Effect of temperature on reproductive development of maiden and repeat spawning Atlantic salmon: understanding the basis for improved egg survival and quality

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Non Technical summary

Maiden (first spawning) and repeat (second) spawning Atlantic salmon were maintained during late summer and autumn over the critical final period of egg growth at temperatures previously shown to be optimal (14^{o}C) for, or detrimental (22^{o}C) to reproductive development in maiden fish. A subset of each age class exposed to the 22^{o}C treatment was given a pellet implant of a synthetic hormone (GnRH) commonly used in the aquaculture industry to synchronise and advance oocyte (egg) development. The twin objectives of the study were to compare the response of maiden and repeat spawning fish to elevated temperature, and to investigate the possible protective effect of hormone therapy in fish exposed to high temperature, with a view to improving the management options for industry in protecting Atlantic salmon from thermal injury in the face of climate variability.

This project provides the first comparison of reproductive development in maiden and repeat spawning Atlantic salmon and shows that there is an extensive period of ovarian reorganisation in repeat spawning fish during summer and early autumn, and that initially, ovarian growth is slower and less ordered in repeats than in maiden fish. There is a catch up phase in February, and by March, both groups of fish are following the same developmental pattern. Repeat spawners have higher absolute fecundity (numbers of eggs) but both age classes make the same relative investment in reproduction.

In both age classes, there is depression of a class of reproductive hormones (chemical factors controlling reproductive development) known as gonadal steroids in fish exposed to 22^{o}C. This is accompanied by successive falls in ovarian size, and oocyte diameter at 22^{o}C, and increased incidence of breakdown of ovarian tissue. The effects tend to be more severe in repeat spawning fish. Accompanying gene expression studies (the genes code for key hormones or structural proteins in the reproductive pathway) showed that suppression of steroid synthesis was reflected in impairment of the downstream processes of yolk and egg shell formation. These are all effects that would be predicted to result in compromised egg viability. As predicted, fish held at 22^{o}C had lower egg size, fertility and survival but in contrast to the hormone and gene measurements, the effects were most marked in maiden fish with intermediate effect being expressed in repeats at 22^{o}C. It is not entirely clear why this should be so, given

the high degree of similarity of effects on the different age classes, but may relate to the fact that eggs of repeat spawners tend to be a little larger and there is some protection provided by egg size, and the greater level of yolk endowment.

Treatment with GnRH was ineffective at offsetting the inhibitory effects of elevated temperature and this is probably due to the fact that there is thermal inhibition of multiple steps in the endocrine cascade that are difficult to overcome with drug therapy. The management implications of the study include confirmation that female broodstock show high sensitivity to high temperature in late summer and autumn, and that these effects are exercised through the hormonal system and the genes that the hormones activate. This means that hatcheries need to engage in strategies that offer thermal protection for fish during summer and autumn. In situations where temperature control is not possible at all, or only available for a portion of the maturing stock, then use of repeat spawning fish offers some level of protection against thermal damage but with the caveat that production losses due to reduced egg fertility and survival are still likely to occur.

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Background

Atlantic salmon (*Salmo salar*) are farmed in Tasmania and limited areas of southern Australia, and support an aquaculture industry of 24,000 tonnes with a gross value in 2006/07 of $272 million (Tasmanian Salmonid Growers Association data). Salmon in Australia are farmed towards the upper limit of their thermal tolerance range with the result that growth occurs at a faster rate and developmental milestones are reached at a younger age than in their Northern Hemisphere counterparts. This includes reproductive development with most fish maturing after a single winter at sea (at 3 years of age) compared with 2 sea-winters in the Northern Hemisphere. The majority of broodstock for egg production for subsequent grow-out cohorts come from these first-spawning or 'maiden' fish due both to the post-spawning mortality that can occur, and the expense of maintaining large broodstock fish for an extra production year. For example, Salmon Enterprises of Tasmania (SALTAS), the largest producer of salmon fry in Australia and the Industry Partner for this project has typically sourced 75% of its egg production from seawater-reared maiden spawners. However, this production strategy has one significant drawback. Survival of embryos to the eyed stage ranges from only 30-50% in seawater-reared maiden fish compared to 60-80% in freshwater-reared repeat spawners (SALTAS production data). The basis for increased mortality appears to be a higher incidence of abnormality in the chorion (egg shell) assembly in the eggs of maiden spawners. Eggs of many fish show holes or breaks in the chorion (King *et al*., 2003) and this is associated with increased egg mortality, primarily due to bacterial invasion. Preliminary studies showed that egg holes were more prevalent in maiden fish (Willis, 1999). This condition is believed to be the same as that described as 'soft shell syndrome' in chinook salmon (*Oncorhynchus tshawytscha*) which is characterised by delicacy of the chorion, increased incidence of holes in the chorion and high mortality (Barnes *et al*., 2003). The finding that anti-bacterial treatment with formalin results in a decrease in egg mortality (Barnes *et al*., 2005; Aberg, 2006) supports the view that egg mortality is related to bacterial invasion.

In recent years, SALTAS has been attempting to manage spawning populations of both maiden and repeat spawning fish by rearing fish in freshwater, rather than the common industry practice of rearing fish in sea cages, and then bringing them to the hatchery when reproductive development is well advanced. Freshwater maintenance

requires significant extra capital investment but allows better environmental control, especially of thermal exposure. In a series of studies on rainbow trout *Oncorhynchus mykiss* (Pankhurst *et al*., 1996; Pankhurst and Thomas, 1998) and Atlantic salmon (King and Pankhurst 2000, 2003, 2004a,b; King *et al*., 2003, 2007; Watts *et al*., 2004, 2005), we have shown that there is high sensitivity of the reproductive process to thermal insult and that controlling temperature is a key component of optimal broodstock management. These findings resulted in a SALTAS decision (at a capital cost of ~$2 million) to shift holding of broodstock from seawater to freshwater. Industry results to date show that egg survival can be increased through thermal management; however, the relationship between stock age (maidens or repeats) and thermal conditions have not been systematically investigated, and the industry management protocols are essentially informed guess work.

An understanding of the endocrine (hormonal) processing regulating ovarian development in teleost fish is a necessary prerequisite for the management of reproduction in culture. Initial reproductive development, and the initiation of subsequent cycles of egg production are both stimulated by the release of hypothalamic gonadotropin releasing hormone (GnRH) which acts on the *pars distalis* of the pituitary to stimulate expression, synthesis and release of the glycoprotein follicle stimulating hormone (FSH) and at later stages luteinising hormone (LH) (Swanson et al., 2003). Both FSH and LH exert their action by binding to membrane receptors on the follicle cells of the ovary resulting in elevation of intracellular cyclic AMP levels and subsequent activation of protein kinase A, and activation or *de novo* synthesis of steroid synthesizing enzymes (Nagahama, 2000). The subsequent steroid cleavage pattern in female fish results in the synthesis of 17β-estradiol (E_2) through the conversion of a series of progestin and androgen intermediates, with each conversion step in the pathway being enzymatically mediated (reviewed in Pankhurst, 1998). A critical step in the production of E_2 is the cytochrome P_{450} aromatase (arom)-mediated conversion of testosterone to E_2, and in Atlantic salmon this appears to be a key step in ensuring high egg fertility and survival (King *et al*., 2003; Watts *et al*., 2004). This stage is also highly sensitive to exposure to elevated temperatures. The explanation for this sensitivity lies in the subsequent actions of E_2 on hepatic protein synthesis. E_2 produced by the ovarian follicle 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 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 radiata* 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 (Berg *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 (King and Pankhurst 2000, 2003, 2004a,b; King *et al*., 2003, 2007; Watts *et al*., 2004, 2005) .

The importance of stock selection (maidens or repeats) rests on the fact that the first cycle of reproduction in maiden spawning Atlantic salmon covers the initial period of reproductive maturation or puberty. Studies on other species suggest that puberty is associated with an increase in hypothalamic GnRH mRNA. In addition, the recently discovered G-coupled protein receptor GPR54 and its ligand the kisspeptin have been shown to have an important role in initiating GnRH secretion and are now considered to be the gatekeepers of puberty (Seminara *et al*., 2004; Nocillado *et al*., 2007). Blockades in the endocrine cascade prior to puberty have been identified at the levels of GnRH and FSH synthesis, and gonadotropin receptor binding at the gonadal level in a variety of species including salmonids (Gur *et al*., 2000; Okuzawa *et al*., 2002; Nocillado *et al*., 2007). The main effect of this appears to be the occurrence of a 'dummy run' with only partial endocrine activation and gonadal response, in the season before the first spawning period occurs, with the phenomenon being described in masu salmon *Oncorhynchus masou* (Amano *et al*., 1992) and rainbow trout (Prat *et al*., 1996). Rapidly maturing Southern Hemisphere Atlantic salmon do not appear to display the dummy run phenomenon but it is possible that part of the spawning failure seen in Tasmanian fish might result from endocrine dysfunction that is similar to that seen in pre-spawning years in other species.

This background resulted in the development of a (non FRDC-funded) preliminary study to determine if there was a differential response to thermal insult based on age

class. The outcomes of this experiment are summarised in Appendix 3 and showed that in concert with earlier experiments on both rainbow trout and Atlantic salmon (reviewed in Pankhurst and King, 2009), exposure to elevated temperature had damaging effects on both maiden, and repeat spawning fish, but that the impacts in terms of egg fertility and survival were greater in maiden fish. This was despite some physiological and endocrine indicators showing that both groups were strongly affected during the yolk accumulation (vitellogenic) phase of egg development (Appendix 3). This raised the following questions:

1. Is the measured effect of temperature on age class consistent?
2. As the effect appears to be generated by endocrine dysfunction, can the effect of thermal insult be reduced by therapeutic hormone treatment?

Accordingly, maiden and repeat spawning fish were set up in an experimental matrix and sampled through the autumn period of maturation until spawning. This provided a data set for egg production and morphometric data, blood plasma for steroid hormone and Vtg analysis, and ovarian, liver and brain expression of key genes involved in the regulation of ovarian development. Egg fertility and survival data were determined from fish that were held under the experimental conditions but allowed to mature through to ovulation and subsequent egg stripping. The thermal manipulation protocol involved 6 treatment groups, 2 age classes of spawning fish (maidens and repeats), 2 temperature regimes and treatment with the hormone, gonadotropin releasing hormone-analogue (GnRH) as below.

Group 1- Maidens held at 14^{o}C
Group 2- Repeats held at 14^{o}C
Group 3- Maidens held at 22^{o}C
Group 4- Maidens held at 22^{o}C with a GnRH pellet implant
Group 5- Repeats held at 22^{o}C
Group 6- Repeats held at 22^{o}C with a GnRH pellet implant

GnRH therapy is a well established procedure for stimulating final oocyte maturation and ovulation through the secretion of LH by the pituitary, and the subsequent synthesis and release of maturational steroids by the ovary, and has been widely used across a

range of salmonid species (reviewed in Pankhurst and King, 2009). There is less information on its use in the stimulation or maintenance of endocrine function earlier in development but in view of its demonstrated capacity to maintain E_2 secretion during vitellogenesis in many species, it was viewed here as a viable strategy for maintaining E_2 levels under conditions of thermal stress.

Need

Strategic R & D plan and priority - Salmon Aquaculture Subprogram:
Currently, variable and unpredictable egg production has a potential opportunity cost for the Tasmanian salmon industry of $15-20 million per annum. Solving this problem is essential for the industry to maintain its continued strong commercial performance; however, the capacity to reach that solution is currently hindered by our limited understanding of the processes that regulate egg shell assembly and how these contribute to chorion and egg abnormalities, and also how these processes change with stock age and rearing temperature. This study addresses that knowledge gap.

Objectives

This project examines the effect of thermal environment on puberty (first reproductive development), egg yolk and chorion (egg shell) formation, and the subsequent effects on egg fertility and survival in farmed Atlantic salmon.
The specific objectives (below) sit in the context of the following consideration of what is known of chorion development in fish.

Specific objectives

1. Determine the effect of age class (maidens or repeats) and thermal regime on reproductive performance (ovarian growth and size, follicle size, time of ovulation, fertility and hatching success) and endocrine parameters (plasma levels of the steroids T and E_2, and vitellogenin). *Objective achieved.*
2. Isolate the cDNAs encoding for the G-coupled protein receptor GPR54 and its peptin ligand KiSS-I and develop quantitative expression assays for GPR54 ,KiSS-I, FSH beta subunit (FSHβ) and egg shell protein (ZP) mRNAs in Atlantic salmon. *Objective achieved.*

3. Measure plasma levels of ZP in maturing fish. *Objective reassigned*. Resources were redirected to development of expression assays and expression measurement of multiple ZP genes (the original proposal was only to measure one gene). Plasma ZP assays are not specific for individual ZP genes families. The alternative approach used here gives greater precision.

4. Utilise DNA microarray technology to screen ~650 known salmon genes for differential expression during puberty and sexual development and in response to change in thermal environment, to identify gene networks associated with the above processes. *Objective in progess*. The technical failure of a freeze-drier during preparation of samples for transport to international collaborator Dr Julian Bobe (INRA, Rennes Cedex, France) resulted in sample degradation during shipment. The sample preparation process is being repeated, and the microarray work will continue as planned.

5. Define histological characteristics and chorionic structure of eggs in relation to stock and rearing conditions. *Objective achieved*. Because of time constraints around processing within the project life cycle, resources were directed to completion of histology from samples already collected in the preliminary experiment (Appendix 3).

6. Assess endocrine intervention and thermal manipulation as a strategy for increasing fertility, and develop outcomes into a management tool for industry use. *Objective partially achieved.* GnRH experiments were completed as planned but outcomes fall short of providing a management tool. This is discussed further under the section heading 'Further Development'.

Methods

Fish Husbandry and Maintenance

Maiden (first spawning 2+ year old fish) and repeat (second spawning 3+ year old fish) adult females from the SALTAS spawning stock were held in 200 (maidens) or 50 (repeats) m^3 circular tanks at ambient photoperiod and temperature under standard conditions of husbandry, at the SALTAS Wayatinah hatchery until January 2009. In mid-January 2009, fish were divided into the following treatment groups (n = 42 per group) and transferred to temperature-controlled $4m^3$ Rathbun tanks under simulated ambient photoperiod. Fish were not fed from the time of transfer to the temperature controlled systems.

Treatment Groups

1. Maidens held at 14°C;
2. repeats held at 14°C;
3. maidens held at 22°C;
4. maidens held at 22°C and treated with a GnRH pellet (see 'sampling protocol' for details);
5. repeats held at 22°C and
6. repeats held at 22°C and treated with a GnRH pellet.

All fish were maintained at the nominated temperature (14 or 22°C) until late March when all fish were exposed to a temperature ramp down to 8°C to induce final oocyte maturation and ovulation (King and Pankhurst, 2000). Temperature profiles for the two temperature regimes are shown in Fig. 1.

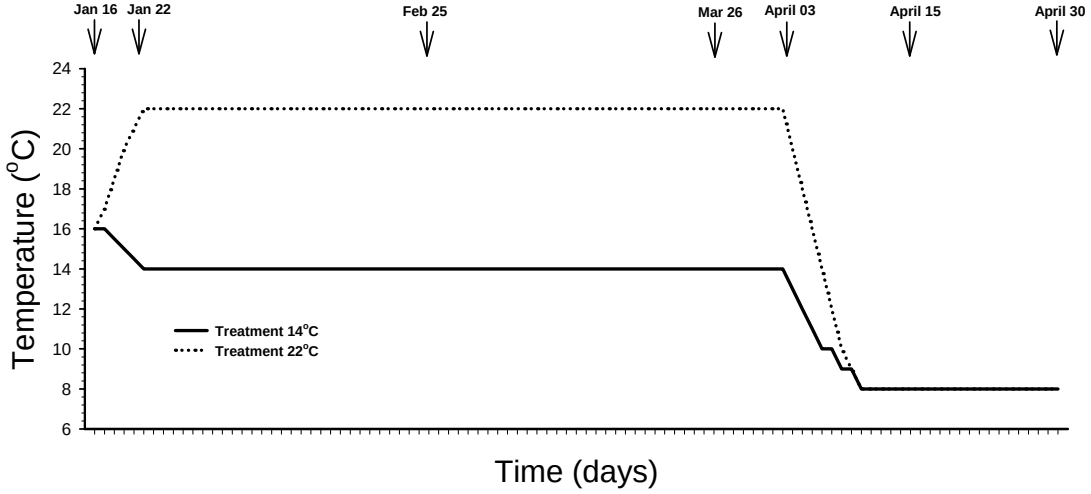

Figure 1. Thermal adjustment protocols for Atlantic salmon maiden and repeat spawners in 14°C and 22°C treatments in 2009 experiments.

