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Effect of elevated temperature on estrogenic induction of vitellogenesis and zonagenesis in juvenile Atlantic salmon (*Salmo salar*)

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In eggs from thermally challenged Tasmanian Atlantic salmon, fertility and embryo survival rates are often low, partly due a reduction in plasma 17β-estradiol (E_2) levels. We used juvenile Atlantic salmon to assess whether hepatic tissue remains responsive to stimulation by E_2 at higher temperatures typically encountered by Tasmanian salmon during summer. E_2 administration stimulated vitellogenin (Vtg) and estrogen receptor alpha transcription at 14 ℃ and 22 ℃, although induction of Vtg occurred more rapidly at 22 ℃. Consequently, plasma Vtg levels increased and reached a plateau more quickly at 22 ℃. Zona pellucida (Zp) B and C transcription was significantly lower in E_2-treated fish at 22 ℃ relative to 14 ℃. This shows that the Vtg gene is E_2-responsive at high temperature unlike Zp B and C genes that displayed traits of thermal inhibition. Therefore, estrogen replacement therapy in adult salmon may offset some, but not all thermal inhibition of reproductive function.

1.0 Introduction

Worldwide, piscine aquaculture will face new challenges as a result of predicted global increases in water temperature. For example, in Tasmanian, farmed Atlantic salmon (*Salmo salar*) already experience warm conditions which accelerates growth rate, and shortens production time (Jungalwalla, 1991). Natural summer water temperatures of 18 ℃ are regularly reported for most farms and higher temperatures (up to and exceeding 22 ℃) have been reported during summer months and are predicted to occur more frequently under conditions of global warming (Battaglene et al. 2008). In both southern and northern hemisphere stocks, it has been previously shown that elevated temperature can reduce reproductive performance in terms of egg fertility and embryo survival (reviewed in Pankhurst and King, 2010). In an attempt to minimise exposure of broodstock to elevated temperature, the salmon industry in Australia is moving towards re-circulating systems that allow complete thermal regulation of broodstock (Battaglene et al. 2008, Pankhurst and King, 2010). Unfortunately, this approach to thermal security requires significant capital investment and may not be a viable option for much of the aquaculture sector due to scale, site and other logistical restrictions (Pankhurst and King, 2010).

Thermal insult which leads to reduced egg quality occurs as a result of temperature induced effects at a number of levels of the reproductive endocrine cascade (Pankhurst and King, 2010). These effects include suppression of plasma levels of 17β-estradiol (E_2) in fish reared at 16 ℃ relative to the more optimal temperatures of 6 and 11 ℃ (King and Pankhurst, 2000), and in fish reared at a more challenging 22 ℃ compared to 14 ℃ (King et al. 2003, King et al. 2007) during vitellogenesis**.** Furthermore, King et al. (2003) reported that testosterone (T) levels during vitellogenesis were generally higher in fish reared at 22 ℃ than in fish reared at 14 or 18 ℃, suggesting that conversion of T to E_2 was also impaired,

probably due to inhibition of P450 aromatase A (CYP19a1a). An *in vitro* study by Watts et al. (2004) further supported this suggestion with increased T production from isolated ovarian follicles in response to 17-hydroxyprogesterone and androstenedione at 14, 18 and 22 ℃, but inhibition of conversion of T to E_2 at 22 ℃. Additionally, an *in vitro* competitive binding study showed that hepatic estrogen receptors (ER) from Atlantic salmon reared at 22 ℃ during vitellogenesis had significantly reduced E_2 binding affinity compared to ER from fish maintained at 14 and 18 ℃ (Watts et al. 2005). This suggests that signal transduction and processes downstream from E_2 synthesis may also be involved in the inhibitory effects of elevated temperature. Measurable downstream effects do include reduction in both hepatic gene expression and plasma levels of vitellogenin (Vtg), and hepatic gene expression of zona pellucida proteins (Zp) (King et al. 2007, Pankhurst et al. 2011), both of which are essential for embryo development and survival. What is less clear is whether these effects are only the result of reduced estrogen tone, or whether other endocrine defects are associated with exposure to high temperature.

An alternative management strategy which could offset the effects of thermal insult might involve administration of exogenous hormones to stimulate the endocrine system and prevent the impairment on endocrine function (Pankhurst and King, 2010). In earlier studies on juvenile salmonids, treatment with E_2 has been shown to induce the expression of hepatically-expressed genes at a variety of temperatures. In juvenile Arctic char (*Salvelinus alpinus*) reared at 10 ℃, Vtg, zona pellucida B (Zpb), zona pellucida C (Zpc) and ER mRNA levels had increased within 12 hours post injection with E_2 (Westerlund et al. 2001), and in brown trout (*Salmo trutta*), treatment with ethinylestradiol (EE_2) resulted in higher levels of Vtg and ERα mRNA in fish held at 19 ℃ compared to 12 ℃ (Körner et al. 2008). Similarly, in E_2-treated juvenile rainbow trout (*Oncorhynchus mykiss*) maintained at 15 ℃, plasma Vtg was detectable 24 h after the initial injection and peaked at >70 $mg.ml^{-1}$ after 10 days,

compared to ~9 mg.ml^{-1} in fish held at 9 ℃ (Mackay and Lazier, 1993). The stimulatory effects of increasing temperature across normal ranges have also been demonstrated *in vitro* in primary hepatocytes from rainbow trout that were maintained at either 14 or 18 ℃ and exposed to E_2 (Pawlowski et al. 2000); Vtg gene expression was significantly higher at 18 ℃ than at 14 ℃. However, whether estrogen-dependent gene expression remains intact at the higher temperatures where endocrine suppression occurs, when circulating E_2 is not a limiting factor, remains to be established. Successful demonstration of maintenance of estrogen sensitivity at high temperature would confirm the potential of endocrine manipulation to offset thermal effects. Therefore using juvenile Atlantic salmon as model animals, the present study investigated whether E_2 implantation could generate circulating E_2 levels that were a good approximation of those observed in developing adults. Accordingly, the E_2 responsiveness of juvenile fish was assessed in terms of hepatic expression of Vtg, ERα, Zpb and Zpc, and plasma levels of Vtg at 14 and 22 ℃ for 14 days.

