A TEMPERATURE SHIFT DURING EMBRYOGENESIS IMPACTS PREVALENCE OF DEFORMITY IN DIPLOID AND TRIPLOID ATLANTIC SALMON (*Salmo salar* L.)

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Temperature shift during embryogenesis

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Abstract

The study investigated effects of a temperature shift during embryogenesis on diploid and triploid Atlantic salmon (*Salmo salar* L.) embryo development and juvenile skeletal deformities. From fertilisation, sibling populations were incubated under one of three temperatures (6, 8 or 11 °C) until 400 °days when all fish were then reared under a common temperature until smolt. Survival was negatively impacted by increasing temperatures irrespective of ploidy. There was no effect of incubation temperatures on growth in diploids, but triploids incubated at 6 °C had improved growth rates (thermal growth coefficient; TGC 6 °C: 1.05, 8 °C: 0.94, 11 °C: 0.48). Fish from 11 °C in both ploidies showed increased jaw and vertebral deformity prevalence. In response to the temperature change at 400 °days post-fertilisation, upregulation of *bmp2*, *bmp4*, *col2a1*, *mmp13*, *opn*, *sparc*, and downregulation of *ocn* further suggest that bone and cartilage formation is compromised after experiencing a thermal shift. The data show that temperature profile during embryogenesis strongly influences future growth and deformity prevalence. Triploids appear to require a lower incubation temperature than the current industry standard of 8 °C to promote better overall performance, however, a thermal shift during embryogenesis was shown to impact expression of important developmental genes.

Keywords: *Atlantic salmon, triploidy, incubation temperature, deformity, development, gene expression.*

1. Introduction

Temperature regimes are routinely adjusted in aquaculture to manipulate production windows and/or promote growth. The ontogeny of embryonic stages, previously described for Atlantic salmon (*Salmo salar* L.) (Gorodilov, 1996), is directly influenced by environmental conditions, in particular, water temperature. Higher incubation temperatures lead to accelerated development (Hayes, Pelluet and Gorham, 1953; Gorodilov, 1996) in relation to an increased metabolic rate (Clarke and Johnston, 1999). Diploid salmon embryos can tolerate temperatures from 0-16 °C (Hayes *et al*., 1953), however, early reports suggested an increase in mortalities in diploid embryos incubated below 4 °C and above 8 °C (Peterson, Spinney and Sreedharan, 1977; Gunnes, 1979; Fraser, Fleming, Poppe and Fjelldal, 2014). Thus, a maximum embryo incubation temperature of 8 °C has been adopted in commercial salmon hatcheries.

The use of elevated temperatures to meet production schedules and supply customers throughout the year is an important component of the Atlantic salmon farming success, however, subsequent negative impacts on stock performances and welfare must be considered. A previous study showed that incubating diploid salmon embryos at an elevated temperature (i.e. 10 °C) until the eyeing stage can produce larger fish throughout the freshwater cycle (MacQueen *et al*., 2008). However, by contrast, those incubated at 2 and 5 °C in the same study had higher growth rates after sea water transfer and the 5 °C treatment reached the same weight as those from the higher temperature treatment at the end of the trial. Somatic growth can be associated with altered muscle development resulting from early exposure to particular temperatures. Lower temperatures appear to promote muscle fibre recruitment in fish with preference towards hyperplasia over hypertrophy as reported with higher myotome counts (Hempel and Blaxter, 1961; Stickland, White, Mescall, Crook and Thorpe, 1988; Usher, Stickland and Thorpe, 1994; Nathanailides, Lopez-Albors and Stickland, 1995; Johnston and McLay, 1997; Johnston *et al*., 2003). A tendency towards hyperplasia can explain initial smaller size in fish incubated at 5 °C fish as energy is directed to generating new fibres rather than growing existing fibres (MacQueen *et al*., 2008). Such an increase in muscle fibre number suggests potential for these fish to grow faster and reach larger weights during hypertrophic growth at a later life stage.

Lower temperatures may reduce skeletal abnormalities in salmon. Recent studies have shown a correlation between higher temperatures, low survival and increased prevalence of jaw (Fraser, Hansen, Fleming and Fjelldal, 2015; Amoroso, Adams, Ventura, Carter and Cobcroft., 2016a; Amoroso *et al*., 2016b) and vertebral (Vågsholm and Djupvik, 1998; Wargelius, Fjelldal and Hansen, 2005; Ytteborg, Bæverfjord, Torgersen, Hjelde and Takle,

2010a) deformities. There is little known about the underlying mechanisms of skeletal abnormalities as a result of elevated temperatures, however it has been suggested that increased muscle mass is poorly supported by under-mineralised bone found in faster growing salmon (Fjelldal *et al*., 2006).

Triploidy in salmon refers to the extra set of chromosomes as a result of retention of the second polar body during meiosis, which in turn, renders the fish sterile. It has been reported that triploids can be initially smaller than diploid siblings but have increased growth potential in later life (Taylor, Preston, Guy and Migaud, 2011; Benfey, 2016; Smedley *et al*., 2018; Sambraus *et al*., 2020). This may be a result of a higher occurrence of hyperplasia in muscle fibres. However, an increased prevalence of skeletal deformities has also been reported in triploids, which is likely associated with faster growth (Fjelldal and Hansen, 2010; Leclercq *et al*., 2011; Taylor *et al*., 2013; Benfey, 2016; Fjelldal *et al*., 2015; Smedley *et al*., 2018). In order to support skeletal reinforcement for this enhanced growth, it is likely that triploids have higher nutritional requirements. Such results have elicited investigations into comparative nutritional requirements between ploidies (Burke, Sacobie, Lall and Benfey, 2010; Fjelldal *et al*., 2015; Taylor *et al*., 2015; 2019; Smedley *et al*., 2016; 2018; Clarkson *et al*., 2017; Sambraus *et al*., 2017; 2020; Vera *et al*., 2017; 2019). Supplementation of nutrients and the development of triploid-specific diets are certainly reducing prevalence of deformities, however, while dietary approaches have been effective in reducing the occurrence of malformation in triploids, pathologies such as lower jaw deformity (LJD) and some vertebral anomalies remain. As such, these malformations are likely to originate from early developmental stages prior to first feeding and have been hypothesised to be related to embryo incubation temperature regimes. This is likely a result from the thermal stress during a sensitive and vulnerable life stage. An upregulation of heat shock protein, *hsp70*, was observed in embryos with both a prolonged incubation temperature of 12 °C and when subsequently exposed to an acute temperature shock (1 °C or 16 °C) for one hour (Takle, Bæverfjord, Lunde, Kolstad and Andersen, 2005). This reaction of *hsp70* suggests a sensitivity to thermal change during embryogenesis.