Sampling Protocol

Fish from both maiden and repeat groups were sampled in late October 2008 to establish a previtellogenic baseline for each group (*Sample 1*), and on the 22nd January 2009 at the time of establishment of the 6 experimental groups (*Sample 2*). Subsequent samples were taken over February 25-27 (*Sample 3*), March 26-27 (*Sample 4*), and April 15-16 (*Sample 5*). Six to seven fish were sampled from each group at each sample time, leaving 7 fish from each treatment to proceed through to ovulation and stripping, after the final destructive sample in April. Fish from treatment groups 4 (maidens at

22°C) and 6 (repeats at 22°C) were implanted with an Ovaplant ™ (Syndel, Canada) cholesterol pellet containing 37μg of the D-Arg^{6},Pro^{9}NEt analogue of salmon gonadotropin releasing hormone (GnRH) on 22nd January at the time of Sample 2, and again over February 26-27 (at Sample 3) to give repeat doses of approximately 7-12μg.kg^{-1}. The dose chosen was designed to stimulate or maintain pituitary secretion of follicle stimulating hormone (FSH), but remain below the threshold likely to stimulate a luteinising hormone (LH) surge and possible premature stimulation of oocyte maturation and ovulation (King and Pankhurst, 2004a,b).

For sampling, fish were netted from the holding tanks, terminally anaesthetised in Aqi-S ™ (Crop & Food, New Zealand), weighed, measured and then blood sampled by caudal puncture using pre-heparinised syringes fitted with 22G needles. Blood plasma was centrifuged at 12000g, and stored frozen at -20°C for later measurement of plasma hormones. Ovaries were excised, weighed and portions allocated to 50ml-pots containing teleost saline or 10% neutral buffered formalin for fecundity estimation and follicle measurement, and histology, respectively. Segments of ovary and liver were transferred to 1-2ml of RNA Later ™ (Qiagen, Germany) to stabilise mRNA for later measurement of gene expression. The cranial cavity was then exposed by lateral incision through the top of the head, and pituitary, forebrain and hypothalamic section of the brain were transferred to RNA Later as before.

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. Follicle size distributions were determined by measuring the horizontal diameter of 100 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 5μm and stained with hematoxylin and eosin.

Plasma Steroid and Vitellogenin Measurement

Plasma levels of estradiol-17β (E_2), testosterone (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, 100µl of plasma were extracted with 1ml ethyl acetate, 100µl 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 74, 74 and 73% 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) 11.4, 6.1 and 7.8 (n=3), 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) 11.3% (n=7). Pooled internal standards were not used here due to the tendency of Vtg to denature following repeated freeze-thaw cycles.

Measurement of hepatic, brain and pituitary gene expression

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. Total RNA was extracted from whole pituitaries and (a minimum of 200mg) ovarian tissue using TRIZOL® reagent (Invitrogen, Carlsbad, CA); the volume of TRIZOL® reagent used was dependent on tissue weight, following the manufacturer's specifications. Four hundred nanograms of liver-derived RNA was 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. The same procedure was also used to produce cDNA from

pituitary and ovarian derived RNA except 1µg of total RNA was used. Following synthesis, cDNA was diluted by a factor of 5 (liver cDNA) or 2 (pituitary and ovary cDNA) with diethylprocarbonate treated water (DEPC) and stored at -20°C until use.

Partial isolation of the ZPC and GPR54 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 1.0). The 25µl PCR reaction contained 17.9µl PCR grade water, 2.5µl 10x PCR buffer, 0.5µl 10mM dNTPs, 2µl 25mM $MgCl_2$, 0.5µl 10µm forward primer, 0.5µl 10µm reverse primer, 0.1µl TAQ (Fisher Biotec, Australia) and 1µl liver derived cDNA template. Thermal cycling consisted of an initial denaturation step at 94°C for 1 min; followed by 34 cycles of 94°C for 30 seconds (s), 49°C for 30 s and 72°C for 4 min; with a final 10 min extension at 72°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 to be confirmed, http://www.ncbi.nlm.nih.gov/Genbank/).

To isolate G-coupled protein receptor 54 (GPR54) mRNA, the GPR54 nucleotide mRNA, sequences from cobia (*Rachycentron canadum*, GenBank accession number DQ790001), flathead mullet (*Mugil cephalus*, DQ683737), Nile tilapia (*Oreochromis niloticus*, AB162143), zebra fish (*Danio rerio*, EF672263) and fathead minnow (*Pimephales promelas*, EF672266) were aligned using ClustalW2 sequence alignment tool (http://www.ebi.ac.uk/) and degenerate primers (GPR54F7 and GPR54R6, Table 1.0) were designed from conserved regions. The fragment was amplified using the same cycling conditions used for ZPC except the annealing temperature was 47°C. The PCR product was then purified, cloned and sequenced as previously described for ZPC.

To isolate and characterise the 5' end of the GPR54 mRNA sequence, 5' amplification was performed using the SMART^{TM} RACE cDNA Amplification Kit (Clontech, Palo Alto, CA) combined with a gene specific reverse primer (GPR54R8, Table 1) according to the manufacturer's specifications. In this instance, the gene specific primer was designed from the partial GPR54 sequence isolated using GPR54F7 and GPR54R6. Once the 5' fragment had been amplified, it was purified, cloned and sequenced as previously described.

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	
GPR54	GPR54F7	CTC ATC ATG CTR GTS GGR CT	194bp
	GPR54R6	CAC ATG AAG TYS CCA AAK ATC CA	
	GPR54R8	TGA CCA GCG AGT TCC CAA TG	TBC

bp = base pairs, TBC = to be confirmed. 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, follicle stimulating hormone (FSH), GPR54, ovarian aromatase (P450aromA); 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 2.0). 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	TBC
	ZPCR4	AAC CTG TCA CTT TGG CAT CG			
FSH	FSHF1	GAG AGG ACT GTC ACG GAA GC	157bp	0.955	AF146152
	FSHR1	GAT GGA CAG CCT TCC AGG TA			
P450aromA	P450F1	TTC CAT CCC GTG GTG GAC TT	106bp	TBC	AF436885
	P450R1	TGC GGC CCA TGT TCA GAA T			
GPR54	GPR54F11	ACG CCT GGC TGG TGC CTC TCT TCT	137bp	TBC	TBC
	GPR54R10	GGC AGC CAG GTT AGC TAT GTA			
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, TBC = to be confirmed

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 4µl 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 5µl SYBR, 0.2µl 10µM forward primer, 0.2µl 10µM reverse primer, 1.6µl PCR grade water and 3µl 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 in-between run variability, respectively. The reference gene selected for expression normalisation was TBP as it showed outstanding 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

Pairwise comparison of means of morphometric and plasma hormone data (Samples 1 and 2) was made using an independent samples t-test, and multiple comparisons (subsequent sample times) using one-way ANOVA with subsequent comparison of means by Tukeys-b using the SPSS (version 15.0) 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/Discussion

Morphometric data

Body length and weight of maiden spawners was consistently lower than that of repeats (Fig. 2), irrespective of treatment group and sample time. This is to be expected given the 1 year difference in age between the spawning groups. Condition factor was fairly consistent among treatment groups with the exception of Sample time 1, when condition factor of maiden spawners at 14^{o}C (Group 1) was higher than that of repeats at 14^{o}C (Group 2) (Fig. 3). There was a general trend of increasing gonad weight, gonadosomatic index and follicle diameter with sample time across all treatment groups (Figs. 3 and 4); as is expected during this period of gonadal growth prior to gonadal maturation. Total fecundity was highest in repeat spawners (a function of body size) but there was no consistent difference in relative fecundity between repeats and maidens, and no discernible effect of temperature regime on fecundity (Fig. 5). In contrast, exposure to 22^{o}C resulted in a reduction in egg size in both maidens and repeats by sample 5 in April (Fig. 4) and there was no apparent ameliorating effect of treatment with GnRH.

Ovulation, Egg Fertility and Survival

Fish from Group 1 ovulated first, followed by a cluster of Groups 3,4,5, and then Group 2 (Fig. 6). Group 6 (repeats at 22^{o}C treated with GnRH) showed markedly delayed ovulation. Egg quality data (taken at ovulation) showed that fish at 22^{o}C (all ages and treatments) had markedly smaller egg diameter and volume (Fig. 7c,d) than fish at 14^{o}C. This is consistent with the smaller oocyte diameter measured from fish at 22^{o}C during autumn. Fertility was higher at 14 than all groups at 22^{o}C and as in the preliminary experiment conducted the year before (Appendix 3), there was less marked reduction in survival in repeats at 22^{o}C. Treatment with GnRH did not appear to have

provided any protective effect and in fact the lowest fertility was recorded from one of the GnRH groups (Group 6). (Fig.7e,f). The delay in progression to ovulation in fish held at 22°C is strongly consistent with earlier studies on both southern and northern hemisphere stocks of Atlantic salmon, and relates at least in part to thermal suppression of synthesis of the maturational steroid 17,20β-dihydroxy-4-pregnen-3-one (17,20βP) (reviewed in Pankhurst and King, 2009).

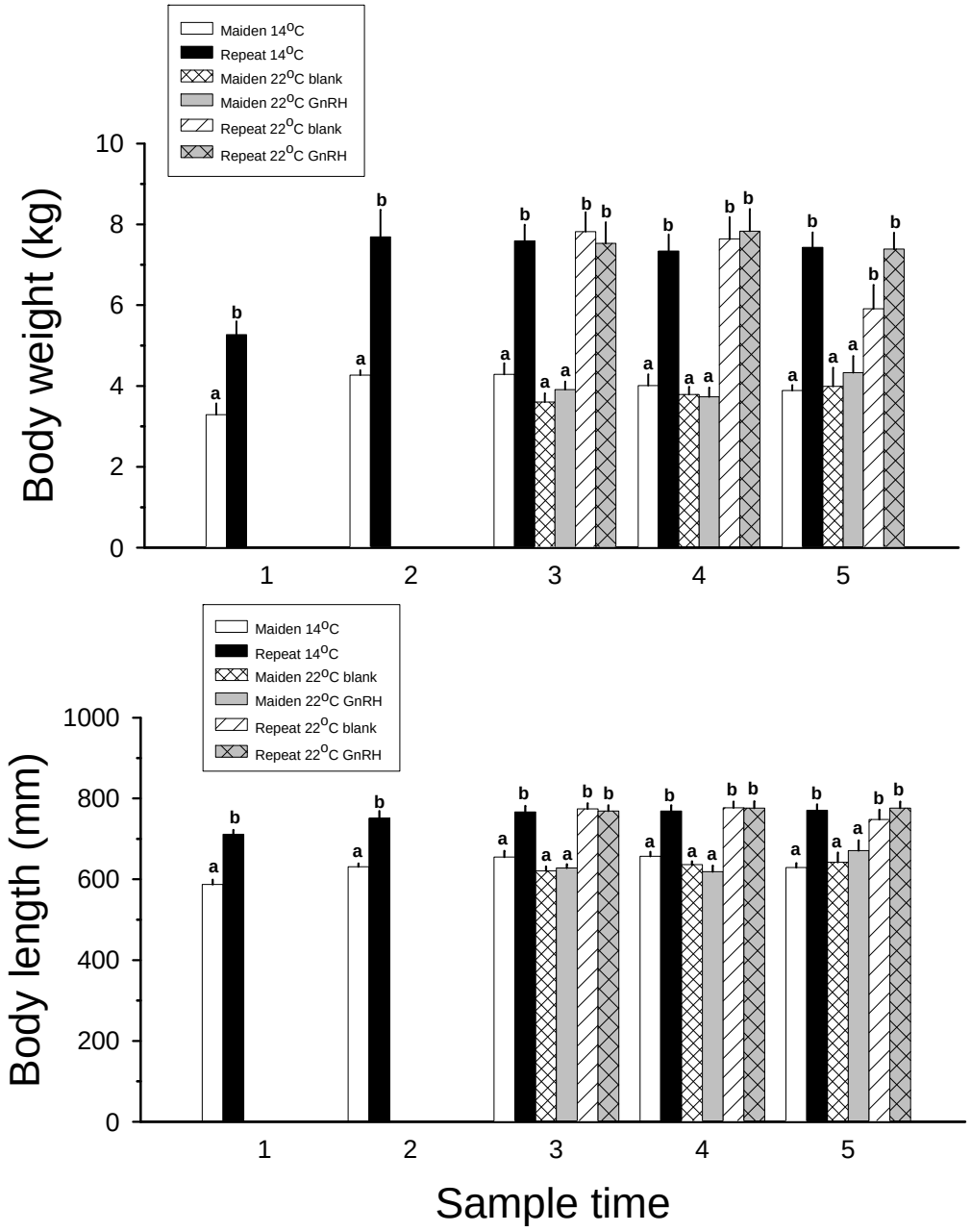

Figure 2. Mean (+ SEM) body lengths and weights of maiden and repeat spawners. **Sample 1 & 2**: fish exposed to normal phototherm prior to application of modified temperature and hormone treatments. **Samples 3-4**: fish exposed to one of two thermal regimes (14°C or 22°C) and either treated with a hormone pellet implant (Gonadotropin Releasing Hormone analogue: GnRH) or a blank pellet implant, since sample time 2. **Sample 5**: fish in all treatments exposed to a reducing temperature profile (thermal recovery) since sample time 4. Different superscripts among sample times denote significant differences.

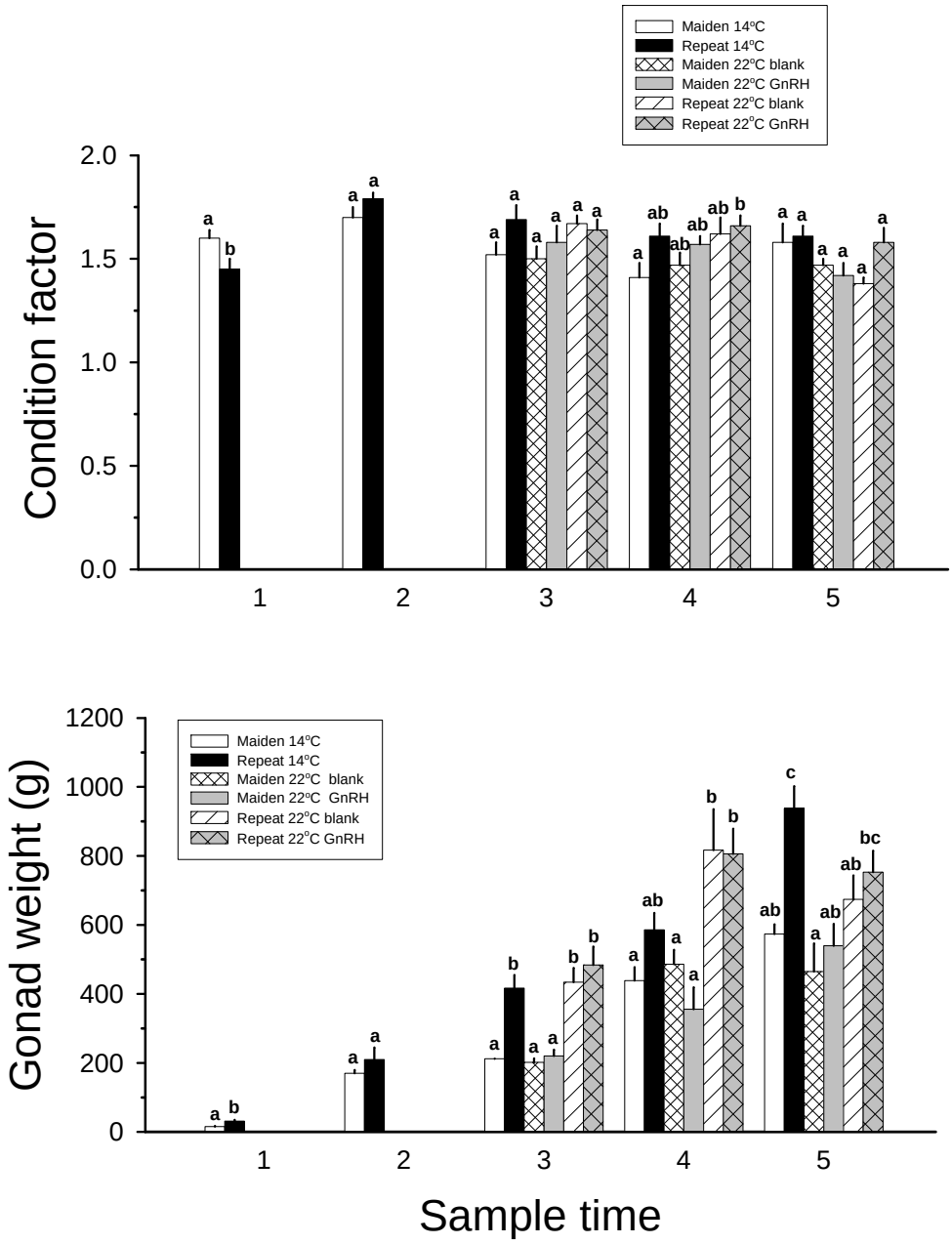

Figure 3. Mean (+ SEM) gonad weight and condition factor of maiden and repeat spawners. Other details as for Fig. 2.