2.0 Materials and Methods

2.1 Fish and sampling

Eighty-four juvenile female Atlantic salmon (mean weight 217.2 g ± 4.68 g) were housed at the Salmon Enterprises of Tasmania Pty. Ltd. (Saltas), Wayatinah Hatchery, Tasmania, Australia, in 4 separate 1000-L tanks (21 fish per tank), with independent recirculating fresh water for each temperature treatment. At day 0, all fish were anesthetised with Aqui-S™ (25 ppm), weighed, implanted with a blank or E_2-containing pellet and placed in thermo-regulated tanks in 1 of 4 experimental groups: blank silastic pellet at 14 ℃, blank silastic pellet at 22 ℃, E_2 pellet at 14 ℃ and E_2 pellet at 22 ℃ (n = 21 per group). Fourteen and 22 ℃ represent cool and warm Tasmanian summers respectively. At 3, 7 and 14 days post

implantation, 7 fish were sacrificed from each group using a lethal dose of Aqui-S™ (50 ppm). Blood was taken from the caudal vasculature using heparinised syringes and centrifuged to separate the plasma component which was then stored at -20 ℃. Sections of liver were dissected, immersed in RNA*later*™ (Ambion) overnight at 4 ℃ then stored at -80 ℃ until processed. Experiments were conducted under approval from the Animal Ethics Committee of the University of the Sunshine Coast (approval number AN/A/07/35).

2.2 E_2 implants

Slow release E_2 pellets were made according to Pankhurst et al. (1986) with some modifications. Briefly, E_2 (Sigma) was mixed with unpolymerised elastomer (Silastic 382 medical grade elastomer, Dow Corning Corporation) at 50 $mg.g^{-1}$ elastomer, and spread into 2 x 2 x 20 mm molds after the addition of 8 µl of accelerant (stannous octoate, Sigma) to give a final E_2 concentration of 2.2 $mg.cm^{-1}$ pellet. E_2 pellets were cut at various lengths to give a total dosage of 10 $mg.kg^{-1}$ body weight; blank or E_2 pellets were then ventrally implanted into the peritoneal cavity using a 15 G needle.

2.3 Plasma E_2 and Vtg measurement

Plasma levels of E_2 were determined by radioimmunoassay. E_2 was extracted from 100 µl of plasma using 1-ml ethyl acetate, and the reagents and procedure described in Pankhurst and Carragher (1992). Extraction efficiency was 78 %, as determined by recovery of ^{3}H-labelled steroid from replicates of a plasma pool. Assay values were corrected accordingly to account for extraction losses. Interassay variability was determined by repeat measurement of a

pooled internal standard and was 7.4 % (CV, n = 3) and the lower limit of detection (LOD) for the assay was 0.41 $ng.ml^{-1}$.

Plasma Vtg levels were measured by enzyme linked immunosorbent assay using the reagents and protocol as described in Watts et al. (2003). Plasma samples were diluted at 1:1000 in assay buffer for measurements. Interassay variability was assessed by repeat measurement of a Vtg standard from the central part of the assay curve and was 13.1 % (CV, n = 7). Pooled internal standards were not used here due to the tendency of Vtg to denature following repeated freeze-thaw cycles. The lower LOD for the assay was 80 $ng.ml^{-1}$.

2.4 RNA isolation and cDNA synthesis

Total RNA was isolated from 15 mg of hepatic tissue using the Illustra RNAspin Mini kit (GE Healthcare) according to the manufacturer's protocol. RNA yield and 260/280 purity ratio were determined using the NanoDrop 2000 (Thermo Scientific). An RNA integrity number (RIN) was determined for a random sample of hepatic RNA (n = 36) using a 2100 bioanalyzer (Agilent) to establish RNA quality. All RNA was stored at -80 ℃ until use.

Four hundred nanograms of liver-derived RNA were used to synthesise cDNA for use in real-time quantitative PCR (qPCR) using the QuantiTect® reverse transcription kit (Qiagen). This kit includes a DNA elimination step to remove potential contamination of PCRs by genomic DNA. Following synthesis, cDNA was stored at -20 ℃ until use.

2.5 qPCR cycling conditions and ERα assay development

qPCRs were conducted on a Rotor-gene 6000 series thermal cycler (Qiagen) using the Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen) master mix and the following cycling conditions: 50 ℃ for 2 min; 95 ℃ for 2 min; 40 cycles of 95 ℃ for 15 sec; 60 ℃ for 15 sec, and 72 ℃ for 20 sec (acquiring). At the end of cycle 40, a melt curve analysis was performed to confirm amplification of a single product as follows: 90 sec preconditioning step at 72 ℃, followed by a temperature gradient up to 95 ℃ at 1 ℃ per 5 sec. The 10 µl qPCR reaction contained 5 µl SYBR, 200 nM each primer, 3.6 µl PCR grade water and 1 µl cDNA template. For every gene analysed no-template controls (NTCs) and a calibrator (which was a pool of randomly selected hepatic cDNA) were included to detect possible contamination, and control for in-between run variability, respectively.

Gene specific primers (GSPs) were designed from the Atlantic salmon ERα mRNA sequence available on GenBank (X89959) to have an optimum annealing temperature of 60 ℃ using Primer3 software (http://frodo.wi.mit.edu/primer3/, Table 1). The ERα qPCR was developed by using serially diluted cDNA to construct a 6 point validation curve. Reaction efficiency was automatically calculated from this curve by Rotor-gene software version 1.7.87 using the equation: $E = [10^{(-1/M)}] - 1$ (Table 1), where E equals efficiency and M is equal to slope. The GSPs were tested for specificity via melt curve analysis (as above), the products were run on a 2 % agarose gel to establish size and gene identity was then confirmed through sequencing. During validation, negative reverse transcription and NTCs were analysed to confirm the absence of genomic DNA and other contamination.