Thermosensitivity of triploid embryos during embryogenesis may therefore be of particular importance. Fraser *et al*. (2015) showed that incubating embryos at 6 °C, rather than 8 or 10 °C, from fertilisation to first feeding can reduce the prevalence of skeletal deformities in triploids, supporting a likely different optimal thermal range compared to diploid siblings. However, no studies to date have investigated how temperature changes during embryogenesis may affect survival and growth performance of triploid salmon. Further, bone regulatory

mechanism discussions, specific to triploid salmon, are limited to later life stages (Fjelldal *et al*., 2015; Amoroso *et al*., 2016b; Smedley *et al*., 2018; Vera *et al*., 2019) and results during embryogenesis have not yet been reported. Ytteborg *et al*. (2010b) described upregulation of genes associated with bone formation (e.g. alkaline phosphatase, *alp*; collagen type 1 alpha 1 chain, *col1a1*; osteocalcin; *ocn*; bone morphogenetic protein 2, *bmp2;* and bone morphogenetic protein 4, *bmp4*) in diploids in response to elevated temperatures during development. Furthermore, Wargelius *et al*. (2005) found that gene expression associated with vertebral development was altered in salmon embryos exposed to an acute heat shock at 12 °C. With a different optimal range, it is likely that triploids require lower incubation temperatures to promote optimal skeletal development. This said, the adoption of lower and continuous hatchery temperatures to ensure optimal performance later in production inevitably slows down fish development and extends the hatchery phase.

The objective of the present study was to compare growth, muscle development and skeletal deformities in diploid and triploid Atlantic salmon smolts having been incubated at different temperatures during the embryo incubation period. Furthermore, each experimental temperature was restricted to the window from fertilisation to 400 °days post-fertilisation and then changed to 8 °C, therefore mimicking a temperature shift commonly experienced when ova are transferred from commercial broodstock facilities to hatcheries.

2. Materials and methods

All experimental procedures were conducted in compliance with the Animals Scientific Procedures Act 1986 (Home Office Code of Practice. HMSO: London January 1997) under project licence PPL70/7916 "Environmental Regulation of Fish Physiology" H. Migaud) in accordance with EU regulation (EC Directive 86/609/EEC). All experimentation performed at the Institute of Aquaculture (IoA) was subject to an ethical review process carried out by the University of Stirling Animal Welfare and Ethical Review Board (AWERB) prior to the work being approved.

2.1. Fish stock and culture conditions

On 8th December 2014, unfertilised eggs from three unrelated dams (2-sea winter) and milt from three unrelated sires (2-sea winter) were provided by Landcatch Ltd. (Ormsary, UK) and transferred to the Institute of Aquaculture (University of Stirling, UK). Eggs were fertilised (30 secs. mixing milt, 60 secs. rinse with 8 °C freshwater) creating three full-sib families and then divided (1 : 1 from each of the three full-sib families) for ploidy treatments and placed into a

water bath at 8 °C prior to triploid induction. Triploidy was induced in one group (655 bar of hydrostatic pressure for 6.25 mins. at 8 °C, 37 mins. post-fertilisation) according to Smedley *et al*. (2016), while the others experienced the handling but did not receive a hydrostatic shock and were maintained as diploids. After water hardening, eggs were further divided into three incubation temperatures (5.8 ± 0.7, 8.3 ± 0.1 or 10.8 ± 0.1 °C, referred to as 6, 8 and 11) and incubated in triplicate in aluminium egg trays (total = 54; 3 trays^{-1} family^{-1} temperature^{-1} ploidy^{-1}) within temperature-specific trough systems (15 mL sec^{-1} flow) in darkness. Temperature was monitored in 30 min intervals with temperature data loggers (HOBO 64K Pendant®, Onset Computer Corporation) submerged in the middle of the incubation trays, central in each trough system. At 400 °days post-fertilisation, ~800 eggs from the two best performing families (400 eggs family^{-1}) were pooled from each treatment (2 ploidies x 3 temperatures) and transferred to single 6 x 0.3 m^3 recirculation system (RAS) tanks and incubated at a constant temperature of 8 °C until first feeding (Fig. 1). One of the three families was removed from the study due to a high mortality rate in both ploidies suspected to be due to poor egg quality. Embryos in all treatments were kept in constant darkness until first feeding at which point fry were given constant light (LL). Three treatments per ploidy (a history of 6, 8 and 11 °C) were created, with eggs incubated at 8 °C referred to as the "control treatment" as routine in commercial practice. Whilst in RAS, pH measurements were regularly checked and maintained (pH 6.8 – 7.2), with the addition of sodium bicarbonate ($NaHCO_3$) as necessary. At 1 g, all fish were transferred to the Niall Bromage Freshwater Research Unit, Stirling, UK and reared in 6 x 1.6 m³ (2 ploidies x 3 temperatures) individual circular tanks under ambient temperature and simulated natural photoperiod (SNP) until smolt (24th April 2016). Due to the incubation temperature differences and subsequent impact on developmental speed, the number of feeding days (d) from first feeding until the end of the trial differed between treatments (6, 364d; 8, 386d; 11, 395d). Diploids were fed a standard commercial diet (BioMar INICIO Plus, 4.9 g kg^{-1} available P) and triploids were fed a triploid-specific diet (BioMar INICIO TRI-X, 7.7 g kg^{-1} available P), with the only difference in the formulation being P supplementation in TriX, and thus in accordance with previously published triploid specific P requirements (Fjelldal *et al*., 2015; Smedley *et al*., 2018; Sambraus *et al*., 2020) and the manufacturer's guidelines (BioMar Ltd, UK).