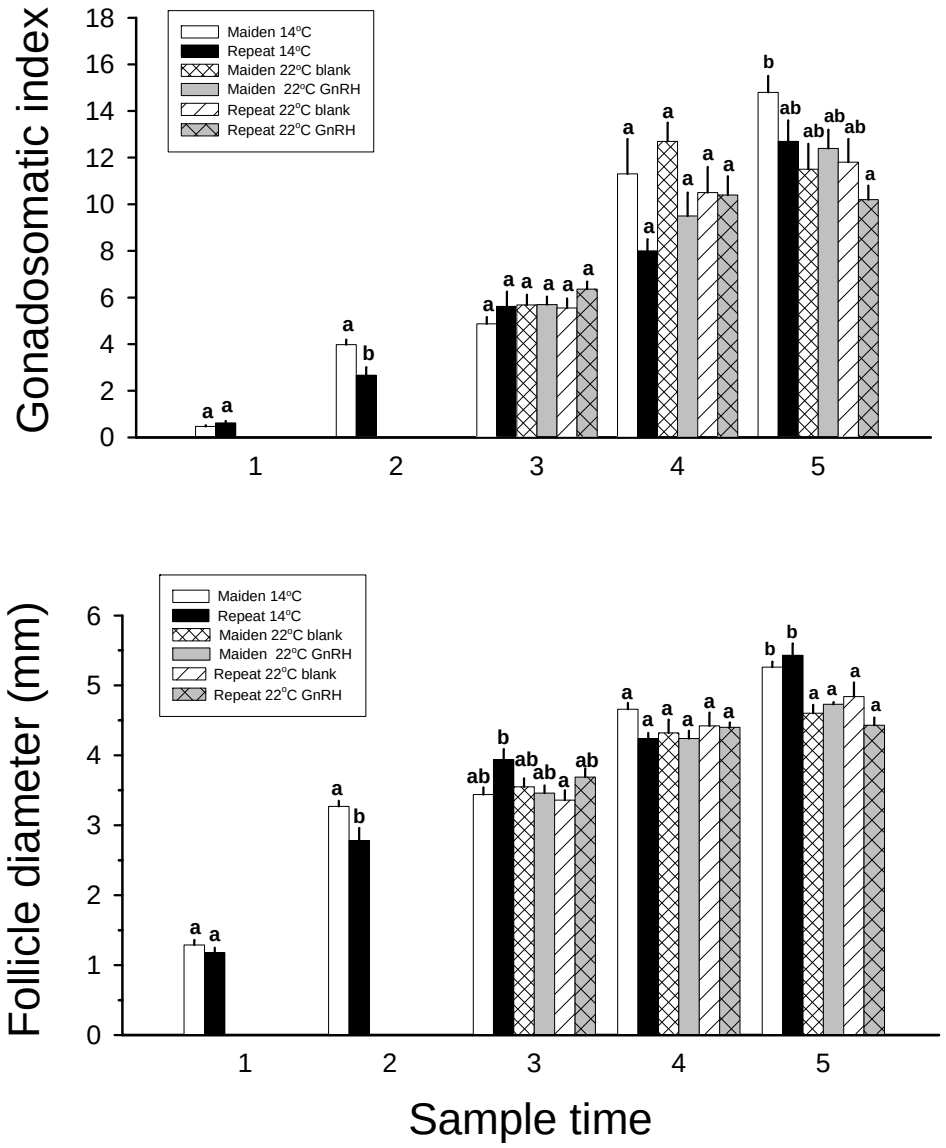

Figure 4. Mean (+ SEM) gonadosomatic Index and follicle diameter of maiden and repeat spawners. Other details as for Fig. 2.

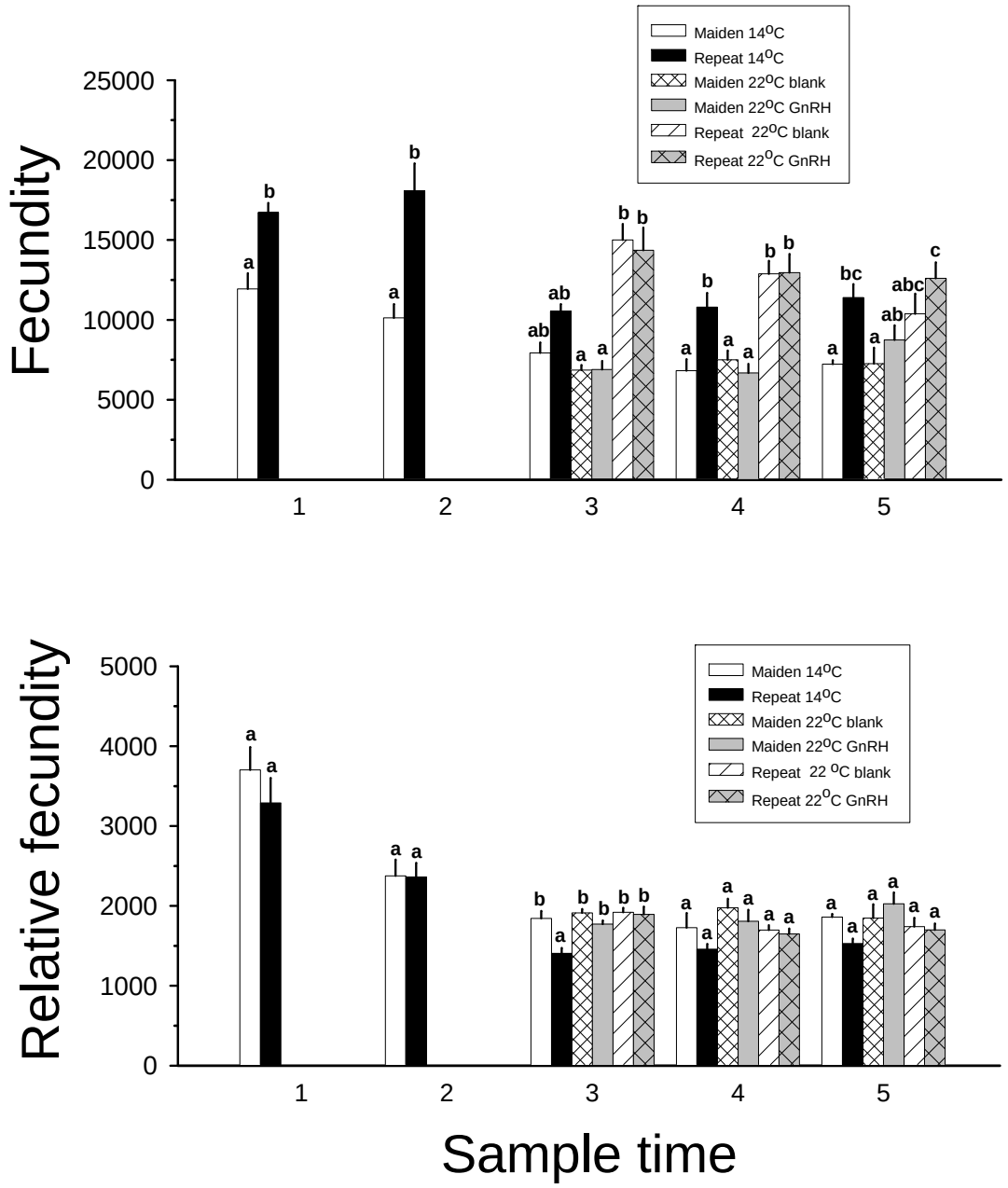

Figure 5. Mean (+ SEM) fecundity and relative fecundity of maiden and repeat spawners. Other details as for Fig. 2

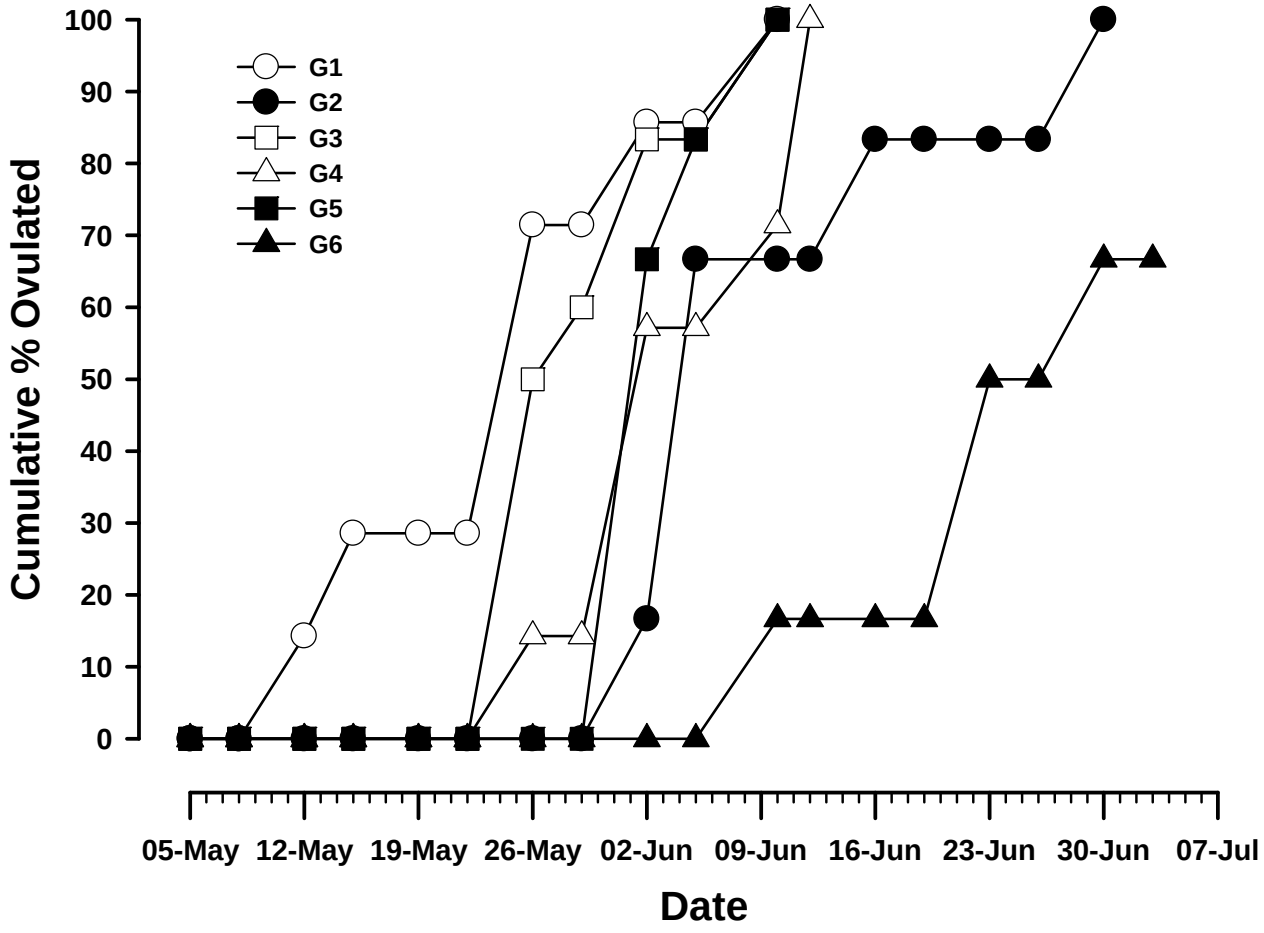

Figure 6. Cumulative ovulation of maiden and repeat spawners. G1 (Group 1) – maidens at 14°C; G2 – repeats at 14°C; G3- maidens at 22°C, G4- maidens at 22°C treated with GnRH; G5 – repeats at 22°C, and G6- repeats at 22°C treated with GnRH.

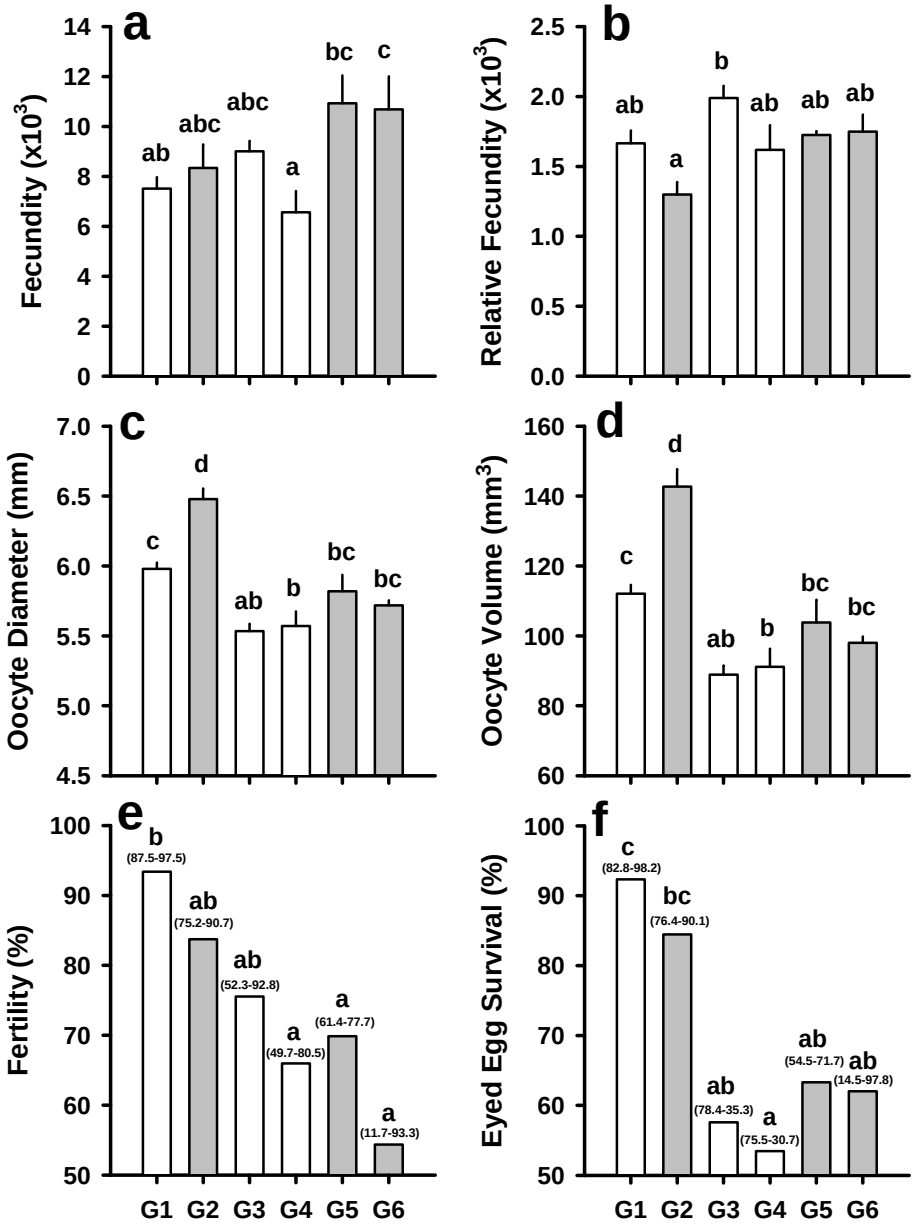

Figure 7. Fecundity, egg size (at time of stripping), fertility and survival data from maiden and repeat spawners. Other details as for Fig. 2.

