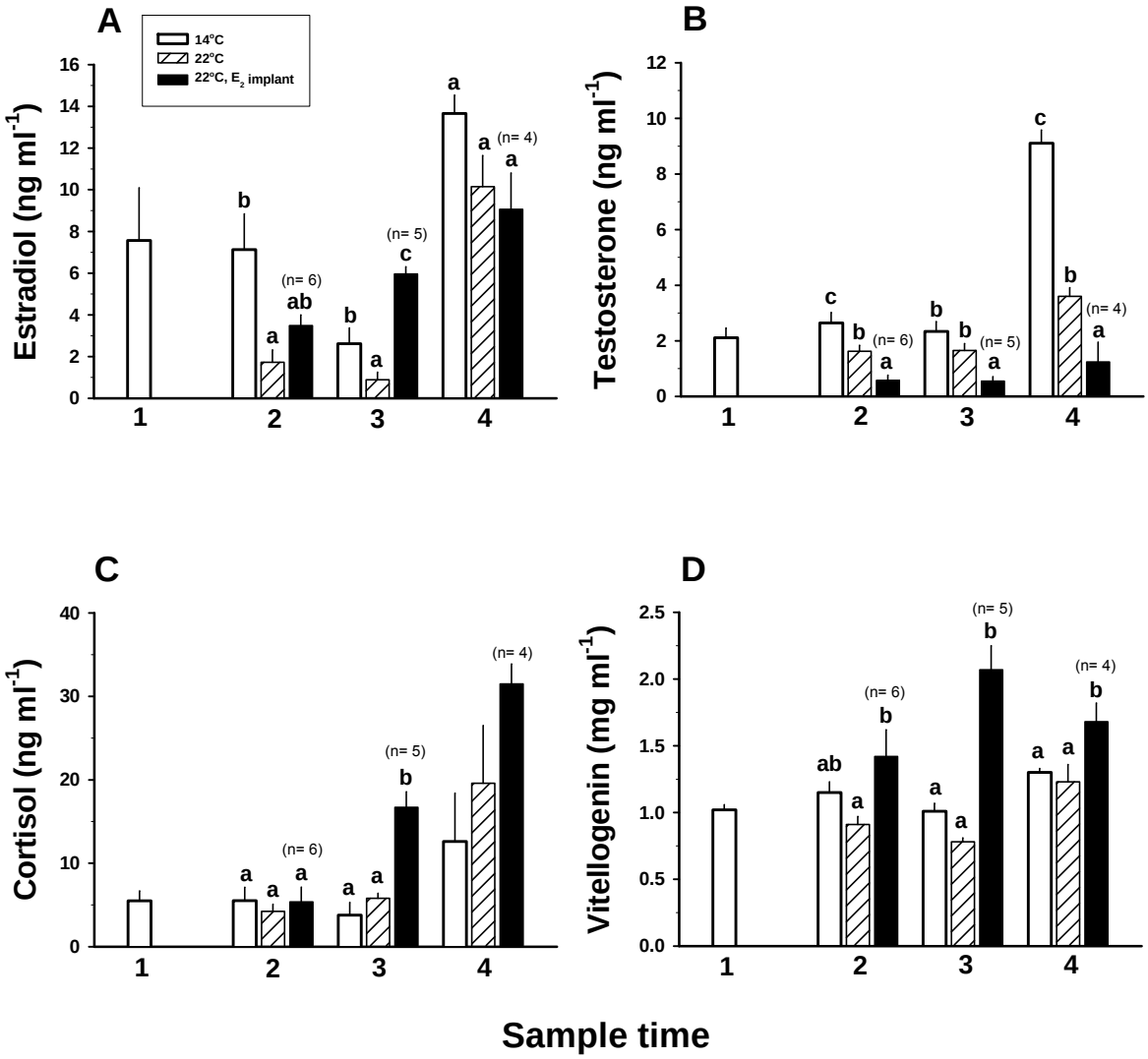

Figure 10. Plasma levels of estradiol, testosterone, cortisol and vitellogenin among maiden Atlantic salmon spawners without hormone pellet implants held at 14°C (open bars) or 22°C (cross-hatched bars), and fish with E_2 pellet implants held at 22°C (black bars). Values are means + SE (n=7 unless stated otherwise). Different alphabetical superscripts among sample times denote significant differences ($P<0.05$).