GSPs for target genes previously optimized and validated for qPCR (Pankhurst et al. 2011) were used to amplify hepatic VtgA (referred to as Vtg), Zpc and Zpb (primers detect both Zpba and Zpbb, collectively referred to as Zpb) while primers developed by this study

were used to amplify hepatic ERα (Table 1). GSPs developed by Pankhurst et al. (2011) were used to quantify the expression of candidate reference genes, namely TATA binding protein (Tbp), hypoxanthine phosphoribosyltransferase 1 (Hprt1) and elongation factor 1 alpha (Ef1α). The suitability of using Tbp, Hprt1 and Ef1α as reference genes for normalisation was assessed using GeNorm (Vandesompele et al. 2002) and BestKeeper (Pfaffl et al. 2004). The output from these algorithms was then independently validated by the method outlined by Setiawan and Lokman (2010). As a result of this analysis (data not shown), expression data from all 3 reference genes was used for accurate target gene normalisation using the Rest© software 2008 package, V2.0.7 (Pfaffl et al. 2002). This package was also used to calculate the level of target gene expression relative to a calibrator sample analysed in every run.

2.6 Statistical Analysis

One-way ANOVA coupled with Tukeys-b was used to test for statistically significant differences in the plasma E_2 and Vtg data ($p \leq 0.05$). Differences in relative gene expression levels were detected non-parametrically using the Kruskal-Wallis test coupled with Bonferroni's Correction to reduce the risk of type 1 error. The P value for Bonferroni's Correction was set at 0.05 before adjustment. All analyses were performed using the SPSS (version 17.0) statistical package.

3.0 Results

3.1 Quantification of plasma E_2 and Vtg

Plasma E_2 levels were low (< 0.4 $ng.ml^{-1}$) throughout the experiment in fish receiving a blank silastic pellet (Fig. 1a). E_2-treated fish had significantly higher plasma E_2 than their respective control fish at all sample times. E_2 levels were also consistently higher at 14 than at 22 ℃ with a general trend of decreasing plasma E_2 concentration over time in E_2 treated fish (Fig. 1a). Plasma Vtg levels were also low but detectable in controls at 3 and 7 day samples, and significantly elevated at those times in fish treated with E_2 (Fig. 1b). At 3 days post-implant, Vtg levels were higher in E_2-implanted fish at 22 ℃ than at 14 ℃; at 7 days there was no difference between treatments and at 14 days post-implant, plasma Vtg was lower at 22 than at 14 ℃. At the 14 day sample, there was also an elevation in plasma Vtg in the blank pellet group at 14 ℃ relative to the 22 ℃ blank group, but to a lower level than in either of the E_2-treated groups (Fig. 1b).

3.2 Hepatic gene expression

At day 3, ERα gene expression in the E_2-treated groups at 14 and 22 ℃ were similar (Fig 2a). Gene expression levels for E_2-treated groups were significantly higher than those observed for control groups at both temperatures on day 3. At day 7, ERα gene expression was elevated in the 14 ℃ blank, 14 ℃ E_2 and 22 ℃ E_2 groups relative to the 22 ℃ blank. By day 14, the levels of ERα gene expression in both E_2-treated groups were significantly elevated over the levels in their respective controls. ERα gene expression was also significantly higher in the 14 ℃ control group than in the 22 ℃ control group on day 14.

At 3 days post-implant, Vtg gene expression was significantly higher in 22 ℃ E_2-treated fish than any other group (Fig. 2b). The mean gene expression level of E_2-treated fish at 14 ℃ was also higher than its corresponding control group. Vtg gene expression in E_2-treated groups was elevated over controls for the remainder of the experiment. Gene

expression was higher in E_2-treated fish at 22 ℃ than that of the E_2-treated group at 14 ℃ at day 7; however, this difference had disappeared by day 14. Gene expression was consistently low in control groups for the duration of the experiment.

At day 3, Zpb and Zpc gene expression levels were elevated in E_2-treated animals at both temperatures relative to their respective controls (Figs. 3a and 3b respectively). Zpc gene expression was also significantly higher in E_2-treated fish at 22 ℃ compared to E_2-treated fish at 14 ℃. Zpb and Zpc gene expression increased over time in the E_2-treated 14 ℃ group. At 22 ℃, Zpb and Zpc gene expression levels were consistently higher than those of the control; however, the level of expression for these genes did not steadily increase over time. Additionally, gene expression was lower at 22 ℃ than 14 ℃ for Zpb at days 7 and 14, and for Zpc at day 14.

4.0 Discussion

Atlantic salmon are routinely reared in Tasmania at temperatures towards the upper limit of thermal tolerance for successful reproduction. Egg fertility and embryo survival rates are often low at high temperatures, partly due to endocrine dysfunction at the level of ovarian E_2 production. Assessment of the role of sustained E_2 levels in this process required a treatment protocol that produced elevations in plasma E_2 in implanted fish that were similar to those in normally maturing adults. By day 3 in the present study, circulating E_2 had risen to ~12 and 9 $ng.ml^{-1}$ in E_2-treated fish maintained at 14 ℃ and 22 ℃ respectively. For the same type of pellet, Pankhurst et al. (1986) observed a rapid fall in plasma E_2 level post-implantation that lasted for approximately 6 hours, after which a more steady decline in plasma E_2 was observed. Based on the plasma E_2 profiles obtained by Pankhurst et al. (1986), it is likely that the plasma concentrations of E_2 in juvenile fish prior to day 3 were initially higher than