Endocrine data

Plasma E_2 levels increased with maturity in a predictable way consistent with earlier studies (Fig. 8). By Sample 3, there was already suppression of plasma E_2 at 22°C in both groups of maiden fish, whereas E_2 levels in repeats were not suppressed at 22°C relative to 14°C. At Sample 4, plasma E_2 levels in maidens remained suppressed at 22°C and there was also suppression at 22°C in repeats treated with GnRH. At Sample 5, all 22°C groups were suppressed relative to maidens held at 14°C. Plasma T levels were less sensitive to exposure to elevated temperature with suppression at 22°C in maidens treated with GnRH at Samples 4 and 5, and both groups of repeats held at 22°C at Sample 5 (Fig. 8). There was no evidence of any ameliorating effect of GnRH on the effects of exposure to 22°C. This indicates that as in earlier studies, there is strong evidence for suppression of aromatase-mediated conversion of T to E_2, but also some impairment of T production. The failure of GnRH to ameliorate the effects of elevated temperature rests either in its lack of capacity to stimulate natural gonadotropin release, or the inability of the ovary to convert steroid precursors to E_2 due to the impairment of gonadal aromatase. Suppression of both E_2 and to a lesser extent, T suggests that both effects are present.

Plasma cortisol levels (Fig. 9) were elevated in maidens relative to repeats at Sample 1 reflecting the mechanics of capture from larger volume holding systems for that sample. There were no differences among groups at sample 2, 3 or 4 and values were generally low and at levels that would be expected from unstressed fish (Thomas *et al*., 1999). At sample 5, plasma cortisol levels were elevated in repeats at 14°C relative to all other groups. In common with data from the year before, differential effects of temperature did not appear to be exercised by activation of the hypothalamic-pituitary-interrenal axis, and subsequent stress suppression (reviewed in Pankhurst and van Der Kraak, 1997).

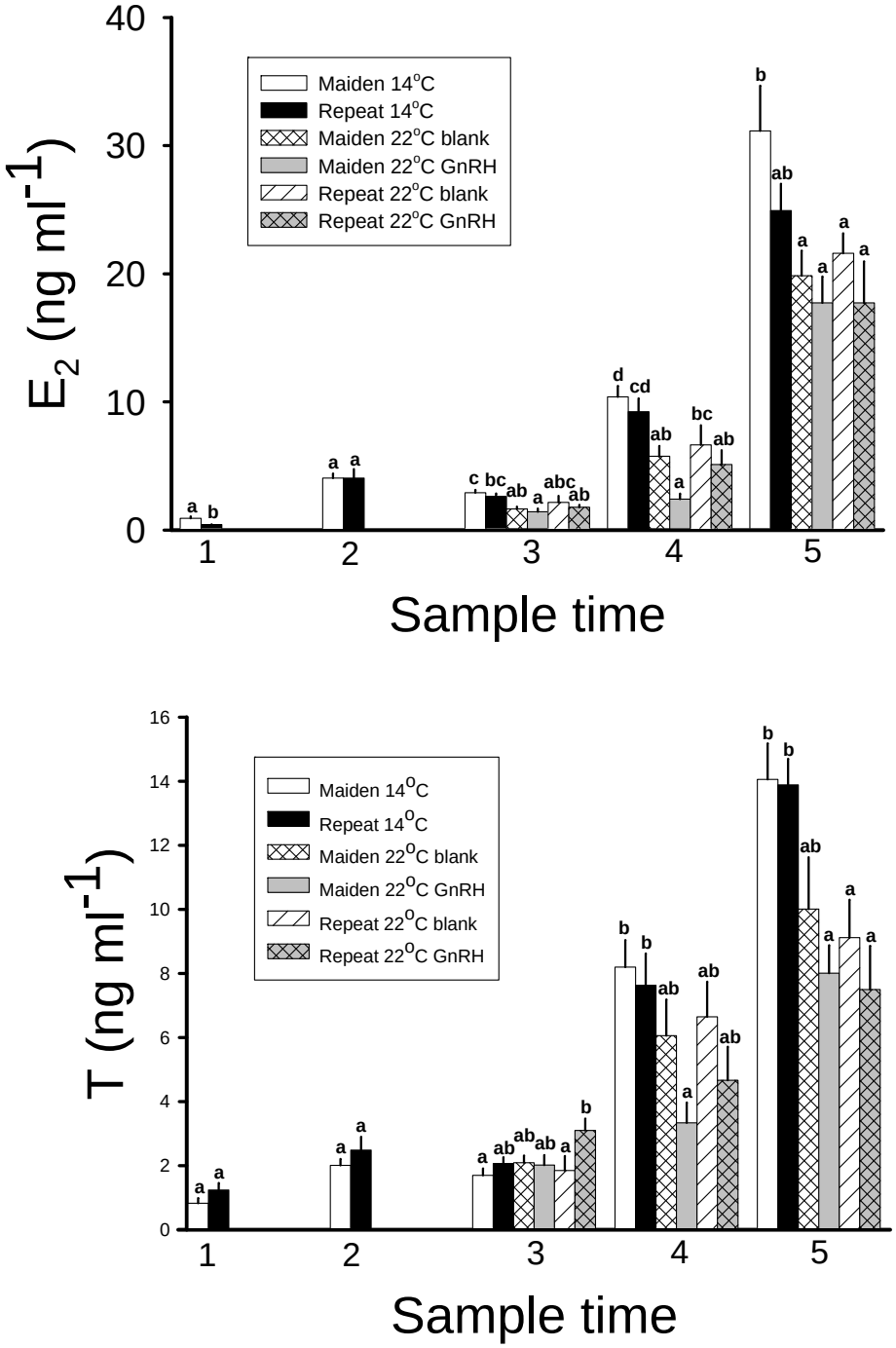

Figure 8. Mean (+ SEM) plasma estradiol (E_2) and testosterone (T) levels of maiden and repeat spawners. Other details as for Fig. 2.

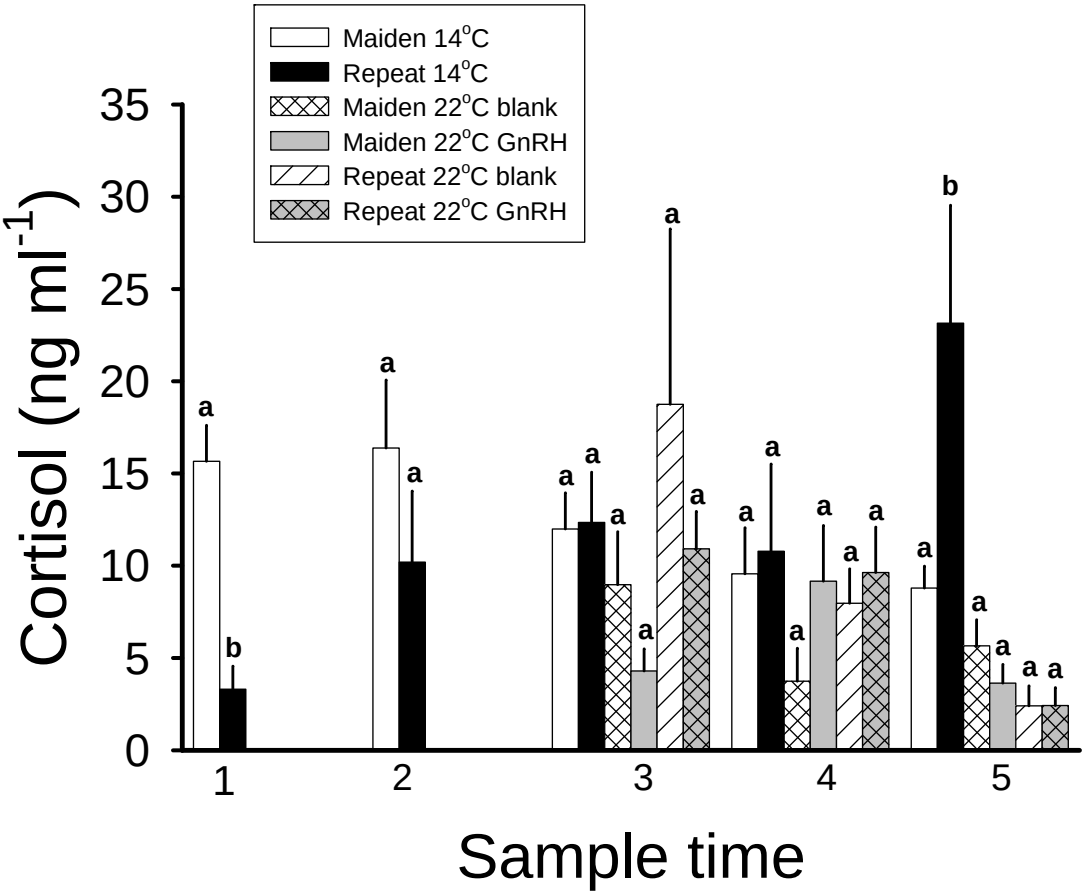

Figure 9. Mean (+ SEM) plasma cortisol levels of maiden and repeat spawners. Other details as for Fig. 2.

Plasma vitellogenin (Vtg) levels increased through vitellogenesis and were similar in maidens and repeats at all sample times until Sample 5, when levels in maidens were higher than in repeats (Fig. 10). At Sample 3, plasma Vtg was suppressed in 3 out of 4 groups at 22°C relative to 14°C fish. There was some recovery at Sample 4 with only group 6 (repeats at 22°C with GnRH) showing suppression. At Sample 5, there were no differences between fish at 14 and 22°C for either age class. These results are consistent with earlier studies where there is an initial suppressive effect of high temperature on Vtg levels in line with falling E_2, then a subsequent catch up in Vtg levels, even when E_2 levels remain suppressed (reviewed in Pankhurst and King, 2009). As with plasma steroid levels, there was no evidence of any protective effect of treatment with GnRH on plasma Vtg levels.

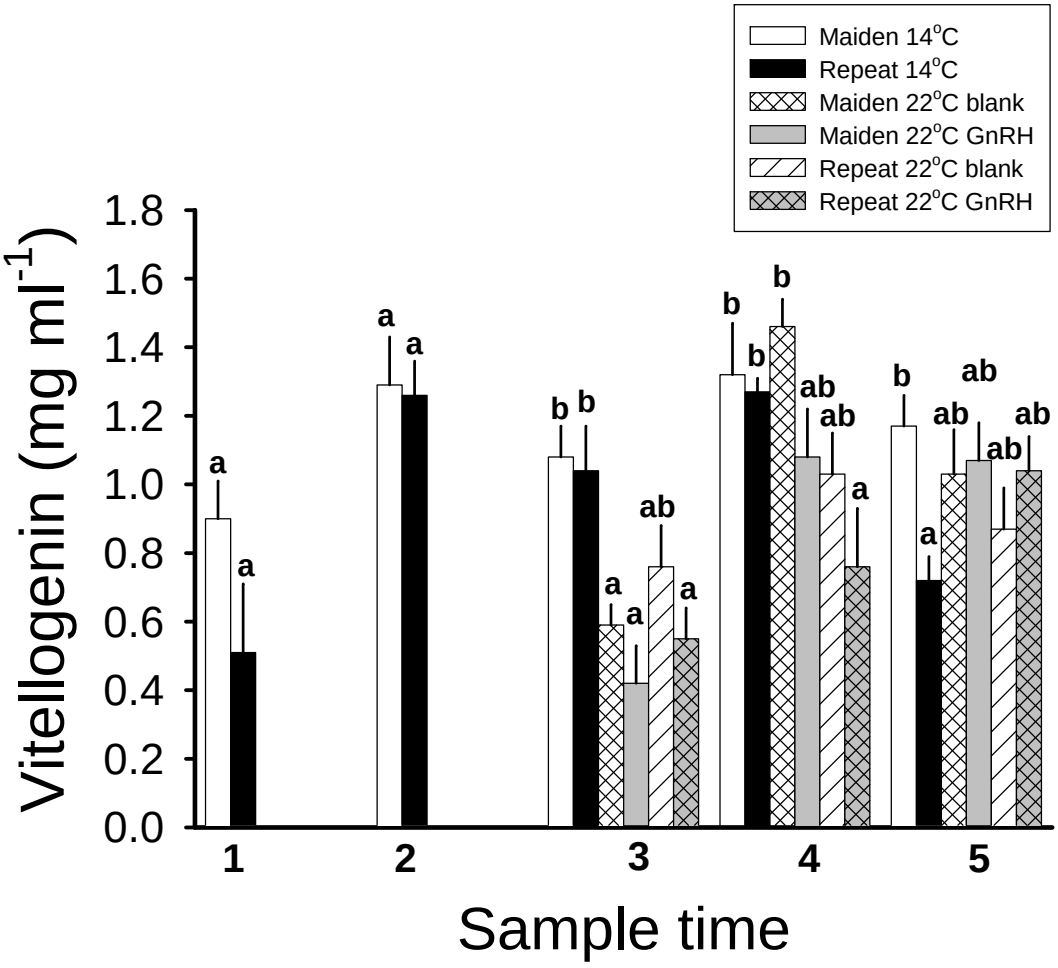

Figure 10. Mean (+ SEM) plasma vitellogenin (Vtg) levels of maiden and repeat spawners. Other details as for Fig. 2.

Gene expression data

The hepatic levels of relative zona pellucida B and C (ZPB and ZPC, respectively) gene expression were determined to assess whether suppressed estrogen production among fish at high temperature was also reflected in reduced hepatic expression of estrogen-responsive genes. In February (Sample 3), ZPB expression was significantly reduced in maiden fish at 22°C receiving GnRH implantation relative to maidens at 14°C, and both groups of repeats at 22°C showed suppression relative to repeats at 14°C. At Sample 4 (March), ZPB expression was suppressed in both groups of maidens held at 22°C, and repeats at 22°C treated with GnRH. At sample 5 (April), there were no differences between treatments (Fig. 11). At Sample 3, ZPC expression was higher in repeats than maidens at 14°C but there were no temperature-related differences in expression. There was suppressed ZPC expression in maidens held at 22°C treated with GnRH, relative to

maidens at 14°C at Sample 4 (Fig. 12). In April (Sample 5), the same effect was present. There were no differences among fish held at 22°C.

Hepatic levels of relative Vtg gene expression increased markedly from sample 1 to 2 (November and January samples) in line with increasing plasma levels of Vtg and increase in oocyte size (Fig. 13). In February (Sample 3), Vtg expression was suppressed in repeats at 22°C but this effect appeared to be offset by treatment with GnRH. This was the only case where GnRH treatment appeared to have any protective effect. At Sample 4 (March), Vtg expression was suppressed in GnRH-treated maiden fish at 22°C, compared to the 22°C maiden control group, but not maidens at 14°C. There were no differences among fish held at 22°C. In April, maidens held at 22°C and treated with GnRH showed suppression relative to maidens at 14°C, and both groups of repeats held at 22°C showed suppressed expression relative to repeats at 14°C.