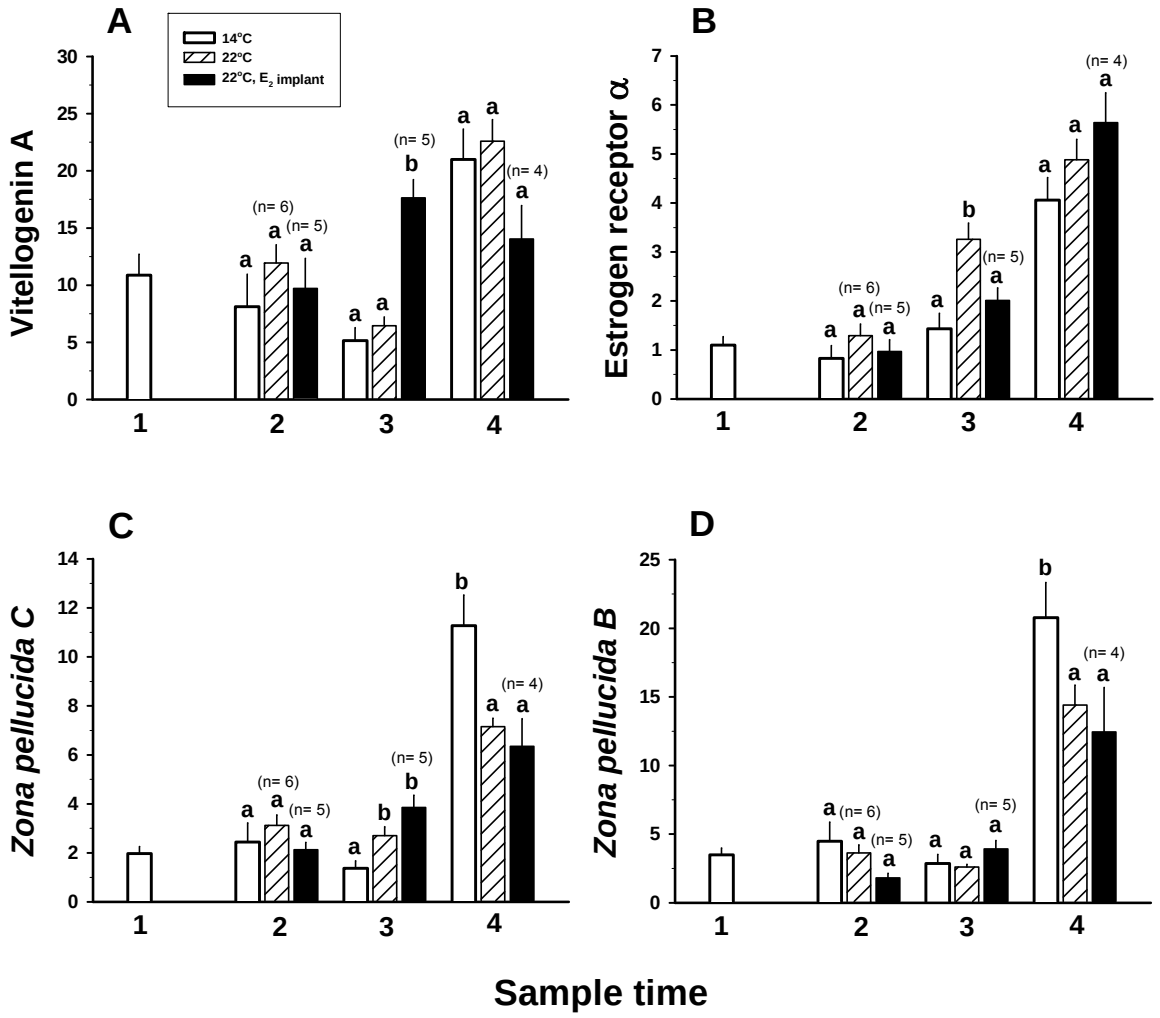

Figure 11. Relative gene expression of vitellogenin A, estrogen receptor α, *Zona pellucida B* and *Zona pellucida C* among maiden Atlantic salmon spawners without hormone pellet implants held at 14°C (open bars) or 22°C (cross-hatched bars), and fish with E_2 pellet implants held at 22°C (black bars). Values are means + SE (n=7 unless stated otherwise). Different alphabetical superscripts among sample times denote significant differences ($P<0.05$).