those observed in fish reared at both 14 and 22 ℃ on day 3. The concentration of plasma E_2 decreased over time for the duration of the present experiment similar to what was observed in other studies using the same type of silastic E_2 implant (Pankhurst et al. 1986). E_2 levels in fish maintained at 22 ℃ were consistently lower at each sampling point relative to fish from the 14 ℃ treatment which suggests that E_2 was utilised and/or cleared at a faster rate at 22 ℃ than at 14 ℃. Similarly, Mackay and Lazier (1993) observed lower plasma E_2 levels in E_2-treated rainbow trout maintained at 15 ℃ compared to 9 ℃, and also suggested that this difference resulted from increased metabolic rate and steroid clearance at the higher temperature. Adult Atlantic salmon reared at 14 ℃ typically have plasma E_2 concentrations of ~4 and 8.5 $ng.ml^{-1}$ during February and March respectively (which coincides with a period of increasing vitellogenesis), while levels in fish held at 22 ℃ remain low at <1 $ng.ml^{-1}$ and 2 $ng.ml^{-1}$ for the same months (Pankhurst et al. 2011). In the present study, the highest E_2 levels in juveniles held at 22 ℃ were measured 3 days after implantation (9 $ng.ml^{-1}$) and decreased to ~3.5 $ng.ml^{-1}$ over a 2 week period. After 2 weeks, the E_2 level observed in thermally challenged juvenile fish was still higher than that typically observed in untreated broodstock reared at 22 ℃ during February and March (Pankhurst et al. 2011). This illustrates that E_2 implantation of thermally challenged broodstock at 10 $mg.kg^{-1}$ during February and March could elevate plasma E_2 levels to a level which is comparable to the physiological levels observed in broodstock reared under optimal conditions, or at the very least could keep E_2 levels above the 1-2 $ng.ml^{-1}$ baseline for a minimum of 2 weeks. This demonstrates the potential of E_2-implantation to at least partially restore circulating E_2 levels in thermally compromised adult broodstock.

For juvenile fish reared at 14 ℃, Vtg gene expression was significantly elevated at 3 days post implantation and this increased further over the course of the experiment. This is broadly consistent with previous observations in juvenile salmon, where

E_2-treatment induced *de novo* synthesis of Vtg mRNA, and gene expression increased over time (Mackay and Lazier, 1993). In E_2-treated fish maintained at 22 ℃, Vtg gene expression was significantly higher than that in the corresponding group of fish reared at 14 ℃ at day 3, indicating that gene expression was induced more quickly at the higher temperature. On day 7, the difference in gene expression was maintained; however by day 14 expression levels were approximately equal. In ectotherms, an increase in temperature causes an increased rate of physiological processing (Sokolova and Lannig, 2008), which in this case appears to have resulted in the faster induction and increased synthesis of Vtg mRNA for the first week of the experiment. Similarly, Mackay and Lazier (1993) reported that juvenile rainbow trout held at 15 ℃ responded more quickly in terms of Vtg mRNA synthesis to E_2 injection than fish maintained at 9 ℃, and had Vtg mRNA levels that were 3-fold higher at the end of the experiment (day 10). Since it has been shown that a rise in temperature can cause an increase in liver somatic index, total protein output (Korsgaard et al. 1986) and basal metabolic rate (Johnston and Dunn, 1987), we suggest that the faster induction and increased Vtg gene expression at higher temperature in the present study can be attributed to more rapid utilisation and clearance of plasma E_2.

Plasma Vtg levels generally reflected Vtg mRNA levels for the duration of the experiment. Circulating levels of Vtg in E_2-treated fish were higher in the 22 ℃ than the 14 ℃ group at day 3 which is consistent with the faster induction of Vtg gene expression at the higher temperature. However, despite the initially more rapid accumulation of Vtg in 22 ℃ E_2-treated fish, plasma Vtg levels were approximately equal in E_2-treated fish at both temperatures by day 7, and by day 14, plasma Vtg levels were higher at 14 ℃. The more rapid production of Vtg at the elevated temperature shows that the rate of short term response to E_2 in terms of Vtg production is modulated by temperature and the maximum response is reached more rapidly at higher temperature. This is consistent with observations of adult

fathead minnows (*Pimephales promelas*) that were exposed to a mixture of estrogenic chemicals and maintained at either 20 or 30 ℃ for 2 weeks, where plasma Vtg was significantly higher after 24 h at 30 ℃ (Brian et al. 2008). Consistent with the present study, no difference in Vtg concentration could be detected between temperature treatments after a longer period of exposure (Brian et al. 2008). Brian et al. (2008) also concluded that temperature alters the short term response of the animal to estrogenic stimulation, but may not have an impact on the final magnitude of response. In contrast, other studies on salmonids have reported different temporal responses to exogenous E_2. Exposure of Atlantic salmon post-smolts and juvenile rainbow trout to elevated temperature resulted in greater total accumulation of plasma Vtg in response to E_2 over time (Korsgaard et al. 1986, Mackay and Lazier, 1993). The apparent difference in plasma Vtg profiles between those and the present study is likely to be a function of dose and route of exposure (implantation in the present study versus injection), and the temperature ranges used in the respective studies. The study undertaken by Korsgaard et al. (1986) utilised temperatures of 3 and 10 ℃ while Mackay and Lazier (1993) maintained fish at 9 and 15 ℃ compared with the upper temperature of 22 ℃ in the present study. Collectively our Vtg mRNA and protein data show that the mechanisms by which E_2 induces and sustains the production of Vtg are still functional in juvenile Atlantic salmon at the relatively high temperature tested in the present study.