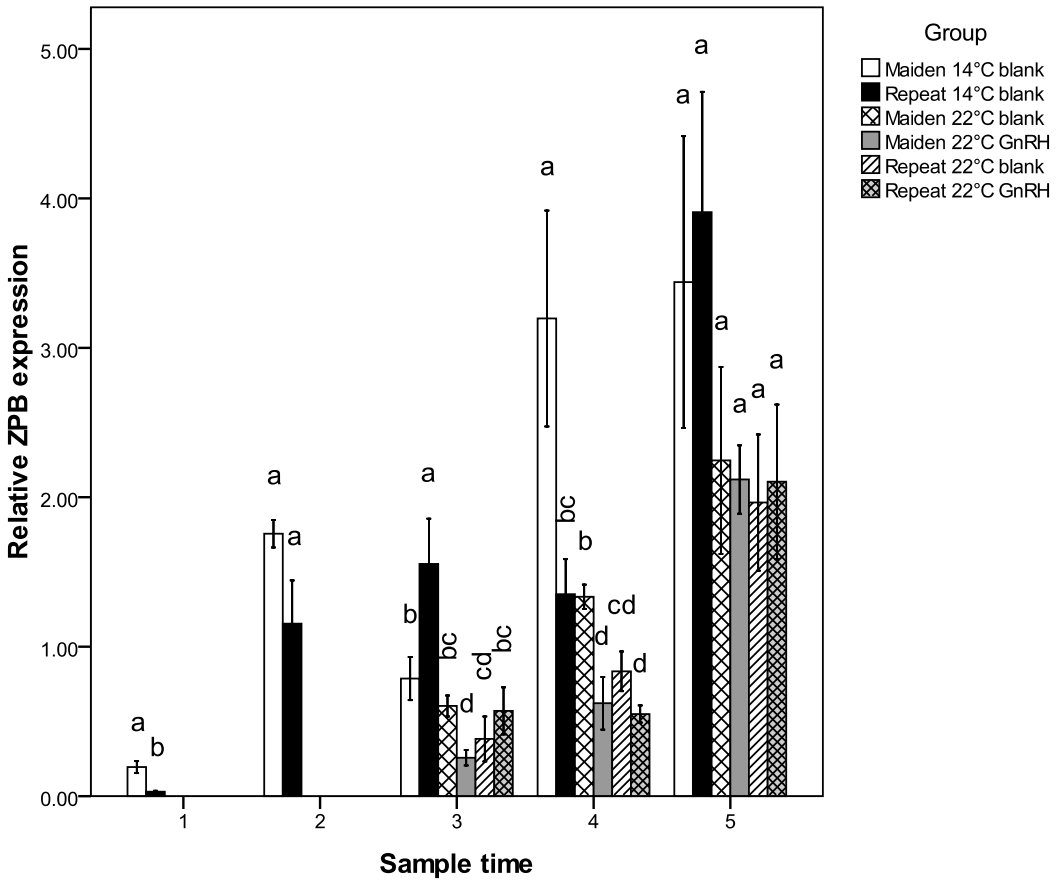

Figure 11. Mean (± SEM) relative zona pellucida B gene expression in maiden and repeat spawners. Other details as for Fig. 2.

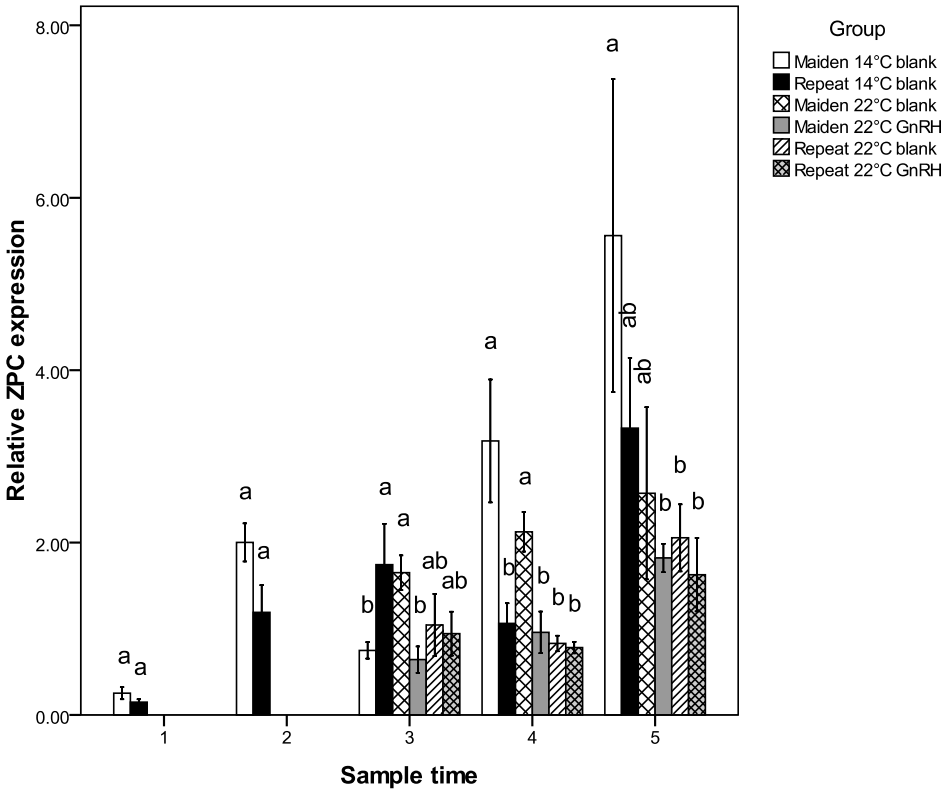

Figure 12. Mean (± SEM) relative zona pellucida C expression in maiden and repeat spawners. Other details as for Fig. 2.

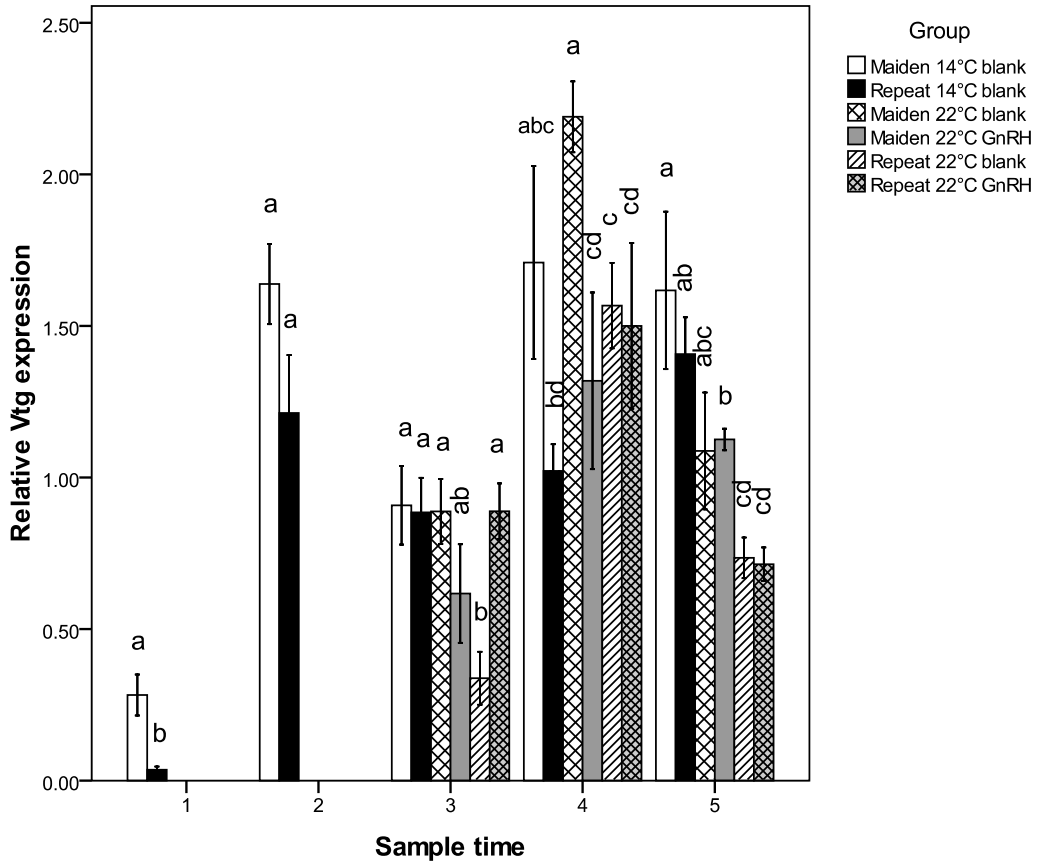

Figure 13. Mean (± SEM) relative vitellogenin expression in maiden and repeat spawners. Other details as for Fig. 2.

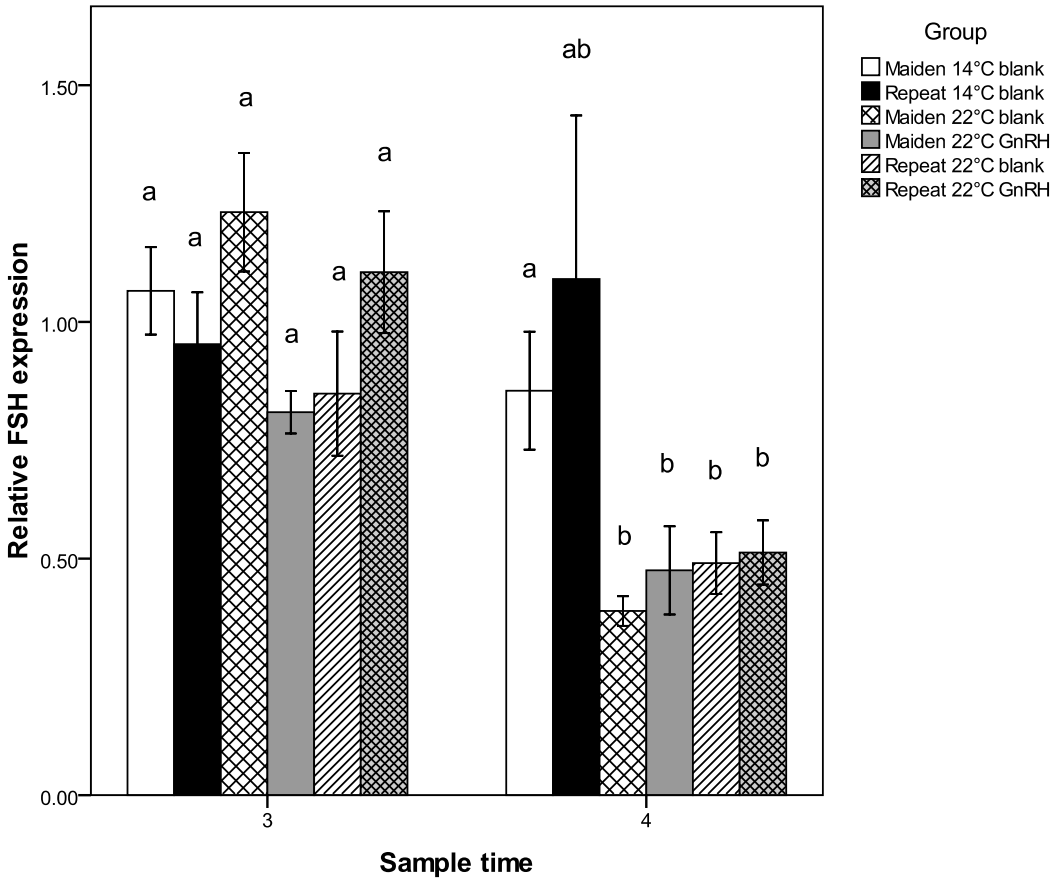

Figure 14. Mean (± SEM) relative FSH expression in maiden and repeat spawners. Other details as for Fig. 2.

The relative gene expression levels of follicle stimulating hormone (FSH) in the pituitaries of maiden and repeat spawning fish are shown in Fig. 14. There were no differences among groups at Sample 3 (February) but there was suppression of FSH expression in all groups at 22°C relative to maidens at 14°C, at the March sample (Sample 4, Fig. 14). Treatment with GnRH implants had no protective effect on FSH expression in either maiden or repeat fish.

Collectively, the expression data show that there is inhibition of estrogen-dependent gene expression that approximately mirrors the falls in plasma E_2 in response to exposure to elevated temperature, and also the reductions in plasma levels of Vtg. These effects could arise from the reduction in circulating levels of E_2 alone, or the additional effects of temperature modulation of estrogen receptor (ER) dynamics and downstream events after E_2-ER binding and translocation to estrogen response elements of target genes. We have already shown that ER dynamics are altered at certain stages of vitellogenesis in fish exposed to high temperature (Watts *et al.*, 2005) but assessment of

the estrogen dependence of impaired ZP and Vtg gene expression requires further investigation. The demonstration of suppression of FSH expression in all groups at 22°C also confirms that events in the endocrine cascade upstream of gonadal steroid synthesis are also impaired by thermal stress. There is limited evidence for this effect in other species. Studies on non-salmonids show that exposure to elevated temperature can reduce expression of genes coding for GnRH and it pituitary receptor, and the LH β-subunit (LHβ) in red sea bream (Okuzawa *et al*., 2003), and LHβ and the ovarian receptor for follicle stimulating hormone (FSH) in pejerrey (Soria *et al*., 2008).

Our intention in the present study was to attempt to offset one of the strong effects of exposure to elevated temperature during vitellogenesis (the inhibition of the production of E_2), by treatment with doses of GnRH below the threshold to trigger maturational events. GnRH is primarily thought to increase plasma levels of LH. For example, Breton *et al*. (1998) found that rainbow trout treated with 40 μg kg^{-1} D-Arg^6 GnRH showed no effect in terms of changes in plasma levels of FSH, but predictable and marked increases in plasma LH, with the largest effect being seen in fish at the end of vitellogenesis. However, *in vitro* studies by Dickey and Swanson (2000) showed that GnRH can stimulate both the expression of FSHβ subunit mRNA and the release of FSH into the medium in perfused pituitary preparations from coho salmon. The means that fish at early stages of maturity probably have the capacity to respond to stimulation with GnRH with an increase in both the synthesis and release of FSH. Under these conditions, follicles should respond with increased production of E_2 and subsequent increased vitellogenin and egg shell protein synthesis by the liver, provided that aromatase activity is not compromised. GnRH may also stimulate E_2 production even without activation of FSH synthesis and release. In both cyprinids (Van Der Kraak *et al.*, 1992) and salmonids (Swanson, 1991), FSH and LH are approximately equipotent in the stimulation of the synthesis of estrogen and androgen synthesis *in vitro*, and GnRH does generate initial increases in plasma E_2 *in vivo* in Atlantic salmon prior to subsequent increases in T and then 17,20βP (King and Pankhurst, 2007). Crim *et al*. (1986) reported a similar effect of GnRH in advancing the vitellogenic state of a Canadian stock of Atlantic salmon.

In this context, the failure of GnRH to provide significant protection in thermally stressed Atlantic salmon in the present study is somewhat unexpected but suggests that

there was possibly impairment of GnRH receptor-ligand interaction as reported by Okuzawa *et al.* (2003) in sea bream, and that GnRH stimulation was unable to overcome the measured thermal depression of FSH expression and aromatase activity. Dose and delivery choices are unlikely to have explained the lack of effect of GnRH given the stable release profile generated by the Syndel product. The Ovaplant ™ pellet has a medium-term release profile with 50% release *in vitro* at 10^{o}C over 3 days and remaining release over a period of 21 days (unpublished Syndel data supplied by Jim Brackett, Syndel). We have yet to determine whether direct administration of exogenous E_2 has the capacity to offset the damaging effects of exposure to high temperature. It is clear from the present experiment that treatment with GnRH alone is not an effective strategy for enhancing endogenous production of E_2 and if as the present study suggests, multiple endocrine events upstream of E_2 production are impaired by exposure to elevated temperature, it is possible that direct administration of E_2 is required.

Benefits and Adoption

The variable spawning success of Atlantic salmon broodstock leaves the Atlantic salmon aquaculture industry with the ongoing problem of lack of predictability of egg production, and a difficult management challenge – increase the number of maiden spawners (with the associated cost of expanded scale of holding and rearing facilities) or increase the hold-over of fish to become repeat spawners with the associated temporal expansion of costs. The most desirable situation would be for industry to hold maiden spawners under conditions that generate egg production rates typical of those seen in repeat spawners. A solution to this problem resolves the annual production uncertainty that currently faces the industry as a result of unpredictable egg quality and survival.