Benefits and adoption

The fragility of spawning success and egg viability of Atlantic salmon broodstock in the face of increasing temperatures leaves the Atlantic salmon aquaculture industry with the ongoing problem of the need to protect broodstock fish from thermal damage, particularly during late summer and early autumn. This carries with it the cost and management impost of the requirement for expanded scale of temperature-controlled holding and rearing facilities, or in the absence of these, acceptance of a high level of unpredictability in annual egg supply. The most desirable situation is for industry to hold fish under temperature conditions that generate optimum egg production rates; however, because of the considerations noted above, this may not always be possible. An alternative solution investigated here was whether some of the known hormonal changes occurring with thermal inhibition could be managed by external supplementation of the affected hormones, to provide a less infrastructure-dependent solution for the industry.

The economic viability of sea cage farming of Atlantic salmon is strongly influenced by the cost of production of smolts. Understanding the mechanisms that can contribute to reproductive failure in spawning fish is an essential component of reducing those industry production costs, and at a broader level, ensuring that there are sufficient smolts produced each year to maintain industry production. The issue has been identified as an industry priority in view of the high cost of unpredictable egg production, and the vulnerability of the industry in the face of climate change. The current survival of eggs to the eyed embryo stage under some circumstances can be as low as 30-50%, compared with 80% for eggs from best performing fish (SALTAS data). The prediction is that failure to protect broodstock fish from thermal insult could produce even lower rates of embryo survival. Modelling of this cost gives direct increases (controlled risk) in smolt production costs of ~$300,000 per annum, but an absolute risk of industry shortfall in production terms of at least $20 million per annum. The current project was directed at managing the risk profile to generate a lower value treated risk for the industry.

The benefits and beneficiaries remain those identified in the original application. Saltas and other smolt suppliers now have unambiguous confirmation that failure to protect broodstock from thermal stress during summer and autumn will have highly detrimental effects on egg production. The attempt to develop a hormone therapy suitable for industry use has not yet been successful, but the understanding that the temperature effects are not easily offset by hormone treatment will allow hatcheries to model and further justify the capital expenditure involved in thermal management of broodstock. This is the approach that SALTAS has taken, with a strategy that encompasses complete onshore management, use of multiple age classes as a hedge against thermal stress in autumn, and the development of large scale temperature controlled systems for thermal management of broodstock through critical periods of development. This will ensure continued certainty of smolt production for the industry.

Further development

An issue remains as to whether combined hormonal manipulation treatments can be used to offset the effects of thermal inhibition during vitellogenesis. The estrogen treatment used in the present study was partially effective at offsetting the effects of exposure to elevated temperate during autumn. Ovarian and oocyte growth was maintained but egg shell assembly still appears to be compromised. This confirms our earlier view that one of the key blockades in the endocrine cascade is in estrogen synthesis through the suppression of aromatase activity, but it appears that other regulatory factors also remain important. We have also previously shown that GnRHa treatment (which activates parts of the endocrine cascade upstream of estrogen synthesis) is ineffective alone. It remains to be tested whether combined GnRHa and estrogen therapy for female broodstock during the critical period of ovarian development during late summer and autumn provides additional protection than either treatment alone. Hormone therapy may

still be a necessary management option for hatchery situations where large scale manipulation of water temperature is not possible.

Planned outcomes

Planned outcome 1: *Capacity to manipulate plasma estrogen levels in adult Atlantic salmon broodstock.* The project has been successful in developing a protocol for treating adult salmon with silastic implants that minimise handling but elevate plasma estrogen to physiological levels for extended periods.

Planned outcome 2: *Understanding of the role of estrogen in ameliorating the effect of thermal stress in female Atlantic salmon.* There is now a clear understanding of the effect of exogenous (externally administered) estrogen on hormonal and reproductive parameters in thermally stressed female salmon broodstock.

Planned outcome 3: *Capacity to use hormone therapy to protect salmon from thermal stress.* This has been partially achieved through the development of a protocol to elevate plasma estrogen in adult broodstock salmon and to maintain oocyte growth in the face of elevated temperature. Development of a complete technical toolbox for hormone therapy for use in hatchery-scale operations remains elusive.