2.2. Verification of ploidy

To confirm ploidy status, blood smears were prepared from samples taken from the caudal peduncle of euthanised fish (20 fish treatment^{-1} ploidy^{-1}; 49.6 ± 25.8 g). Air dried slides were

fixed in 100 % methanol and then placed into Giemsa stain for 10 mins. Slides were digitised using a slide scanner at 20x magnification (Axio Scan Z1, Zeiss) and erythrocyte length and diameter was determined by Fiji software (ImageJ). A total of 30 randomly chosen nuclei per slide were measured to the nearest 0.01 μm and a mean taken for presumed diploid and triploid fish. Diploid control groups had significantly smaller erythrocyte nuclear lengths, with no overlaps with the triploid groups (2N, 7.2 - 8.6 μm; 3N, 9.2 - 11.1 μm) confirming that all sampled fish that were subjected to hydrostatic pressure shock were likely to be triploids.

2.3. Sampling procedures

At smolt, fish were selected by sweeping the full depth of the tank with a net and removing the first 50 from the net and sacrificed using an overdose of anaesthetic (Tricaine, Pharmaq; 1000 ppm). Fish were assessed for externally visible jaw (i.e. shortening or curvatures) and vertebral (i.e. lordosis, kyphosis, scoliosis, shortened trunk (STR) or tail (STA)) deformities and then frozen flat at -20 °C for later radiological deformity analysis (50 individuals $treatment^{-1}$ $ploidy^{-1}$) and to assess whole carcass mineral composition (3 pools; 3 individuals $pool^{-1}$ $treatment^{-1}$ $ploidy^{-1}$). Additional smolts were selected using the same method and sacrificed (Tricaine, Pharmaq; 1000 ppm) for myogenic morphology assessment (6 individuals $treatment^{-1}$ $ploidy^{-1}$). A 6 mm thick cross section was excised from the trunk immediately anterior to the dorsal fin. Cross sections were then mounted onto cork using optimal cutting temperature (O.C.T.) compound and frozen in isopentane cooled to -170 °C in liquid nitrogen and subsequently stored at -70 °C until processing.

Samples for expression of genes associated with lipid metabolism, muscle and bone formation were collected as whole individuals at eyeing and at first feeding (6 individuals $treatment^{-1}$ $ploidy^{-1}$). No further samples were collected to analyse lipid metabolism as subsequent samples were tissue specific for remaining genes. At smolt, muscle-related genes were analysed in dissected muscle from the Norwegian Quality Cut (NQC) region and the vertebral column under the dorsal fin (2 cm, ~10 vertebrae) was sampled for bone associated genes (6 individuals $treatment^{-1}$ $ploidy^{-1}$). All samples for gene expression analysis were collected into 'RNA Later', stored at 4 °C for 24 hours and then frozen at -20 °C until processing.

2.4. Growth assessment

Growth performance was assessed between first feeding (i; initial) and smolt (f; final). Following 24 hrs. of fasting, 30 individuals per tank were sedated (Tricaine, Pharmaq; 50ppm)

for body weight (BWi and BWf) and fork length (FL) measurements. Growth rate was calculated using the thermal growth coefficient (TGC, % BW °C d^{-1}).

2.5. Myogenic morphology assessment

Muscle fibre analysis was investigated according to Johnston, Strugnell, McCracken and Johnstone (1999). A cryostat (Leica CM1860 UV, Leica Biosystems, Nussloch, Germany) cooled to -18 °C was used to process 7 µm sections which were mounted onto charged microscope slides and stained with haemotoxylin and eosin (H&E). Slides were digitised (Axio Scan Z1, Zeiss) and subsections (2 x 1 mm areas) were selected from seven different myotome blocks to ensure analysis of ~1000 muscle fibres from different areas across the cross section. Morphometric analyses were carried out using Fiji Software (ImageJ).

2.6. Radiological deformity analysis

Right lateral radiographs were taken of smolts from each treatment (50 individuals $treatment^{-1}$ $ploidy^{-1}$) using a Faxitron UltraFocus Digital Radiography System (Faxitron Bioptics LLC., Arizona, USA) exposing individuals for 1.8 mA at 26 kV. Radiographs were digitalised (Faxitron UltraFocus100; Daax, UK) and subsequently examined using ClearCanvas Workstation (Personal Edition, Synaptive Medical, Toronto, Canada) by two independent blind evaluations. Severity of deformities was classified according to Hansen, Fjelldal, Yurtseva, and Berg (2010). Regions of the vertebral column were defined according to Kacem, Meunier and Bagliniere (1998); Cranial Trunk (R1), v1–8; Caudal Trunk (R2), v9–30; Tail (R3), v31–49; Tail Fin (R4), v50–58/59/60.

2.7. Mineral composition

Mineral composition was determined from whole smolt carcass (3 pools; 3 individuals $pool^{-1}$ $treatment^{-1}$ $ploidy^{-1}$) using the nitric acid (HNO_3) digestion technique. Pools of fish were homogenised, and oven dried at 75 °C for 24 hrs. and subsequently powdered using a mortar and pestle. Samples were digested in Kheldal digestion tubes with 69 % nitric acid using a MARS microwave digestion system (CEM MARSXpress, CEM ltd., Buckingham, UK) using the following program: 10 mins. heating phase to 190 °C, maintain 190 °C for 20 mins., cooling phase to 21 °C for 60 mins. Samples were then diluted with distilled water to 2 % HNO_3 and analysed for mineral content via Inductively Coupled Plasma Mass Spectrometry (ICP-MS; Thermo X series II; Collision cell technology). Due to a technical error during processing, no data for either ploidy of 11 °C are available.

2.8. Gene expression analysis

2.8.1. RNA extraction and cDNA synthesis

Samples were added to TriReagent® (Sigma-Aldrich, Gillingham, UK) at a ratio of 100 mg mL^{-1} reagent according to the manufacturer's protocol. Total RNA (totRNA) concentration was determined using a Nanodrop spectrophotometer (ND-1000; Labtech Int., East Sussex, UK) and quality of samples was confirmed by assessing the integrity of 28S and 18S ribosomal RNA (rRNA) with agarose gel electrophoresis (1 %). To eliminate genomic DNA (gDNA) contamination, samples were treated with DNA-free™ (Applied Biosystems, UK) as per the manufacturer's guidelines. cDNA was subsequently synthesised using 1 µg total RNA and a high capacity reverse transcription kit (without RNase inhibitor) (Applied Biosystems, UK). Final cDNA 10 µL reactions were diluted 1:10 in nuclease-free water to a total volume of 100 µL and 2.5 µL was used for each 10 µL (2.5 ng μL^{-1}) qPCR reaction.