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 with the stated SALTAS aim of reducing the reliance on repeat spawning fish for egg production. The potential cost of failing to solve the problem is high. The survival of eggs to the eyed embryo stage can be as low as 30-50%, compared with 80% for eggs from best performing fish (SALTAS data). Modelling of this cost gives direct increases in smolt production costs of $225,000 per annum, but a potential industry shortfall in production terms of $15-20 million per annum.

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. Use of repeat spawning fish will offset this to some extent, but the understanding that the effects are exercised on all age classes will allow hatcheries to model and 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 hormonal manipulation can be used to offset the effects of thermal inhibition during vitellogenesis. The GnRH treatment used in the present study was apparently ineffective despite the fact that FSH synthesis was maintained in fish held at 22^{o}C. This confirms our earlier view that the key blockade in the endocrine cascade is in estrogen synthesis, probably through the suppression of aromatase activity. It remains to be tested whether this can be offset by estrogen therapy for female broodstock during the critical period of ovarian development during late summer and autumn. 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: *Understanding of the basis of reproductive and egg shell development in maiden and repeat spawning salmon.* The comprehensive morphometric and endocrine description of reproductive development in maiden and repeat spawning fish produced here provides the first comparison of reproductive performance in maiden and repeat spawning Atlantic salmon.

Planned outcome 2: *Understanding of the effect of thermal stress on salmon broodstock of different age classes.* The project has clearly demonstrated the differential effects of thermal stress on fish of different age classes and highlighted the greater sensitivity of maidens to thermal stress in terms of egg survival.

Planned outcome 3: *Capacity to manipulate salmon stocks to achieve desired fertility outcomes.* Differential age class sensitivity provides another management strategy for decisions in relation to thermal protection of fish, especially where facility capacity is limited, or where temperature control is not an available option.

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 greater certainty of sustained, high quality egg production for the salmon industry.

Planned outcome 5: *A management toolbox relevant to salmonid culture at any location where broodstock are potentially affected by climate change.* This has been partially achieved in terms of identification of timing and nature of physiological insult, the physical conditions required to protect fish from such insult and the level to which different age classes are likely to be effective. Development of an effective hormonal therapy for managing thermal exposure still remains to be completed.

Planned outcome 6: *Uptake of technical knowledge gained into the salmon production sector*. This has occurred through industry adoption through onsite technology transfer at SALTAS and further dissemination through FRDC Atlantic Salmon Aquaculture Subprogram meetings with industry and other research providers.

Conclusion

This project has generated the first comparison of morphometric and hormonal development in maiden and repeat spawning Atlantic salmon. It has identified that there is an extensive period of ovarian reorganisation in repeat spawning fish during summer and early autumn, and that initially, ovarian growth is slower and less ordered in repeats than in maiden fish. There is a catch up phase in February, and by March, both groups of fish are following the same developmental pattern. Repeat spawners have higher absolute fecundity as a result of their larger size but there is no difference in relative fecundity, i.e. both age classes are making the same relative investment in reproduction.

In both age classes, there is depression of the plasma steroids E_2 and to a lesser extent T in February and March in fish exposed to 22^{o}C. This is accompanied by successive falls in ovarian size, and oocyte diameter at 22^{o}C and increased incidence of ovarian atresia. The effects tend to be more severe in repeat spawning fish. Gene expression studies showed that suppression of estrogen synthesis was reflected in impairment of the downstream processes of vitellogenin synthesis and release, and the assembly of zona radiata (egg shell) proteins. These are all effects that would be predicted to result in compromised egg viability.

Exposure of fish in late March to a reducing temperature profile lead to some degree of ovarian recovery with all fish advancing to oocyte maturation and ovulation, but with considerable delay in fish exposed to high temperature. Fish held at 22^{o}C had lower egg size, fertility and survival but in contrast to the endocrine parameters, the effects were most marked in maiden fish with intermediate effect being expressed in repeats at 22^{o}C. It is not entirely clear why this should be so, given the high degree of similarity of endocrine and morphometric effects on the different age classes, but may relate to the fact that eggs of repeats tend to be a little larger. There may simply be protection

provided by egg size, and the greater level of maternal endowment through Vtg that this carries.

Treatment with GnRH was ineffective at offsetting the inhibitory effects of elevated temperature and this is probably due to the fact that there is thermal inhibition of multiple steps in the endocrine cascade that cannot be overcome by stimulation of endocrine processes upstream of aromatase conversion of T to E_2. GnRH was chosen as a management option because of the relative ease with which treatment can occur in the hatchery situation. Its lack of effect means that direct treatment with E_2 now needs to be tested as an alternative.

The management implications of the study include 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 means that hatcheries need to engage in strategies that offer thermal protection for fish during summer and autumn. In situations where temperature control is not possible at all, or only available for a portion of the maturing stock, then use of repeat spawning fish offers some level of protection against thermal damage but with the caveat that production losses due to reduced egg fertility and survival are still likely to occur.

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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:

- Description of the mode and timing of reproductive development in repeat spawning fish from the Tasmanian stock of Atlantic salmon;
- Description of the structural changes occurring during ovarian development and recrudescence in maiden and repeat spawning fish, respectively;
- The relative effects of thermal stress on morphometric development, ovarian structure, plasma steroid and vitellogenin (Vtg) levels, ovarian expression of Vtg and zona radiata protein (ZP) genes, and pituitary expression of the FSH gene in maiden and repeat spawning Atlantic salmon;
- The differential sensitivity of maiden and repeat spawning fish to thermal stress in terms of egg fertility and survival;
- The lack of effectiveness of GnRH 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.

Appendix 2: Staff

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Appendix 3: Summary of preliminary experiment

A preliminary experiment was conducted in advance of FRDC project 2008/217: Effect of temperature on reproductive development of maiden and repeat spawning Atlantic salmon – understanding the basis for improved egg survival and quality. This project was funded directly by SALTAS, Griffith University and the University of the Sunshine Coast, and established the basis for the subsequent FRDC project. This work is currently under preparation for publication but is presented here in summary form.

Methods

Groups of maiden or repeat spawning fish were set up at Saltas Wayatinah in July 2007 under ambient conditions in 200m^3 and 50m^3 tanks, respectively. Seven fish from each group were destructively sampled on 31/8/07, 2/11/07 and 7/1/08 to establish the developmental profile of each fish cohort under normal ambient spring conditions. At each sample time, blood, ovary and liver samples were collected. Plasma was separated from blood for analysis of plasma steroids and liver samples were stabilised in RNA Later™ for analysis of gene expression. Ovarian tissue was sampled for histology and the direct measurement of ovarian follicle size and fecundity. After the January sample, each group (maidens or repeats) was further divided into maintenance at 14^{o}C or 22^{o}C through until April when all fish were dropped back to a spawning temperature of 8^{o}C (Fig. 1). Subsequent destructive sampling of 7 fish from each of the four groups occurred on 14-15/2/08, 27-28/3/08, and 24-25/4/08. A remaining sample of fish was allowed to complete ovulation (June and July 2008) for generation of cumulative % fish ovulated, fecundity, fertility, oocyte diameter and volume, and egg survival data. Sampling protocols and analytical methods are as described in the body of the report.

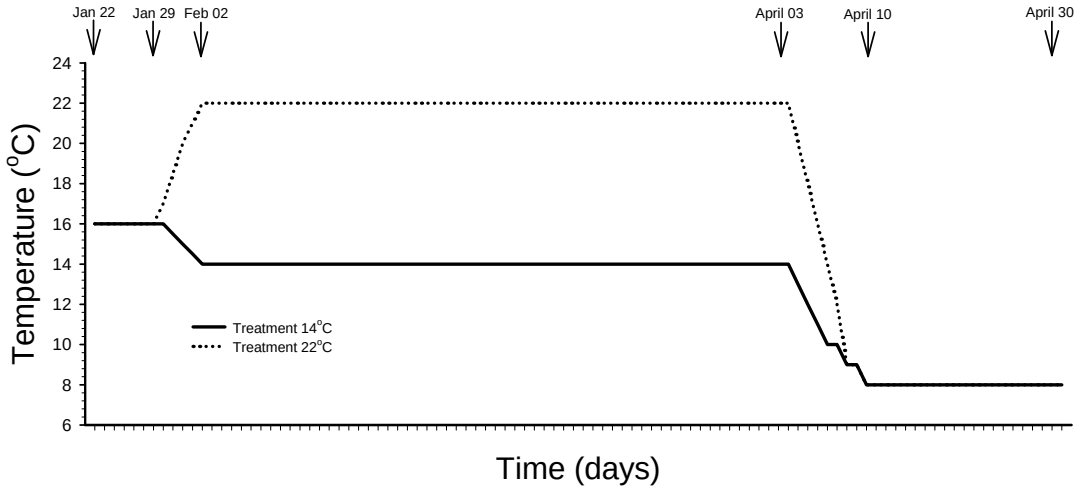

Figure 1. Thermal adjustment protocols for Atlantic salmon maiden and repeat spawners in 14^{o}C and 22^{o}C treatments in 2008 experiments.

Results and Discussion

There was no difference in body weight within groups for maidens and repeats, with repeats being consistently larger than maidens (Fig.2). Condition factor (CF) was initially poorer in the repeats than the maidens but these fish had recovered by sample 3 and there was no difference in CF thereafter (Fig. 3). Gonad weights were consistently higher in repeats than maidens, and there was a depressive effect of elevated temperature that became apparent in samples 5 and 6 (Fig. 3). Gonadosomatic index (GSI) was depressed in repeat spawners at 22^{o}C by sample 6, and follicle diameter was smaller in both groups at 22^{o}C at sample 5, and in repeats at 22^{o}C at sample 6 (Fig. 4) indicating that elevated temperature was having a suppressive effect on oocyte growth, and that the effect was more marked in repeat spawners.

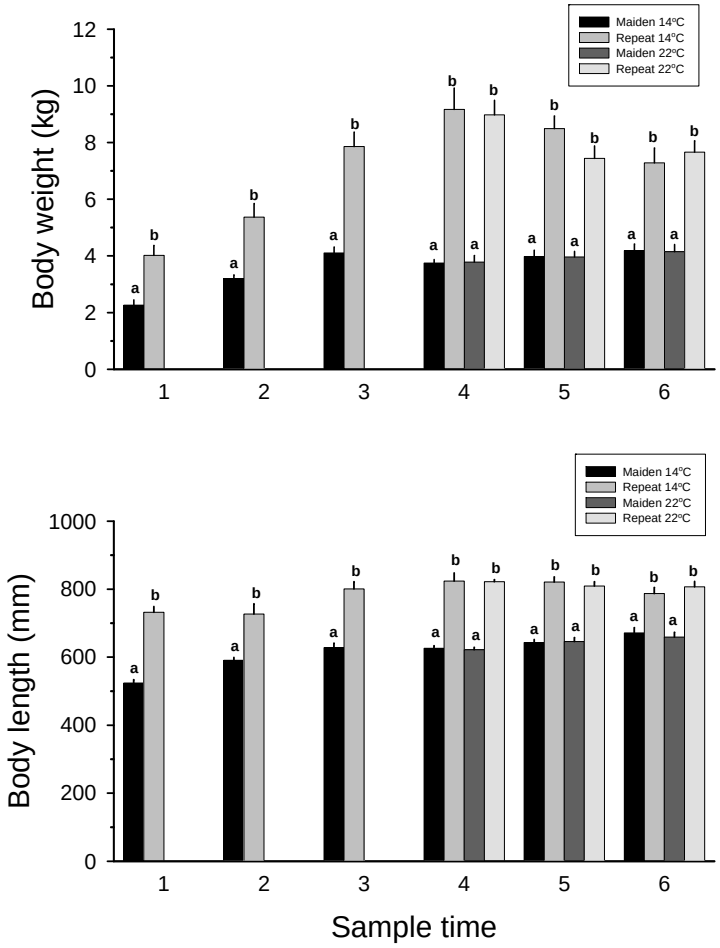

Figure 2. Mean (± SEM) body lengths and weights of maiden and repeat spawners exposed to one of two thermal regimes (14°C or 22°C), prior to spawning. Note that temperatures for all fish were adjusted downward to 8°C commencing after sample 5. Different superscripts among sample times denote significant differences.

Absolute fecundity determined from ovarian tissue samples was higher in repeats at all times except in fish held at 22°C by sample 6. Relative fecundity (egg.kg^{-1}) determined from ovarian tissue samples was variable but again showed depression in repeats at 22°C by sample 6 (Fig. 5). This is consistent with the egg size data and suggests that repeats might be less robust in the face of elevated temperature than maidens. Sample follicle size distributions are shown for maiden and repeat spawning fish held at 14°C (Fig. 6) and 22°C (Fig. 7). There are no obvious differences between Groups, or in response to thermal exposure; however, there appears to be a high level of oogenic activity and follicle recruitment occurring in January.

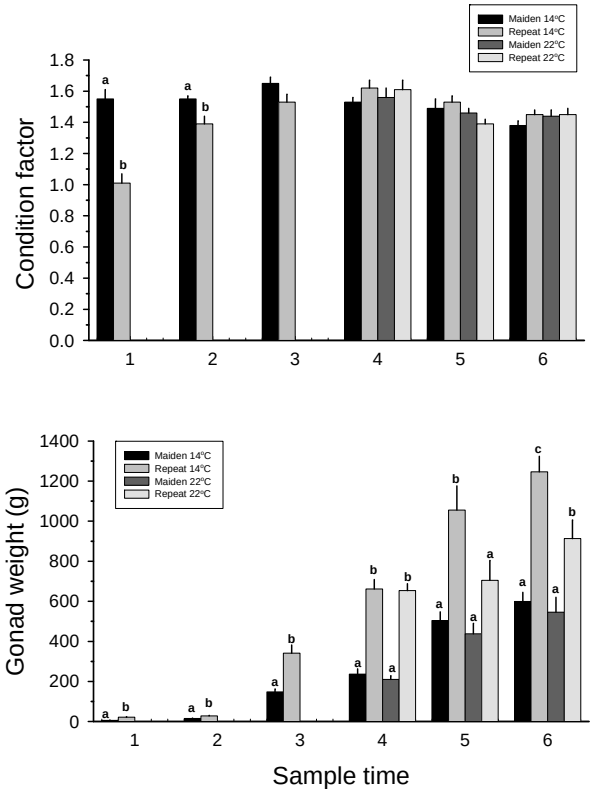

Figure 3. Mean (± SEM) Gonad weights and Condition Factors of maiden and repeat spawners exposed to one of two thermal regimes (14°C or 22°C), prior to spawning. Other details as for Fig. 2.

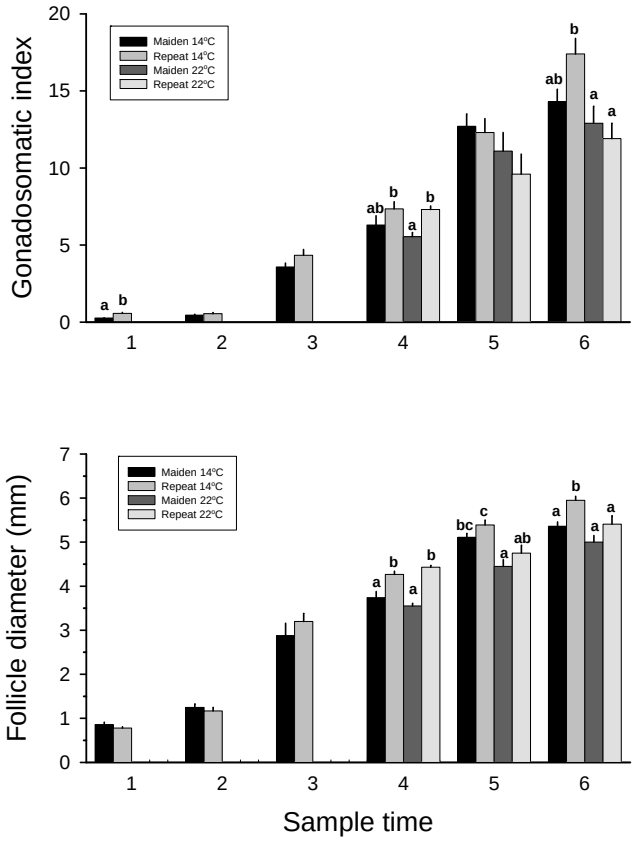

Figure 4. Mean (± SEM) Gonadosomatic index and Follicle diameter of maiden and repeat spawners exposed to one of two thermal regimes (14°C or 22°C), prior to spawning. Other details as for Fig. 2.