The relative expression of Zpb and Zpc genes in the present study was up-regulated in a time-dependent fashion by E_2-treatment in fish reared at 14 ℃, which is consistent with previous reports on salmonids. In juvenile Arctic charr and rainbow trout, E_2-treatment significantly up-regulated the gene transcription and translation of Zps at temperatures of 10 and 16 ℃ respectively (Celius et al. 2000, Westerlund et al. 2001). In the present study, consistent with the broad effects of increased rate of metabolic processing, E_2-treatment

caused a more rapid of induction of Zpc at 22 ℃ relative to 14 ℃. Zpb gene expression was also induced at day 3 at high temperature, but unlike Zpc the level was not greater than that observed in the corresponding group at 14 ℃. From days 7 to 14, gene expression of both Zps was higher in the E_2-treated group at 22 ℃ than its corresponding control group, but was consistently lower than the level observed for E_2-treated fish reared at 14 ℃. Furthermore, there was an apparent difference in the expression profiles as gene expression for both Zpc and Zpb at 22 ℃ did not increase over time as observed for E_2-treated fish at 14 ℃. This suggests that unlike Vtg, the responsiveness of juvenile Atlantic salmon to exogenous E_2 in terms of Zp transcription is partially compromised at 22 ℃, which may have implications for the usefulness of E_2 therapy in maintaining reproductive function in thermally challenged adult broodstock.

The promoter region of Vtg genes contain estrogen responsive elements (Eres) and enhancer sequences that appear to be relatively simple (Teo et al. 1998, Bouter et al. 2010), and regulation of Vtg is under strict E_2 control (Lubzens et al. 2010). However, it has been suggested elsewhere that the regulatory mechanism of Zps may differ to that of Vtg (Berg et al. 2004). Due to the range of different reproductive strategies and sites of Zp gene expression among fish species, it is quite difficult to make generalisations about the regulatory mechanisms of genes in the Zp family (reviewed in Babin et al. 2007). For example, in zebrafish (*Danio rerio*) where Zps are exclusively expressed in the ovary, Zp expression cannot be induced by E_2, and the promoter region of these genes lack Eres (Mold et al. 2001, Liu et al. 2006). It is thought that in zebrafish, Zp expression is at least party regulated by nuclear transcription factor Y through CCAAT boxes (Mold et al. 2009) and through E-boxes (Mold et al. 2001) that bind basic helix-loop-helix proteins such as factor in the germ cell α. The promoter region of medaka (*Oryzias latipes*, liver Zp expression) contains multiple E-boxes (Kanamori et al. 2003) and perhaps other regulatory elements, and

the promoter region of winter flounder (*Pseudopleuronectes americanus*, liver Zp expression) contains imperfect Eres and CCAAT and TATAAA boxes (Lyons et al. 1993). While hepatically expressed Zps are inducible by E_2 (Berg et al. 2004, Knoebl et al. 2004), it has recently become apparent that many factors besides E_2 could potentially be involved in the regulation of Zps. Furthermore, no data is available on the promoter regions of Atlantic salmon Zp genes, or whether potential regulating factors of these genes are temperature sensitive. To our knowledge, this is first report on the inhibitory effect of elevated temperature on the expression of Zp genes in an E_2-treated salmonid. This suggests that thermal sensitivity of the complex factors regulating Zp, but not Vtg gene expression, may have resulted in the differential effect of elevated temperature on Vtg and Zp gene expression observed in the present study. However, characterisation of the 5'-flanking promoter regions of the Zp genes from Atlantic salmon, and subsequent thermal exposure trials quantifying the expression of regulatory factors are required to clarify this possibility.

Exposure to E_2 at 14 ℃ significantly up-regulated ERα gene expression by day 3 in the present study relative to the corresponding control group. Similarly, both *in vitro* and *in vivo* studies have demonstrated that transcription of hepatic ER can be induced by E_2 administration in salmonids (Flouriot et al. 1996, MacKay et al. 1996, Westerlund et al. 2001). Exposure of E_2-treated juvenile fish to 22 ℃ in the present study did not significantly alter the level of hepatic ERα gene expression relative to fish reared at 14 ℃ for the duration of the experiment. In contrast to the present study, other studies have described a modulating affect of temperature on ER transcription. Higher levels of ER mRNA were detected in isolated hepatocytes from rainbow trout in response to E_2 at 18 ℃ compared to 14 ℃ (Pawlowski et al. 2000), and juvenile brown trout treated with EE_2 showed significantly enhanced ERα gene expression at 19 ℃ compared to 12 ℃ (Körner et al. 2008). As increases in temperature are only stimulatory up to a certain point, the use of a higher

temperature in the present study may account for the apparent lack of ERα stimulation compared to previous studies. Even though ERα expression was unaffected by high temperature treatment in the present study, other *in vitro* studies on Atlantic salmon and tilapia (*Oreochromis aureus*) have shown that exposure to high temperature can decrease hepatic ER binding affinity (Watts et al. 2005, Tan et al. 1999) and potentially affect E_2 signal transduction. The fact that E_2-treatment still significantly elevated Vtg gene expression at 22 ℃ in the present study suggests that any negative affects on receptor affinity and therefore signal transduction were not sufficient to significantly decrease the transcription of other E_2-dependent genes. E_2-treatment resulted in high and sustained plasma E_2 levels so that any reductions in receptor affinity may have been offset by high ligand concentration and the effects of increased metabolic rate. It is still possible that signal transduction might be impaired at the lower ligand concentrations typically observed at elevated temperatures.

The expression of ERα was significantly elevated in control fish in the present study at day 7 and 14, at 14 ℃. This may indicate that there was waterborne estrogen in the 14 ℃ recirculating system which stimulated ERα gene expression in the control group. When ERα expression was elevated at day 7, the expression levels of both Zp and Vtg genes were also higher in the 14 ℃ than the 22 ℃ control group. This was also observed on day 14 for Zpb gene expression and plasma Vtg levels, further supporting the possibility that unintended E_2 exposure may have occurred. Fish-to-fish transfer of steroid hormones has previously been demonstrated by Budworth and Senger (1993) where plasma T levels were found to be significantly elevated in the blood of saline-injected trout whose tank received water from a recirculating system that also held fish injected with T. The mechanism for fish-to-fish transfer appears to be loss and uptake across the gill epithelium (Scott et al. 2005, Vermeirssen and Scott, 1996). Therefore the unexpected gene expression detected in the 14

℃ control group could be due to loss of E_2 via the gills in E_2-treated fish, subsequent contamination of the 14 ℃ recirculating system and uptake of E_2 by the control group. The level of ERα expression was not different between the 14 ℃ control and E_2 treated group at day 7, while in the same fish, Vtg and Zp expression was elevated in the E_2 pellet group relative to controls. This difference could be due to differential sensitivity of these genes to E_2 stimulation. In rainbow trout a higher dose of E_2 was needed to stimulate the *in vitro* hepatic expression of Vtg compared to ER (Flouriot et al. 1996).