2.8.2. Sequence information and primer design

Sequence-specific primers for genes were based on registered sequence data in Atlantic salmon from the National Centre for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov). Sequence information was then subjected to BLAST analysis against an Atlantic salmon genome and transcriptome (NCBI). Primer pairs were manufactured by MWG Eurofins Genomics (Ebersberg, Germany) and sequences with associated information are detailed in Table 1. Each primer product was purified by GeneJET PCR Purification Kit (Thermo Scientific, UK) according to manufacturer's instructions. Products were then cloned using the pGEM®-T Vector System (Promega) and plasmids were harvested using a GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich, UK) according to manufacturer's instructions. Each resulting plasmid was subsequently sequenced via LIGHTrunTM sequencing (GATC, Cologne Germany) to confirm identity. Plasmids were then linearised by enzymatic digest and standards for qPCR assays were generated using a serial dilution from 10^8 copies to 10 copies of each gene investigated.

2.8.3. Quantitative PCR (qPCR)

Absolute quantification qPCR assays were designed for genes and performed in accordance with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin *et al*., 2009). qPCR was performed in duplicate on individual samples using a Lightcycler® 480 II (Roche Diagnostics, West Sussex, UK) with relevant

primer pairs, cDNA template, nuclease-free water, and Luminaris™ Color HiGreen qPCR Master Mix (Applied Biosystems, UK) in a total reaction volume of 10 µL. Amplification was achieved in 384-well plates and conducted in a thermo cycling program consisting of a pre-incubation of 95 °C for 10 mins. followed by 40 cycles of; 95 °C for 15 secs., TA °C for 30 secs., and 72 °C for 30 secs. This was followed by a temperature ramp from 60 to 90 °C for melt-curve analysis to verify that no primer–dimer artefacts were present and only one product was generated from each qPCR assay. Quantification was achieved by a parallel set of reactions containing standardised plasmids described above.

Results from cDNAs were normalised by relating expression data to geometric mean of two reference genes with stable gene expression levels across samples; *β-actin* and *elf-α*. Gene activity was expressed as a fold change from 8 °C (current industry incubation temperature) specific to each ploidy. As it remains unknown how the extra set of maternal chromosomes in a triploid animal impacts on the transcriptome, gene expression analyses were assessed separately for each ploidy.

2.9. Statistics

Data were analysed using Minitab (Version 17.0, Minitab Inc., Pennsylvania, USA) statistical analysis software. Normality and homogeneity of variance in the data were confirmed using Kolmogorov-Smirnov and Levene's tests, respectively, with any percentage data being arcsine transformed. Two-way ANOVAs were used to test ploidy, temperature treatment and their interaction when assessing growth and weight, muscle morphometric and mineral composition. Post-hoc tests were determined by Tukey's multiple comparisons. One-Way ANOVAs were used to test the temperature treatment on gene expression for individual ploidy. Gene expression data were log2 transformed prior to statistical analyses. The likelihood of a treatment experiencing increasing deformity severities was determined using ordinal logistic regression (OLR), using the 8 °C treatment as the reference within respective ploidy analyses. For mortality and gross deformity, 95% confidence intervals were estimated according to the central limit theorem (CLT; Feller, 1968, 1971) and overlapping standard deviations between treatments were considered not significantly different. Use of this approach is referred to as "Analysis of CLT" throughout. All significance was accepted at $p < 0.05$.

3. Results

3.1. Mortality

Within all temperature treatments, triploids had a higher cumulative mortality than their diploid counterparts (Table 2). Irrespective of ploidy, there was an increase in cumulative mortality in response to increasing embryo incubation temperatures. Diploids appeared to have a linear increase in cumulative mortality (~10 % between each temperature), whilst triploids had a more prominent increase from 6 °C (49.3%) to 8 and 11 °C (68.7 and 72.9%, respectively).

Triploid embryos had a consistently higher mortality compared to diploids under all temperature regimes between fertilisation to 400 °days, however no significant differences were observed due to the large variation between families (Table 2).

No ploidy difference was also shown in mortality between 400 °days to hatch in 6 or 11 °C treatments, however triploids had a significantly higher mortality than diploids when incubated at 8 °C (Table 2). In diploids, mortality was comparable in 6 and 8 °C treatments, however a significant increase was shown in embryos incubated at 11 °C. In triploids, a significant increase in mortality was found in both 8 and 11 °C compared to 6 °C.

Between hatch and first feeding, no ploidy differences were observed in any temperature treatment (Table 2). Within diploids, there was a significant increase in mortality from 6 to 8 °C, however mortality in 11 °C showed comparable rates to both 6 and 8 °C. Triploids showed a similar trend with mortality significantly increasing between 6 to 8 °C. However, mortality in 11 °C was comparable to 8 °C, but not 6 °C.

During the final window (first feeding to smolt), triploids had a higher mortality than diploids in all treatments (Table 2). Both ploidies showed the same trend of increasing mortality between 6 and 8 °C, and comparable mortality between 8 and 11 °C.

3.2. Growth performances

An overall effect of ploidy ($p < 0.001$, 2N > 3N) but no overall temperature effect was observed ($p = 0.234$) on first feeding weight (BWi). No difference between ploidies was observed for BWi at 6 °C, however, diploids had a significantly higher BWi than triploids in 8 and 11 °C treatments (Fig. 2a). Within each ploidy, no differences were found in BWi between temperature treatments.

There was a significant effect of both ploidy ($p < 0.001$, 2N > 3N) and temperature ($p < 0.001$, 8 > 6 > 11 °C) on final smolt weight (BWf) and a significant interaction between ploidy and temperature ($p < 0.001$). BWf was comparable between ploidies in fish at 6 °C, however, diploids had significantly higher BWf than triploids in 8 and 11 °C treatments (Fig. 2b). Within diploids, there was an increase in fish BWf between 6 and 8 °C treatments, and

then comparable thereafter. Conversely, BWf of triploids was comparable between 6 and 8 °C treatments, but lower in fish at 11 °C.