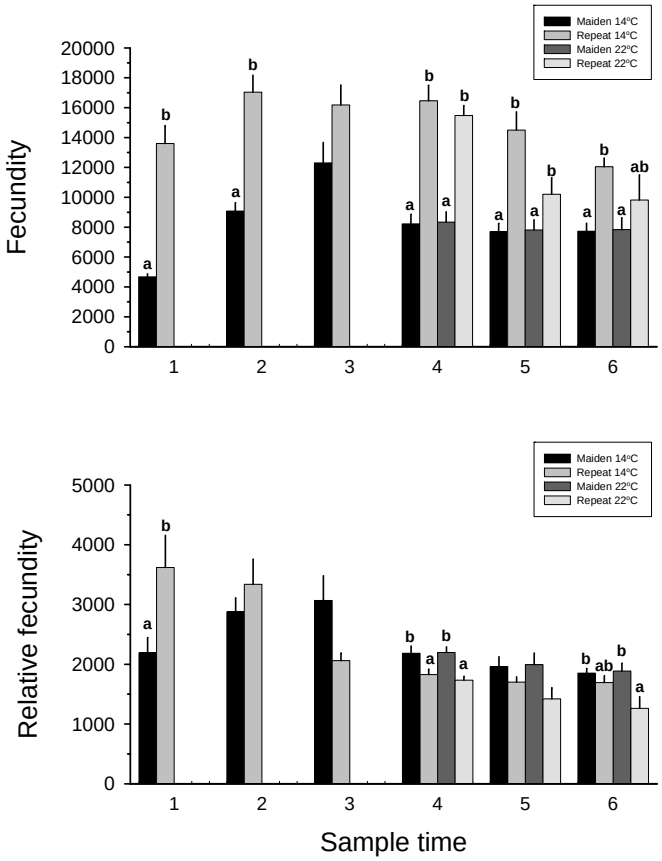

Figure 5. Mean (± SEM) Fecundity and relative fecundity of maiden and repeat spawners exposed to one of two thermal regimes (14ºC or 22ºC), prior to spawning. Other details as for Fig. 2.

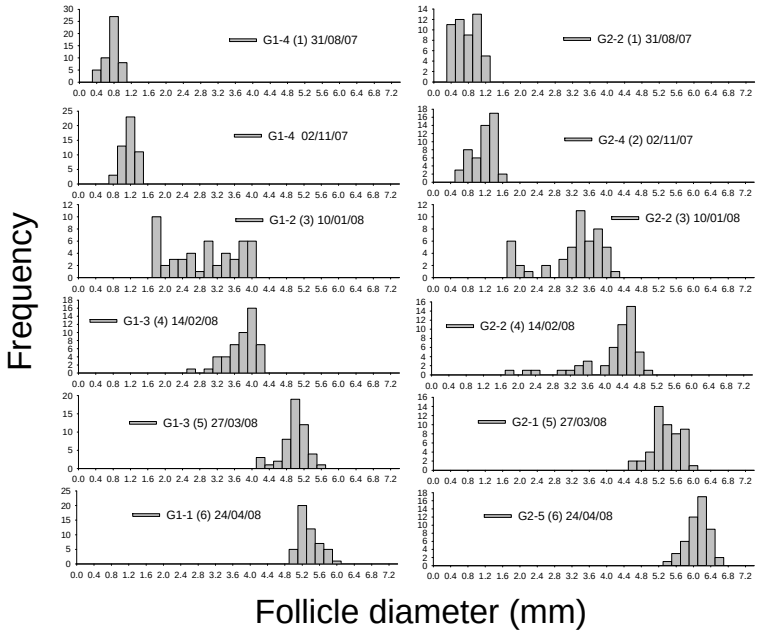

Figure 6. Follicle size distribution for individual maiden and repeat spawners held at 14ºC. Fish shown have mean follicle diameters that approximate the group mean for that sample time. G1-maidens 14ºC, G2-repeats 14ºC.

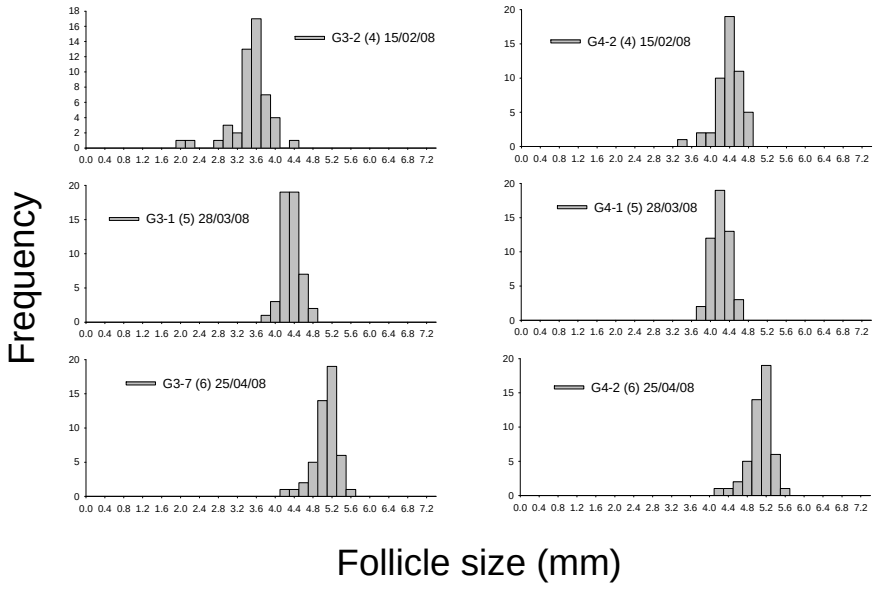

Figure 7. Follicle size distribution for individual maiden and repeat spawners. Fish shown have mean follicle diameters that approximate the group mean for that sample time. G3-maidens 22ºC, G4-repeats 22ºC.

Plasma levels of estradiol-17β (E_2) and testosterone (T) were measured as primary markers of endocrine response to elevated temperature. Plasma E_2 levels were strongly depressed in both maidens and repeats exposed to 22°C at samples 4, 5 and 6 (February, March and April) but with some evidence of recovery in E_2 levels in maiden fish at the April sample (Fig. 8). The same effect was seen in T levels; however, here repeat fish at 14°C also show some evidence of depression of T levels in March (Fig. 8). The thermal effects of elevated temperature in depressing plasma E_2 levels are strongly consistent with our previous work on maiden fish (King et al., 2003, 2007; Watts et al., 2004), and show that there is no apparent extra protection provided by using larger, repeat spawning fish.

Plasma cortisol levels were measured as an indicator of the primary stress response. Stress can itself have marked inhibitory effects on reproductive processes (Pankhurst and van Der Kraak, 1997), raising the question as to whether thermal effect simply reflected the effects of stress on exposed fish. Plasma cortisol levels were only elevated in maiden fish at 14°C in the early stages of the experiment and this related to crowding of fish prior to capture (Fig. 9). In thermally manipulated groups in samples 4-6 (when fish were showing signs of thermal inhibition of reproduction), cortisol levels were low and typical of levels in unstressed fish (<10 $ng.ml^{-1}$) (Thomas et al., 1999) and not different between treatment groups. This indicates that the effects of elevated temperature are not simply being exercised through the effects of stress.

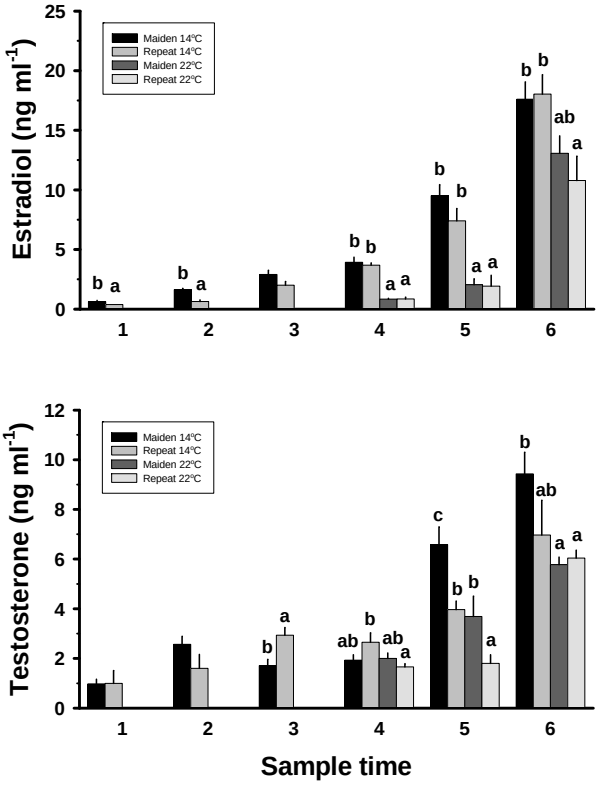

Figure 8. Mean (± SEM) plasma levels of Testosterone and Estradiol among maiden and repeat spawners exposed to one of two thermal regimes (14°C or 22°C), prior to spawning. Other details as for Fig. 2.

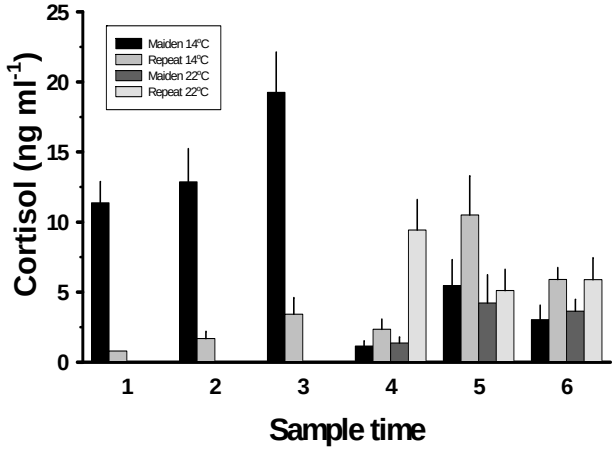

Figure 9. Mean (± SEM) plasma levels of cortisol among maiden and repeat spawners exposed to one of two thermal regimes (14°C or 22°C), prior to spawning. Other details as for Fig. 2.

Gonadal histology was assessed in order to examine the effect of temperature on seasonal changes in gonadal structure in both maidens and repeats. At Sample times 1 to 3, there was a gradual transition in gonadal stage in both maiden and repeat spawners from gonads which had a mixture of small pre-vitellogenic and early-stage vitellogenic follicles (sample 1, Fig. 10), to gonads in which larger vitellogenic follicles predominated (sample 3, Fig. 10). This indicates the onset of vitellogenesis (the endowment of vitellogenin into the follicles) at this time. The gonads of maiden and repeat spawners were similar in appearance with the exception that the gonads of repeats retained remnants of mature follicles (atretic follicles) not ovulated in the previous spawning season (Fig. 10). Generally, only the thick, highly refractile egg membrane (*zona radiata*) of these atretic follicles remained. The process of 'atresia' (the breakdown and resorption of follicles, thus sequestering resources back into maternal nutrient stores) is a normal process which occurs at the end of a reproductive season in repeat spawning fish and at other times during gonadal development in response to abnormal follicle development, or impairment of reproductive processes resulting from an extraneous stressor. No atretic follicles were observed in the gonads of maiden fish at sample times 1 to 3.

By sample 4, gonads of repeat and maiden spawning fish from both temperature treatments were characterised by the predominance of large vitellogenic follicles, consistent with the follicle growth and enlargement stage of gonadal development. The gonads of some repeat spawners had atretic follicles, which on the basis of the thickness of the zona radiata, were likely to be products of the previous spawning season. In contrast, 2 of 7 fish in both the maiden and repeat 22^{o}C treatments had atretic follicles which on the basis of zona radiata thickness, were from the current season's recruitment of follicles. This may indicate onset of thermal impairment of gonadal development.

At sample 5, irrespective of thermal regime, gonadal tissue was characterised by enlargement of vitellogenic follicles, consistent with changes in follicle size-class distribution reported previously for this experiment. The gonads of both 14^{o}C and 22^{o}C treatments showed atresia within the current season's follicles. This may not be unexpected during this highly accelerated, follicle-growth phase approaching gonadal maturation and spawning. What was of note; however, was that in the 14^{o}C treatments (maidens and repeats), 2 out of 7 fish had atretic follicles, but the prevalence of these within the sample of tissue sectioned was low (usually 1-2 follicles). In contrast, 3 of 7 fish in the maidens and 5 of 7 fish in the repeat spawning fish held at 22^{o}C showed follicle atresia, but the prevalence of atresia was high, with multiple follicles within the sample tissue affected. At sample 6, three weeks after the onset of the thermal recovery regime, ovaries were similar in appearance to the previous sample. Two of 7 fish in the 14^{o}C maiden and repeat treatment groups showed low prevalence of follicle atresia (1-2 follicles), whereas 5 of 7 fish in both the 22^{o}C maiden and repeat treatment groups, had multiple atretic follicles present.

This shows that elevated temperature (22^{o}C) during the follicle growth phase resulted in thermal impairment of follicle development, at least in terms of prevalence of follicle atresia in affected fish. This corresponded to differential post-ovulatory measures of fertility and egg survival in maidens and repeats held at the higher temperatures (see below).

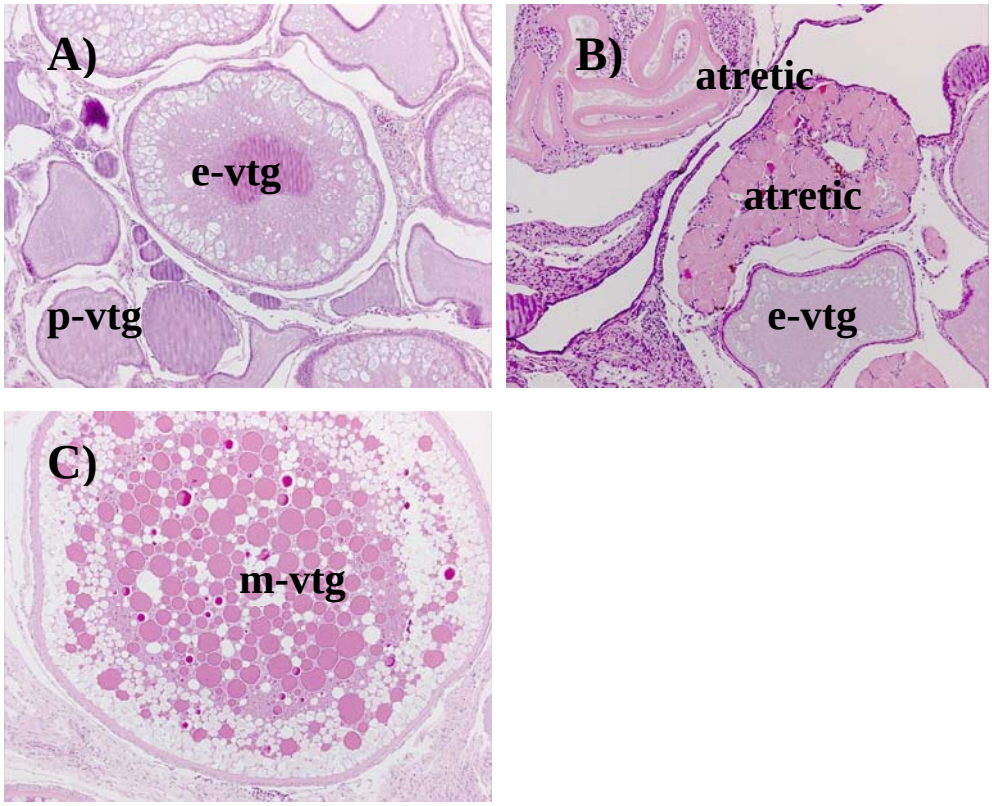

Figure 10. Micrographs of gonadal histology of A) a maiden spawner, sample time 1, showing pre-vitellogenic follicles (p-vtg) and early-stage vitellogenic follicles (e-vtg); B) a repeat spawner, sample time 2, showing 2 late-stage atretic follicles in which only the zona radiata remains, these being remnants of the previous spawning season; C) a repeat spawner, sample time 3, showing a mid-vitellogenesis stage follicle (m-vtg).