Thermal management of broodstock holding facilities is the most effective way to avoid endocrine dysfunction and ensure egg quality in Atlantic salmon during periods of high ambient water temperature (Pankhurst and King, 2010). However, for facilities where temperature control is not a desirable or viable option, E_2 therapy has been suggested as an alternative approach for maintaining endocrine function and subsequent egg quality. In the present study we have demonstrated that juvenile Atlantic salmon remain responsive to E_2-treatment at 22 ℃ in terms of Vtg and ERα gene expression, and Vtg protein production. Our results indicate that the machinery by which E_2 exerts its action is still intact at 22 ℃, and suggests that the compromised levels of Vtg mRNA and protein observed in our previous study of adult animals (Pankhurst et al. 2011) occurred mainly as a result of reduced E_2 tone and not impairment of other downstream processes such as reduced E_2 signal transduction. We have also confirmed previous reports on the modulating effect of temperature on the speed of induction and duration of Vtg production which could have implications for the frequency of E_2-treatments in a commercial production setting. For the first time we have demonstrated that the zonagenic response to E_2-treatment in juvenile Atlantic salmon is significantly reduced at 22 ℃. While the molecular basis for the observed dysfunction is unclear, we suggest that it may occur as the result of thermal impairment of (non-Ere) factors co-regulating the expression of both Zps but not Vtg. We conclude that the usefulness of E_2

therapy warrants investigation in thermally challenged adult broodstock as the liver remains responsive to E_2 at high temperature in terms of Vtg production, ERα gene expression and to a lesser extent Zp gene expression.

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Table 1. *qPCR primers and sequence information*

Gene name	Primer	Sequence (5'→3')	Prod. size	E*	Source seq.
Vtg	VtgF4	AAC TTT GCC CCT GAA TTT GC	95 bp	0.984	DQ834857
	VtgR4	GCT CTA GCC AGA CCC TCC TC			
Zpb	ZpbF1	GTT TCC AGG GAT GCC ACT CT	113 bp	0.937	AJ000664, AJ000665
	ZpbR1	TGG TAG ATG GCA AAG GCA GA			
Zpc	ZpcF5	GTC CCC CTG CGT ATC TTT GT	121 bp	0.969	GU075906
	ZpcR4	AAC CTG TCA CTT TGG CAT CG			
ERα	ERαF1	AAG CAT GCC GCC TCA GAA AG	150 bp	1.003	X89959
	ERαR2	TCC TGT GCT CCA GGT CAC CA			
Tbp	TbpF1	TCC CCA ACC TGT GAC GAA CA	117 bp	0.981	BT059217
	TbpR1	GTC TGT CCT GAG CCC CCT GA			
Hprt1	Hprt1F1	GAT GAT GAG CAG GGA TAT GAC	165 bp	0.963	BT043501
	Hprt1R1	GCA GAG AGC CAC GAT ATG G			
Ef1α	Ef1αF2	GCA CCA CGA GAC CCT GGA AT	94 bp	0.969	AF321836
	Ef1αR2	CAC GTT GCC ACG ACG GAT AT			

*E = efficiency, bp = base pairs

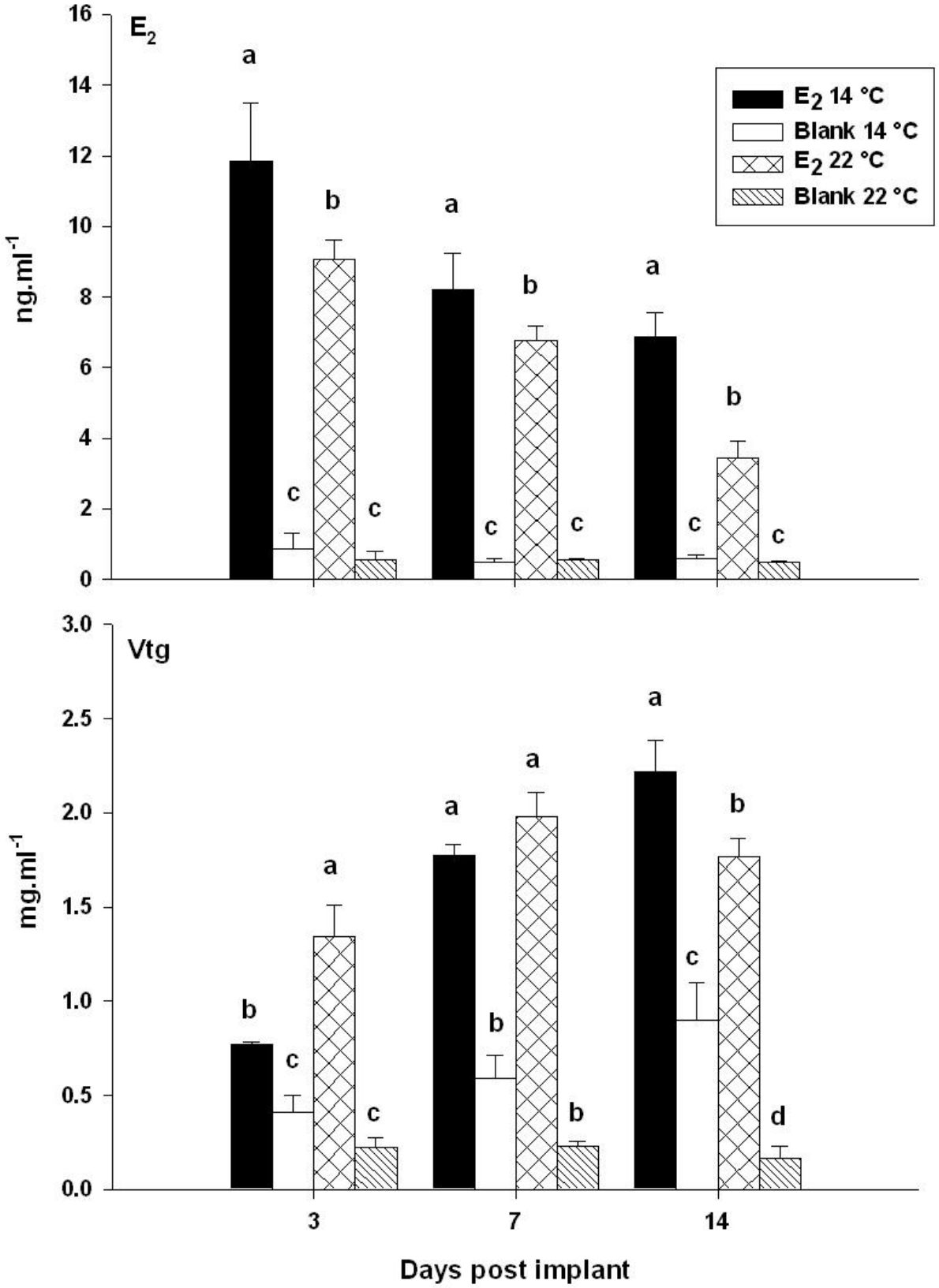
E2
ng.ml-1
E2 14 °C
Blank 14 °C
E2 22 °C
Blank 22 °C
Vtg
mg.ml-1
Days post implant
3
7
14