Although statistical differences could not be determined, TGC of both diploid and triploid fish at 6 °C appeared to be similar (Fig. 2c). However, triploids appeared to have a lower TGC at 8 and 11 °C compared to diploids.

3.3. Muscle analysis in smolts

Diploids had an overall higher final fibre number (FFN) compared to triploids, however within temperature treatments, differences were significant only in fish from the 6 °C treatment with a higher (+23 %) FFN in diploids than triploids (Table 3). An overall temperature effect was observed with FFN in fish from 8 °C being significantly greater than 11 °C, but both comparable to FFN in fish at 6 °C. In diploids, there was no significant difference in FFN between any temperature treatments, while in triploids, fish at 8 °C had significantly more fibres per mm^2 compared to 6 °C, but both were comparable to 11 °C.

Triploids had an overall higher muscle fibre cross-sectional surface area than diploids, however within temperature treatments, differences were significant only in fish from the 6 °C treatment with a higher (+23 %) area in triploids than diploids. There was a significant interaction between ploidy and temperature on muscle fibre cross-sectional surface area whereby no differences were found within diploids, while triploids had significantly higher area at 6 °C compared to 8 °C, but both were comparable to 11 °C.

3.4. Deformity analysis

Triploids had a significantly increased visible external deformity prevalence in 8 °C (8 *vs.* 0 %) and 11 °C (50 *vs.* 18 %), however no difference was found in 6 °C (Table 4a). Within diploids, visible external deformity prevalence was significantly greater in 11 °C compared to 8 °C but not different from 6 °C. Triploids also had increased deformity prevalence in 11 °C compared to 8 °C, but also significantly higher when compared to 6 °C. These deformities were mainly comprised of jaw malformation as externally visible vertebral deformities were negligible. Triploids had higher jaw deformity prevalence in 8 °C and 11 °C compared to diploids but no difference between ploidy was evident at 6 °C. In diploids, jaw deformity prevalence was significantly higher in 11 °C compared to 8 °C, with those in 6 °C showing intermediate prevalence. However, in triploids, jaw deformity prevalence was significantly higher in 11 °C compared to both 6 and 8 °C.

There was no difference in the number of vertebrae per fish, assessed radiologically, between ploidies or treatments (58.4 ± 0.2; $p > 0.05$). Similarly, there were no significant differences observed in the number of radiologically deformed vertebrae (dV) in deformed individuals between treatments or ploidy (Table 4b).

Triploids had a significantly higher deformity prevalence than diploids in 8 °C (72 vs. 24 %), and 11 °C (88.2 vs. 40 %) treatments, but prevalence was comparable between ploidies at 6 °C (Table 4b). Within diploids, there was no significant difference in the prevalence of radiologically deformed individuals with increasing incubation temperatures. Within triploids a comparable deformity prevalence was exhibited between 8 and 11 °C, however those from 6 °C had a significantly lower prevalence.

Deformed individuals were categorised by severity bands (Fig. 3; None: 0 dV; Mild: 1-5 dV, Moderate: 6-9 dV or Severe: ≥10 dV). Within diploids, in comparison to the control treatment, incubated at a constant 8 °C, the likelihood of fish from 11 °C treatments having increased deformity severity was significant (OLR CoEf = -0.91, $p = 0.036$), however, no differences were found when compared to fish from treatments 6 °C (OLR CoEf = -0.01, $p = 0.991$). With reference to triploids incubated at a constant 8 °C, there was a significantly reduced likelihood of deformity occurrence in fish from 6 °C treatments (OLR CoEf = 1.15, $p = 0.004$) and a significantly increased likelihood when originating from 11 °C treatments (OLR CoEf = -1.75, $p < 0.001$).

The most common location for these deformed vertebrae was in the tail fin (R4) irrespective of ploidy or temperature (Fig. 4a, b, c). In triploids, there is a common peak at v54 in all temperatures, however, in diploids there was a shift in the peak with increasing temperatures (6, v54-55 < 8, v54-56 < 11 °C, v56-57). The prevalence in this region appeared to be consistent in diploids in each temperature treatment (~10 %), however an increase was observed in triploids in response to increasing temperature treatments (6, ~20 % < 8, ~30 % < 11 °C, ~50 %). Both diploids and triploids showed a similarly increased deformity prevalence in the caudal trunk (R2) when incubated at 11 °C, however triploids had a much larger increase in the cranial trunk (R1) compared to diploids (35 *vs*. 10 %).

3.5. Mineral composition

Overall, triploids had higher levels of calcium (Ca; 4581.8 – 4765.2 *vs.* 3852.1 – 3897.4 µg mg^{-1}, $p = 0.002$), phosphorous (P; 4521.1 – 4548.0 *vs*. 4067.7 – 4078.0 µg mg^{-1}, $p = 0.008$) and Ca:P (1.00 – 1.05 *vs*. 0.94 – 0.95, $p = 0.002$) compared to diploids. No temperature difference was found between 6 and 8 °C, irrespective of ploidy.

440

441 3.6. Gene expression analysis

442 *3.6.1. Genes associated with lipid utilisation*

443 No significant differences in expression were observed in any of the genes associated with lipid
444 utilisation at the eyeing stage in both ploidy (Fig. 5a, b).

445 Conversely, at first feeding (after the temperature shift), an upregulation of *fas* was
446 found in diploids from 6 and 11 °C treatments (~2 fold), and an upregulation of *srebp1* was
447 found in both ploidy from 6 and 11 °C (4 – 8 fold) compared to those in 8 °C (Fig. 5c, d). A
448 significant downregulation of *lxr* was observed in triploids at 6 °C compared to 8 °C, with 11
449 °C being intermediate. Furthermore, no differences in expression were found between
450 temperature regimes in *lxr* for diploids, and *fas* for triploids.