Planned outcome 4: *Protection and enhancement of the egg producing capacity of the salmon industry, and with that; increase in the profitability of the sector and its capacity for further expansion through reducing production costs and the delivery of a reliable and predictable supply of smolts (in turn a function of the predictability and sustainability of the production of high quality eggs);and security for the Tasmanian Atlantic salmon farming industry contribution to rural employment through the direct and indirect generation of employment for ~2,000 Tasmanians.* This outcome is supported through confirmation that thermal

protection remains the key to sustained, high quality egg production for the salmon industry.

Conclusion

This project has generated the first description of morphometric and hormonal development in maiden spawning Atlantic salmon treated with physiological doses of estrogen designed to offset the effects of thermal stress. It has confirmed the inhibitory effects of maintenance at higher temperature on egg growth, development, ovulation, fertility and survival and shown that estrogen treatment provides partial protection against the growth inhibiting effects of exposure to high temperature. Estrogen treatment alone; however, is not sufficient to provide full protection for maintenance of egg viability under conditions of exposure to high temperature.

As in previous experiments, there was depression of the plasma steroids E_2 and to a lesser extent T in fish exposed to 22^{o}C. This is accompanied by reduced ovarian size and oocyte diameter, and increased incidence of ovarian atresia. Gene expression studies showed that suppression of E_2 synthesis was reflected in impairment of the downstream processes of vitellogenin synthesis and release, and the assembly of ZP (egg shell) proteins. Treatment with exogenous estrogen restored plasma E_2 levels to values similar to those seen in fish held at 14^{o}C, enhanced ovarian growth and egg size but did not restore egg fertility and survival. This is probably due to the failure of estrogen treatment to fully restore the expression of the genes coding for ZP (and subsequent effects on egg shell integrity). E_2 treatment did increase expression of vitellogenin genes, showing that a key step in egg viability rests with the correct assembly of egg shell proteins.

The fact that treatment with E_2 was not fully effective at offsetting the inhibitory effects of elevated temperature is thought to be due to there being thermal inhibition of multiple steps in the endocrine cascade, including some above the

production of E_2 from androgen precursors, and suggests that estrogen therapy may need to be combined with treatment with GnRHa (which activates higher levels of the endocrine cascade). The management implications of the study include further confirmation that female broodstock show high sensitivity to thermal insult in late summer and autumn, and that these effects are exercised through the hormonal system and the genes that the hormones activate. This was further emphasized by inhibitory effects on the whole experimental stock from the unintended effects of summer maintenance at high temperatures over a period of development which previous experiments had suggested might not be that critical in terms of sensitivity to high temperature. The current study suggests that this assumption needs re-evaluation and that it remains critical for hatcheries to maintain strategies that offer thermal protection for fish earlier in summer and autumn.

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Appendix 1: Intellectual Property

As described in the original application, neither the authors, nor the industry partner seek to protect project IP for the purposes of further commercial development, and outcomes will be made available to a wider audience through publication in the primary literature in due course.

Significant information advances identified as a result of project IP are:

- Further description of the mode and timing of reproductive development in spawning fish from the Tasmanian stock of Atlantic salmon;
- Description of the structural changes occurring during ovarian development and recrudescence at 14 and 22^{o}C, respectively;
- The relative effects of thermal stress and estrogen therapy on morphometric development, ovarian structure, plasma steroid and vitellogenin (Vtg) levels, ovarian expression of Vtg, *zona pellucida* protein (ZP) and estrogen receptor (ER) genes in maturing Atlantic salmon;
- The protective effects of estrogen therapy of egg growth in fish exposed to high temperature;
- The failure of estrogen therapy to maintain expression of ZP genes and subsequent egg fertility and viability;
- The lack of effectiveness of estrogen treatment as an amelioration strategy for the management of thermally stressed fish;
- Confirmation for industry that thermal protection of broodstock remains a key success factor for continued and predictable egg production, and smolt supply to the grow-out sector of the Atlantic salmon aquaculture industry;
- And that continued investment in high-cost infrastructure for thermal management of broodstock is justified and essential for protecting production in the face of climate variability.

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