Figure. 1. Plasma levels (mean + SE, n = 7) of (a) E_2 and (b) Vtg in juvenile Atlantic salmon given a blank implant at 14 ℃ or 22 ℃, or an E_2 implant (10 $mg.kg^{-1}$) at 14 ℃ or 22℃. Fish were sampled at 3, 7 and 14 days post implantation. Different superscripts at each sampling time denote significantly different means ($p \leq 0.05$).

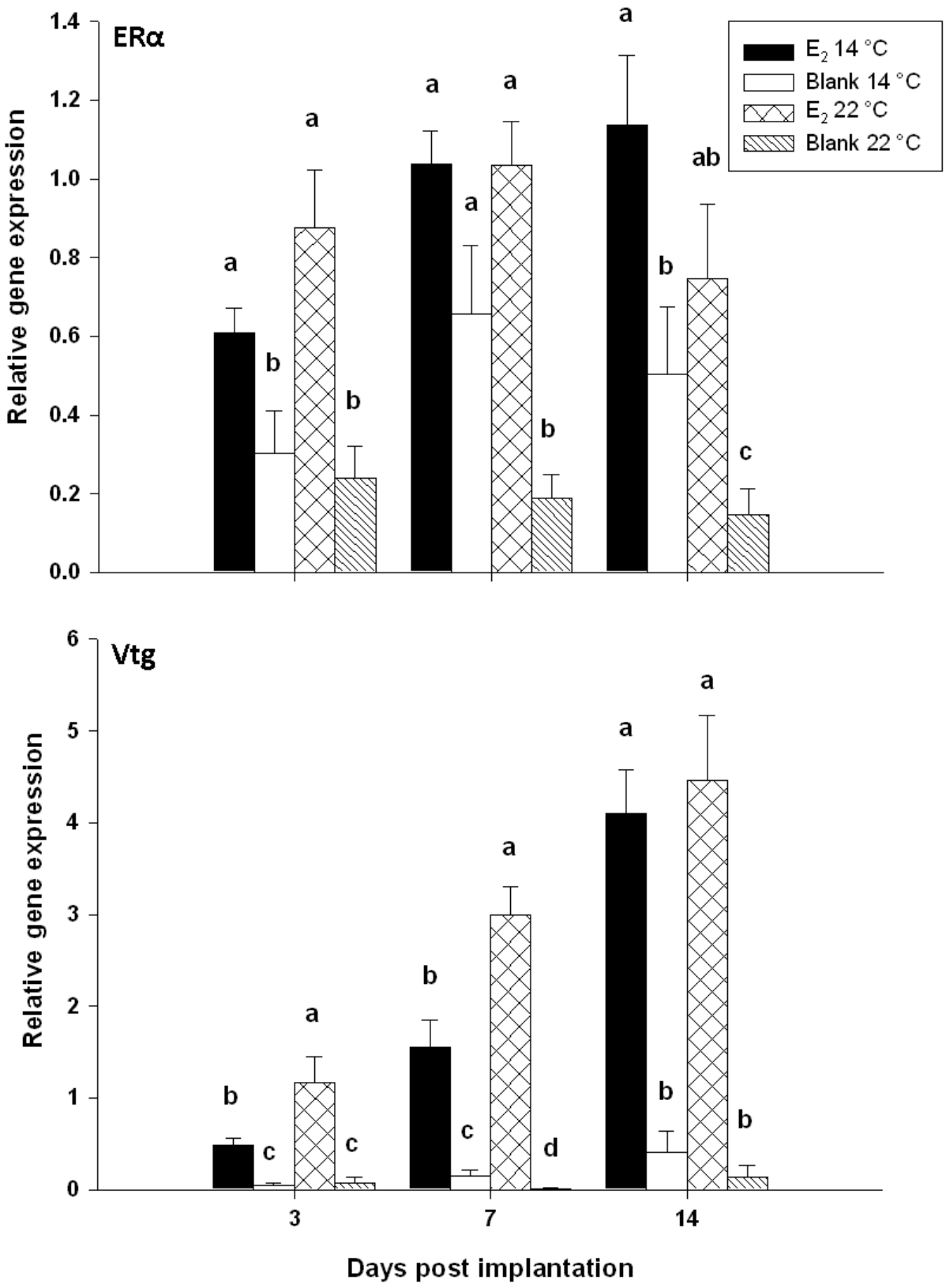
ERα
Relative gene expression
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
b
a
b
a
a
b
a
b
ab
c
E2 14 °C
Blank 14 °C
E2 22 °C
Blank 22 °C
Vtg
Relative gene expression
6
5
4
3
2
1
0
b
c
a
c
b
c
a
d
a
b
a
b
3
7
14
Days post implantation