451

452 *3.6.2. Genes associated with myogenesis*

453 At the eyeing stage, there was an upregulation in *igfbprp* found in ova from 11 °C in both
454 diploids (~4.5 fold) and triploids (~3 fold) compared to 8 °C but were only significantly greater
455 than expression at 6 °C within respective ploidies (Fig. 6a, b). No other differences were found
456 between temperature treatments in diploids, however there was a significant downregulation
457 of *igf1* and *myod* in triploid ova from 6 °C compared to 8 °C.

458 At first feeding, there was a significant upregulation in *igf1* (~3.5 fold), *igf2* (~6.5 fold)
459 and *igfbrp* (~8.5 fold) found in fish from both 6 and 11 °C treatment compared to 8 °C in
460 diploids (Fig. 6c). Conversely, *igf1r* showed lower expression in diploids from 6 and 11 °C
461 treatments compared to 8 °C. In triploids, *igf1* had significantly greater expression in fish from
462 6 °C (~4 fold) and 11 °C (~8 fold) compared to 8 °C (Fig. 6d). Further, upregulation in fish
463 from 11 °C was significantly higher than in 6 °C. Like in diploids, *igf1r* expression was also
464 reduced in triploid fish from 6 and 11 °C compared to 8 °C. *igf2* expression in triploids was
465 significantly greater in 6 than 8 °C, with 11 °C intermediate. *igfbprp* expression in triploids
466 was significantly greater in 6 and 11 °C (~4 fold) compared to 8 °C. No differences were found
467 in expression of either *myf5* or *myod* in either ploidy.

468 At smolt, there were also no differences found in expression of any of the genes
469 involved with muscle development in diploids (Fig. 6e). In triploids, a significant difference
470 between temperatures was only observed in *igf2*, with fish from 6 °C showing an upregulation
471 (~2 fold) compared to those from 8 °C (Fig. 6f).

472

473 *3.6.3. Genes associated with bone formation and mineralisation*

Diploid ova at the eyeing stage showed a significant upregulation of *alp* in 11 °C (~1.7 fold) compared to 8 and 6 °C (Fig. 7a). Both *col1a1* and *col2a1* expression was significantly higher in diploids of 11 °C compared to 6 °C, although were comparable to 8 °C. In triploids, there was a significant downregulation of *alp*, *col2a1*, *opn* and *sparc* in 6 °C compared to both 8 and 11 °C (Fig. 7b). No other differences were observed.

At first feeding in diploids, no difference in *alp* expression was observed (Fig. 7c), however a significant upregulation in first feeding triploids from 11 °C (~2 fold) compared to 8 °C was found (Fig. 7d). A significant upregulation of *bmp2*, *bmp4*, *col2a1*, *mmp13*, *opn* and *sparc* occurred in first feeding fry from the 6 and 11 °C treatments, irrespective of ploidy (Fig. 6c, d). An upregulation was also found in *col1a1* expression in diploids from 11 °C compared to 8 °C (~2 fold), however no differences were found between any of the temperature treatments in triploids. *ocn* was the only gene associated with bone formation to show a significant downregulation in 6 and 11 °C compared to 8 °C, and this occurred in both ploidies.

At smolt, there were no differences found in expression of any of the genes associated with bone formation within each ploidy (Fig. 7e, f).

4. Discussion

The present study showed that incubation temperature during embryonic development had an evident effect on both diploid and triploid Atlantic salmon survival, growth and skeletal health. Results confirmed that triploids have a lower optimal thermal range than diploids, and they showed an improved survival when incubated at ~6 °C during embryogenesis pre-hatch. Moreover, growth rate was comparable between ploidies incubated at 6 °C, but triploids had poorer growth compared to diploids at 8 and 11 °C. This study intends to build on results from Fraser *et al*. (2014 and 2015) and investigate the effect of thermal switching during embryogenesis.

As reported previously in other trials, mortality rate during embryogenesis was overall higher in triploids compared to diploid siblings (Sutterlin, Holder and Benfey, 1987; O'Flynn, McGeachy, Friars, Benfey and Bailey, 1997; Benfey, 2001; Taylor *et al*., 2011; Fraser *et al*., 2015; Amoroso *et al.*, 2016a). However, triploids incubated at a lower temperature (~6 °C) during embryogenesis showed an increased survival as previously reported (Fraser *et al*., 2015). Indeed, comparable survival between ploidies were observed within each period prior to first feeding, when incubated at 6 °C, supporting the theory that triploids have a lower optimal thermal range than diploids. Results from Fraser *et al*. (2014) suggested that increased mortality in embryos incubated at elevated temperatures may be linked with aplasia of the

septum transversum, a malformation of the heart in which the cavity and the abdominal cavity are separated. The analysis during freshwater up to smolt also showed mortality correlating with incubation history, with those exposed to a higher temperature during incubation having the highest mortality rates. This was true for both ploidies, highlighting the need for lower temperatures during this developmental stage.

Embryonic growth was not affected by the differing incubation temperature regimes as shown by comparable first feeding body weight (BWi) within each ploidy. The authors acknowledge the lack of replication in treatments after 400 °days due to limited facility availability. Further studies should be conducted to validate the effect of such temperature regimes on the parameters analysed. As reported in previous studies (Galbreath, St. Jean, Anderson and Thorgaard, 1994; McGeachy, Benfey and Friars, 1995; Taylor *et al*., 2011), BWi for all temperature treatments in this study were higher in diploids than in triploids, and significantly so in 8 and 11 °C. This was supported by the lack of differences in ploidy-specific expression of *myf5* and *myod* between treatments during embryogenesis, both genes being involved in the initial stages of muscle development. Moreover, there was an altered expression in genes associated with the somatotropic axis in both ploidies, whereby *igf1*, *igf2*, and *igfbprp* all showed increased expression in fish from 6 and 11 °C treatments relative to the 8 °C control at first feeding (after the temperature change). This supports the idea of direct thermal influence on gene expression, particularly the temperature shift experienced during the late stages of embryogenesis. Although there was an upregulation in the 6 and 11 °C treatments, it is likely that the BWi assessment came too early post temperature change to see any effect on growth. There was a significant downregulation of *igf1r* for both ploidies in 6 and 11 °C treatments. This receptor binds and thereby regulates the action of insulin-like growth factors (IGFs) and therefore having a lower expression may account for an over expression in the IGFs. As this is prior to any exogenous feeding, it is clear that utilisation of the yolk is impacted by incubation temperature. Previous studies in salmonids suggested that thermal regime during embryo incubation plays a major role in the catabolism of the yolk reserves (Marr, 1966; Peterson *et al*., 1977; Heming, 1982; Peterson and Martin-Robichaud, 1995; Ojanguren, Reyes-Gavilán and Muñuz, 1999), which is likely linked to thermosensitive enzymes. Fagotto (1995) described that pH and enzymatic latency are the most likely determinants of yolk utilisation and it is well established that temperature has a direct impact on enzyme activity and the ionisation of a solution, subsequently altering the pH. Therefore, it is highly likely that temperature not only affects somatic metabolism, but more fundamentally, the utilisation of yolk during embryogenesis.