Hepatic levels of zona pellucida B and C (ZPB and ZPC, respectively) gene expression were determined to assess the response of maiden and repeat spawning fish to elevated temperature. ZPB gene expression was significantly inhibited in both maiden and repeat spawning fish reared at 22°C from February to April (Fig. 11). However, there is evidence to suggest recovery of ZPB gene expression in thermally challenged fish after temperature reduction to 8°C (Fig. 11). Expression of ZPC followed roughly the same pattern as ZPB; except the differential effect of temperature on gene expression was not seen until March and April (Fig. 12). At 14°C in March and April, ZPC gene expression was higher in repeat compared to maiden fish though not by a statistically significant amount; while gene expression in thermally exposed fish was suppressed compared to all fish at 14°C (Fig. 12). Again, there is evidence to suggest recovery of gene expression in thermally exposed fish after temperature reduction to 8°C (Fig. 12). There is a clear and significant reduction in zona pellucida gene expression in both maiden and repeat spawning fish as a result of thermal insult.

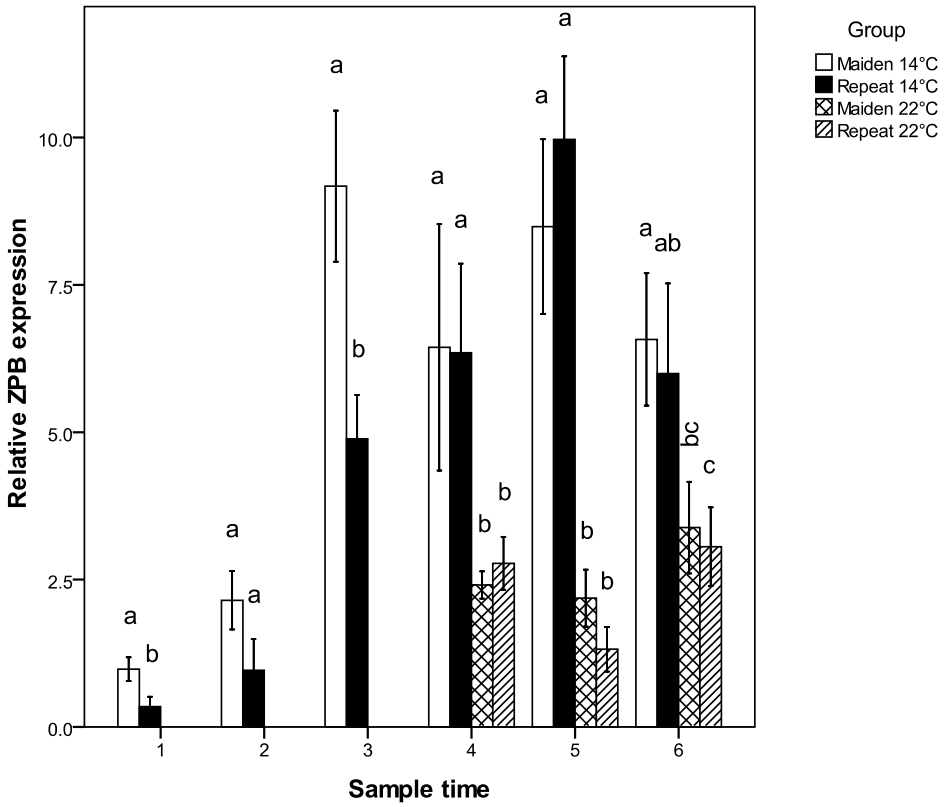

Figure 11. Mean (± SEM) relative zona pellucida B expression among maiden and repeat spawners exposed to one of two thermal regimes (14ºC or 22ºC), prior to spawning. Other details as for Fig. 2.

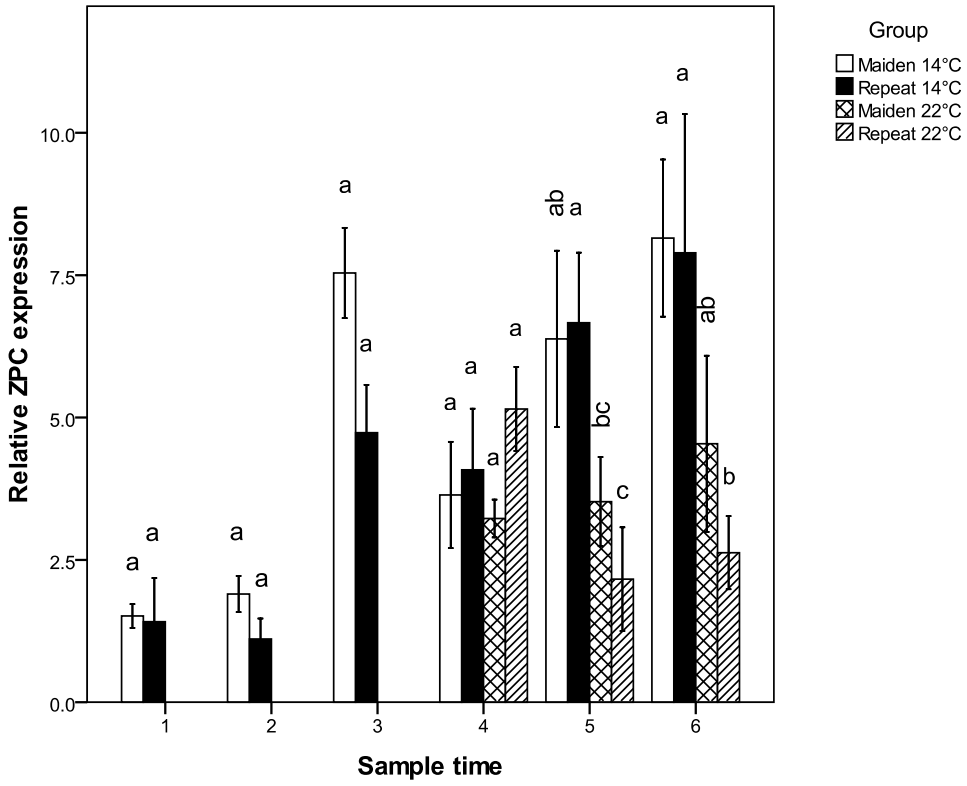

Figure 12. Mean (± SEM) Relative zona pellucida C expression among maiden and repeat spawners exposed to one of two thermal regimes (14ºC or 22ºC), prior to spawning. Other details as for Fig. 2.

Hepatic levels of relative vitellogenin (Vtg) gene expression were significantly affected by elevated temperature. Vtg expression was lower in both maiden and repeat spawning fish exposed to 22°C relative to 14°C during March and April (Fig. 13). Unlike the ZPB and ZPC expression profiles, there is no evidence to suggest recovery of Vtg expression after temperature reduction to 8°C in repeat or spawning females (Fig. 13).

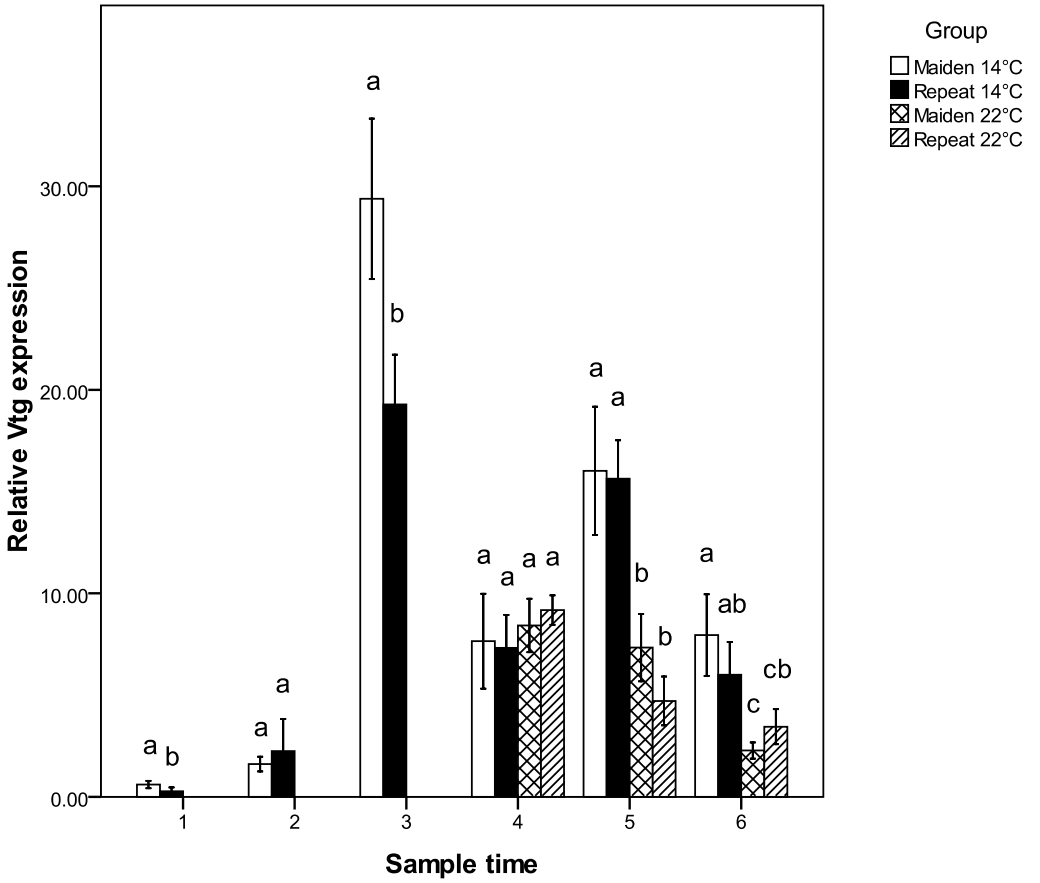

Figure 13. Mean (± SEM) relative vitellogenin expression among maiden and repeat spawners exposed to one of two thermal regimes (14ºC or 22ºC), prior to spawning. Other details as for Fig. 2.

Ovulations were complete in all groups at approximately the same time; however, maidens at 14ºC began ovulating first followed by repeats at 14ºC, then repeats at 22ºC followed by maidens at 22ºC (Fig. 14). This is consistent with the delaying effects of elevated temperature on ovulation seen by us and others in several salmonid species including Atlantic salmon (Pankhurst and Thomas, 1998; King and Pankhurst, 2007; Vikingstad et al, 2008).

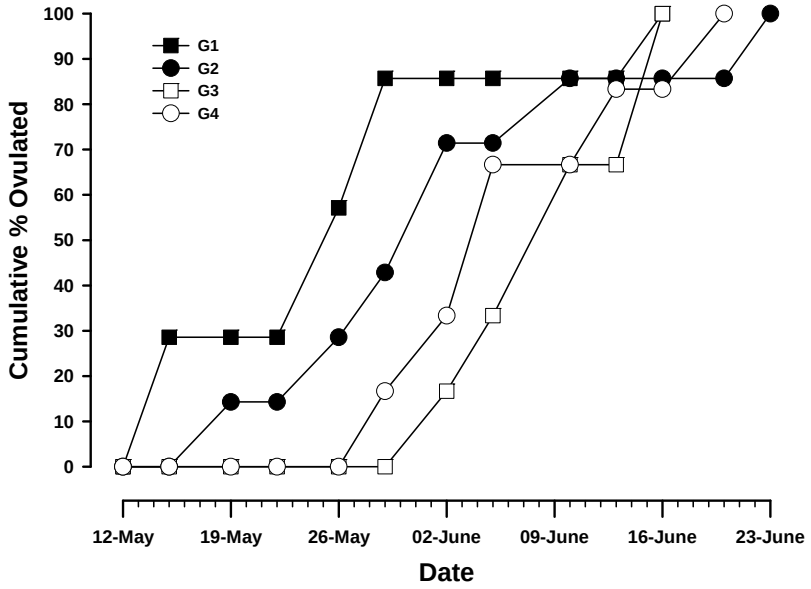

Figure 14. Cumulative ovulation in maidens at 14°C (G1), repeats at 14°C (G2), maidens at 22°C (G3) and repeats at 22°C (G4).

There were no statistical differences in fecundity among groups of ovulated fish, although repeats tended to have higher egg production than maidens. Relative fecundity was also not affected by treatment (Fig. 15). However, there was markedly reduced fertility in maidens at 22°C (G3) relative to both maidens and repeats at 14°C (G1 and G2). Repeats at 22°C showed intermediate fertility between 14°C fish and 22°C maidens. Survival to the eyed egg stage was highest in maidens at 14°C, significantly suppressed at 22°C in maidens, and at intermediate levels in repeats at both temperatures (Fig. 15).

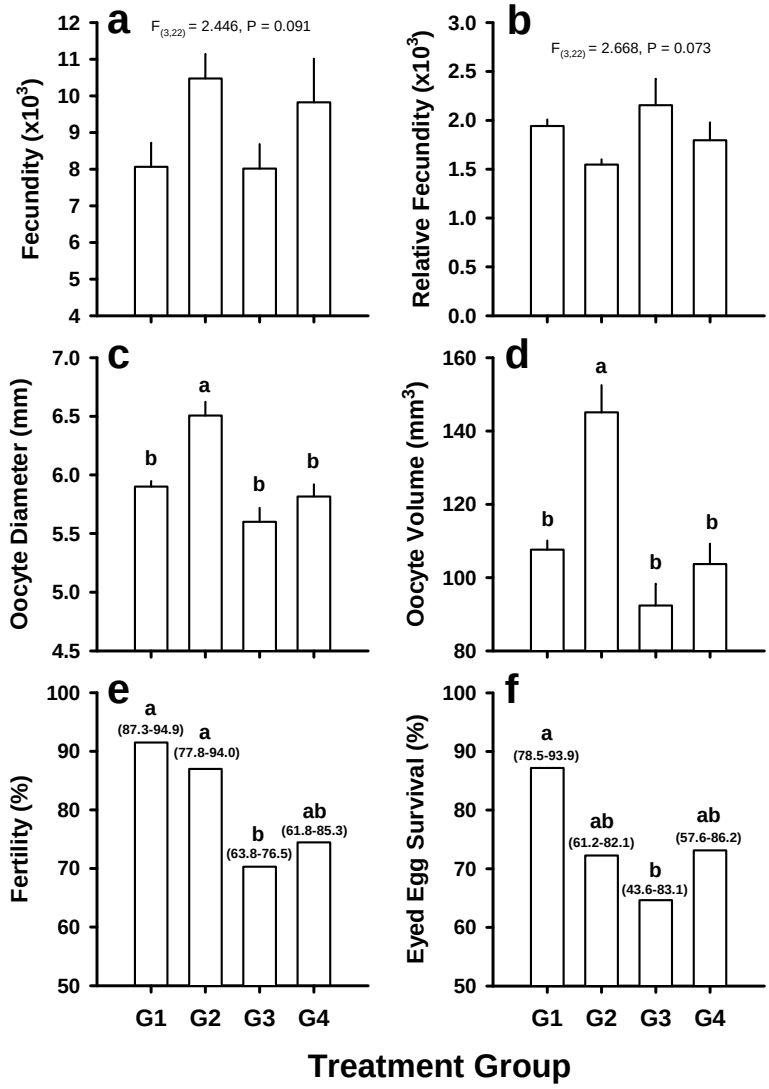

Figure 15. Fecundity and survival data from maiden and repeat spawning fish in 2008.

These outcomes offer the helpful possibility that despite lower fertility and egg survival than maidens at 14^{o}C, repeat spawners may be more robust in the face of thermal insult. This combined with their larger size and egg production could make the use of repeat spawning fish desirable under situations where there was any threat of exposure to higher than normal temperature. These possibilities are examined further in the body of the report along with assessment of the scope for hormone therapy to rescue the more highly compromised maiden fish.

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Pankhurst, N.W., and Van Der Kraak, G. (1997). Effects of stress on growth and reproduction. *In* Fish Stress and Health in Aquaculture (eds. G. K. Iwama, A.D. Pickering, J.P. Sumpter and C.B. Schreck), pp. 73-93. Cambridge University Press, Cambridge.

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