Figure. 2. Relative gene expression levels (mean + SE, n = 7) of hepatic (a) ERα and (b) Vtg in juvenile Atlantic salmon given a blank implant at 14 ℃ or 22 ℃, or an E_2 implant (10 $mg.kg^{-1}$) at 14 ℃ or 22℃. Fish were sampled at 3, 7 and 14 days post implantation. Gene expression levels were normalised to Tbp, Hprt1 and Ef1α. Different superscripts between groups at each sampling time denote significantly different means ($p \leq 0.05$).

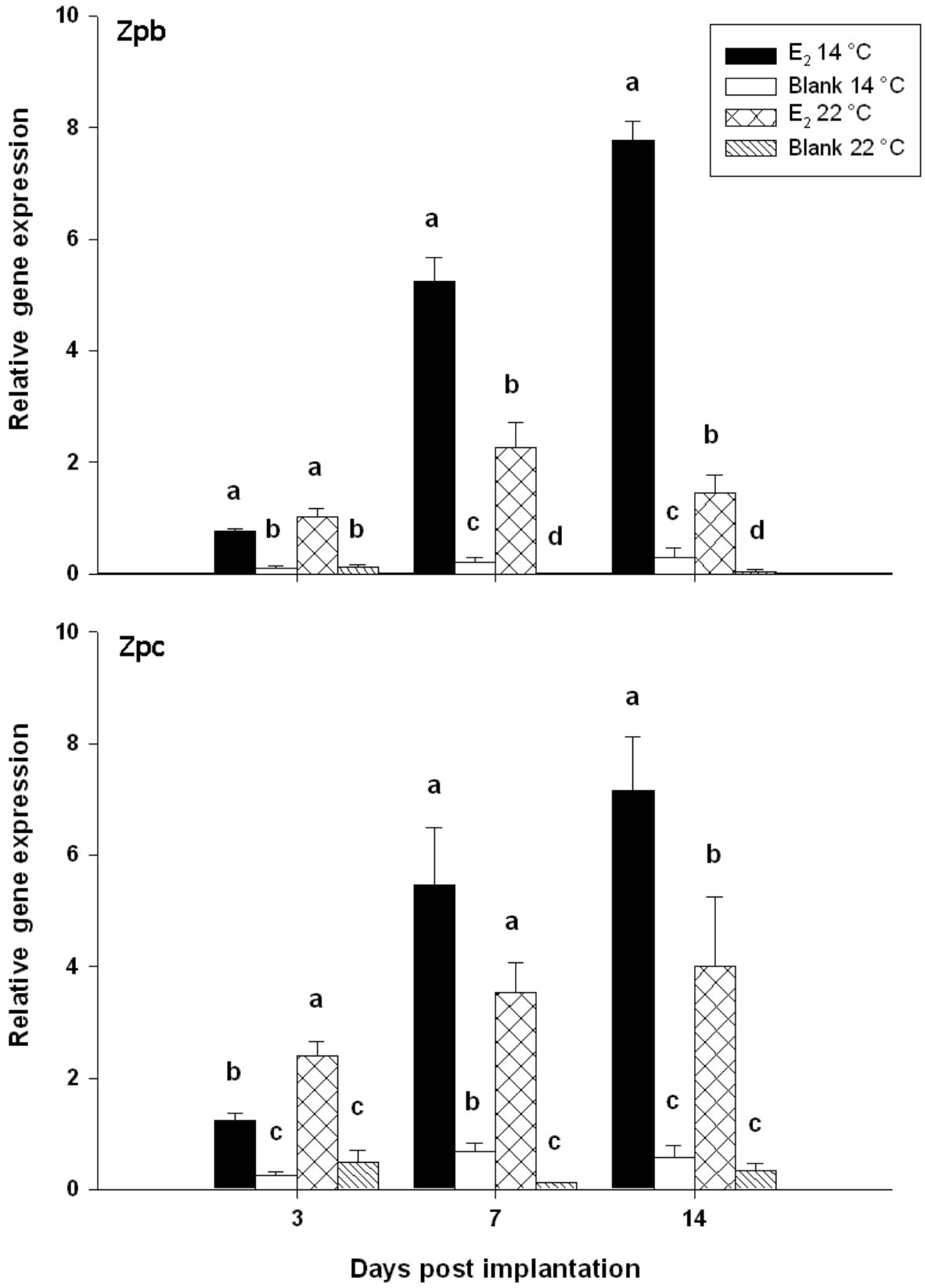
Zpb
Zpc
Relative gene expression
Relative gene expression
10
8
6
4
2
0
E2 14 °C
Blank 14 °C
E2 22 °C
Blank 22 °C
a
b
a
b
a
b
c
d
a
c
b
d
b
c
a
c
a
b
a
c
b
c
a
c
b
c
3
7
14
Days post implantation

Figure. 3. Relative gene expression levels (mean + SE, n = 7) of hepatic (a) Zpb and (b) Zpc in juvenile Atlantic salmon given a blank implant at 14 ℃ or 22 ℃, or an E_2 implant (10 $mg.kg^{-1}$) at 14 ℃ or 22℃. Fish were sampled at 3, 7 and 14 days post implantation. Gene expression levels were normalised to Tbp, Hprt1 and Ef1α. Different superscripts between groups at each sampling time denote significantly different means ($p \leq 0.05$).