In the present study, we observed no differential expression in genes associated with lipid utilisation between incubation temperatures at the eyeing stage (~250 °days) in both ploidies. However, the ~2 °C temperature change at 400 °days clearly influenced the ability to utilise the yolk later during embryonic development as individuals from both the 6 and 11 °C treatments showed upregulation of *fas* in diploids and *srebp1* in both ploidies at first feeding (~900 °days). The expression of these genes, involved with fatty acid biosynthesis and subsequent storage, appears to be significantly increased as a result of the temperature shift, suggesting that acute changes in temperature has a direct influence on the energy status of a developing fish. Although the gene expression pattern appeared to be the same in fish incubated at 6 and 11 °C, the resulting growth was very different. Furthermore, a downregulation of *lxr* was found in triploid first feeding fry previously incubated at 6 °C, whist no differences were observed in those from 11 °C or in diploids from either experimental temperature. This suggests that the two ploidies show different metabolism in response to different temperatures from an early life stage and is supported by the difference in first feeding weights between ploidy (2N > 3N). Published studies in rainbow trout (*Oncorhynchus mykiss*) and red crucian carp (*Carassius auratus*) have shown higher protein absorption in triploids compared to their diploid siblings and several studies have suggested that enhanced growth may be associated to an upregulation of glutamate dehydrogenase and oligopeptide transporter (Olivia-Teles and Kaushik, 1990; Liu et al., 2012; 2014). Yolk proteins are metabolised and used to build muscle through a combination of hyperplasia (fibre recruitment) and hypertrophy (growth of present fibres).

This initial difference in first feeding weight would certainly impact the subsequent growth trajectory of the cohorts. Due to fertilisation of all treatments occurring on the same day (to control for parental effect) and each treatment experiencing different thermal regimes, the first feeding dates ultimately differed. As a result, treatments experienced a different number of feeding days up to the point of smoltification. This differential grow out duration in part resulted in different final body weights (BWf) at smolt between temperature treatments that could not be compared directly. However, when comparing ploidies, the growth rate, and therefore BWf, was reduced in triploids from the 8 and 11 °C treatments relative to diploids. This agrees with the theory that triploids have a lower thermal tolerance than diploids and concurs with a recent study where metabolic rate and aerobic scope in triploids suffered at 10.5 °C but was comparable to diploids at 3 °C (Riseth, Fraser, Sambraus, Stien and Hvas, 2020). Nevertheless, triploids have also been reported to have better growth rates than diploids under different experimental incubation temperatures (Taylor *et al*., 2011; Fraser *et al*., 2015). In the

present study, both ploidies had comparable growth rates when incubated at 6 °C, suggesting that triploids held for this short duration at the lower temperature was enough to match the growth performance of diploids.

In order to standardise smolt weights for comparison, final weights were predicted using TGC in each treatment to adjust to the same number of developmental grow out days (Fig. 8). Diploid smolts were predicted to be in the range of 72 - 82 g with no clear effect of incubation temperature. Triploids at 6 °C were predicted to match their diploid siblings (71.7 and 72.6 g, respectively), however, they were predicted to be much smaller at 8 °C (53.5 g) and 11 °C (10.7 g), further supporting the detrimental effect of increased egg incubation temperatures on triploids. Moreover, the temperature shifts that fish from the 6 and 11 °C treatments experienced may have contributed to reducing their respective optimal growth supporting the thermal impacts during embryogenesis reported in a previous study, albeit in diploid Atlantic salmon (Takle *et al*., 2005).

Previous studies in diploid Atlantic salmon have reported a preference for muscle growth through an increase in hypertrophy over hyperplasia when embryos were incubated at 10 or 11 °C compared to 1.6 or 6 °C, respectively (Stickland *et al*., 1988; Usher *et al*., 1994). This may be an energy preservation strategy as nuclear division (hyperplasia) requires more energy expenditure than hypertrophy. This theory coincides with reports of fewer, yet larger fibres and fewer nuclei present in muscle samples of fish incubated at high temperatures (Usher *et al*., 1994). In the present study, a temperature effect was observed in triploids as smolts from the 6 °C treatment had a significantly lower FFN and higher fibre area compared to 8 °C. It is important to note that fish from each temperature treatment were at different developmental stages at this sampling point due to their earlier differences between thermal profiles, yet this would be the transition to seawater in a commercial setting (i.e. end of parr-smolt transformation). This means that although a significant difference was observed at smolt in triploids incubated at 6 °C, they had less developmental days than the 8 °C (22 days less) and 11 °C (31 days less) treatments. However, comparing ploidies within a temperature treatment shows that triploids had a lower FFN and higher fibre area relative to their diploid counterparts in the 6 °C treatment, but comparable in 8 and 11 °C treatments. Johnston *et al*. (1999) reported a lower density of satellite cells in triploids which are important precursors to muscle fibre recruitment which contrasts with the current result. This may be a result of the different freshwater temperatures experienced in each of the studies during embryonic development (fluctuations between 10 – 3 °C vs. constant 6 °C then 8 °C) or differing temperature maximums reached during the parr stage (21 vs. 14 °C). Fish with a higher proportion of newly

recruited fibres are likely to have a greater potential for growth through subsequent hypertrophy after seawater transfer however the present trial could not be continued into seawater stages to confirm this.

A higher prevalence of externally observable deformities was found in the highest incubation temperature treatment in both ploidies, with jaw malformations (LJD) being the most common. This would suggest that jaw malformation manifests itself during embryogenesis and is not solely a dietary deficiency, as ploidy-specific treatments were fed the same diet. In recent years, both vertebral and jaw deformity results in triploid trials have sparked investigation into nutritional requirements and triploid specific diets have been trialled with supplementation of minerals, in particular P (Burke *et al*., 2010; Fjelldal *et al*., 2015; Taylor *et al*., 2015; Smedley *et al*., 2016; 2018; Sambraus *et al*., 2020). In the present trial, all triploids were fed a high P diet, in accordance with previously published triploid specific P requirements (Fjelldal *et al*., 2015; Smedley *et al*., 2018; Sambraus *et al*., 2020). However, LJD was still observed in the higher temperature treatments, suggesting that lower incubation temperature during embryogenesis had a greater effect on favourable jawbone development than diet. This theory agrees with results from Fraser *et al*. (2015) who also reported a reduction in jaw and skeletal deformity in triploid Atlantic salmon when reared at lower incubation temperatures, albeit using a commercial diet with no P supplementation from first feeding.

Ca and P are both key minerals for bone mineralisation (Fjelldal, Nordgarden and Hansen, 2007) and thus it appears that incubation temperature (or the temperature shift) had no impact on mineralisation within a given ploidy. There was an overall ploidy effect in whole body Ca, P and Ca:P ratio, with triploids having significantly higher concentrations, however, this most likely reflected the ploidy-specific diets used to ensure optimal nutritional requirements were met. Nonetheless, whole body P and Ca levels in both ploidy and treatments were within normal accepted ranges generally accepted to be reflective of good bone mineralisation (Bæverfjord, Åsgård and Shearer, 1998). Collectively, these observations suggest that the deformities experienced in response to increased incubation temperature may be related to impaired cartilage development rather than extracellular matrix (ECM) mineralisation itself. This would support the findings of Amoroso *et al*. (2016b) who linked temperature induced LJD with cartilage impairment rather than disruption of ECM mineralisation directly. Amoroso *et al*. (2016b) also observed a significant downregulation of *gphb5* and *col2a1* in smolts with known LJD suggesting possible hormonal involvement and cartilaginous impairment, thus concluding these genes to be reliable biomarkers of LJD. In contrast, in the present study, a significant upregulation of *col2a1* was observed at first feeding

in fish that experienced a temperature switch at 400 °days compared to the constant 8 °C control. Furthermore, there was an increase in prevalence of jaw malformation in fish exposed to 11 °C for both ploidies. In accordance with previous studies (O'Flynn *et al*., 1997; Benfey, 2001; Fraser *et al*., 2015), there was a higher occurrence of LJD in triploids compared to diploids, however, this was only evident in 8 and 11 °C treatments whereas no difference was observed at 6 °C. This further supports the suggested lower optimal thermal range of triploids relative to their diploid counterparts, and that triploids can perform similarly to diploids when incubated at 6 °C or below. It should be noted that results reported from Amoroso *et al*. (2016b) were obtained from dissected jaw tissue from smolts with known LJD whereas in the current study, vertebral tissue was analysed, and deformity status was unknown at the point of sampling which may in part explain the contrasting results between both studies. Nonetheless, *col2a1*, associated with cartilage formation, is likely associated with LJD and a temperature change during embryogenesis may alter normal cartilage development. Further investigations into these biomarkers for specific malformation aetiology using individuals that possess vertebral deformities should be explored, in addition to histological verification of differences in the ECM and collagen development.

A clear correlation between radiologically assessed vertebral deformity prevalence in triploids and increasing temperature was also evident. This was mostly comprised of increasing incidences of deformed vertebrae in region 4 and region 1 (the latter to a lesser extent), both of which are regions that have been previously reported as susceptible to morphological variations (Kacem *et al*., 1998). Again, within all temperature treatments, triploids had a higher prevalence relative to diploids, however, were not considered deficient in Ca or P according to their whole-body mineral composition. According to Sambraus *et al*. (2020), it was suggested that there may be critical windows of elevated P requirements in early life stages and therefore the single sampling point in the current study may have missed valuable information of a prior nutritional deficiency. When assessing deformity prevalence, all malformed vertebrae were considered although these are not necessarily detrimental to the health and welfare of the fish. With that in mind, severities of vertebral deformities are a more relevant parameter for comparison. According to Hansen *et al*. (2010), a low prevalence of deformed vertebrae (<6 dV) at harvest would not have detrimental effect on the performance and welfare of the fish although severity of the malformation cannot be overlooked. In the present study, vertebral deformity assessment was conducted at smolt, therefore how these deformities may progress in seawater remains unknown. However, there was a clear correlation between severity of vertebral deformity and increasing incubation temperature in both ploidies which concurred

with results from Fraser *et al*. (2015). Vertebral deformities considered as severe (i.e. ≥10 dV) were absent in the 6 °C treatments for both ploidies, except for one single diploid fish, which is very promising considering historical studies reporting increased deformities in triploids. It can therefore be assumed that an increase in severe vertebral deformities associated with sub-optimal egg incubation conditions, particularly in triploids, will have significant implications for fish welfare and growth performance (Grini, Hansen, Berg, Wargelius and Fjelldal, 2011; Fraser, Fjelldal, Hansen and Mayer, 2012; Fraser *et al*., 2015; Amoroso *et al*., 2016a). Moreover, accelerated growth observed at higher temperatures has been associated to altered gene transcription, particularly those coding for osteoblast development and chondrocyte growth (Ytteborg *et al*., 2010a). Temperature had little or no impact on the expression of genes associated with bone formation and mineralisation in both ploidies at first feeding as expression of all genes, except *col2a1*, were comparable in both 6 and 11 °C treatments. However, a significant differential expression pattern was apparent in both ploidies between these two temperature "shifted" regimes relative to the constant 8 °C control, suggesting that the temperature alteration experienced at 400 °days post-fertilisation had a direct influence on the expression of these genes.

5. Conclusions

The present study confirmed that an incubation temperature of 6 °C until 400 °days is favourable to maintain good development of diploid and triploid Atlantic salmon embryo. This lower temperature during embryogenesis reduced mortality rates and severity of deformity historically associated with triploids and matched the growth performance of their diploid siblings. This investigation agrees with previous results, suggesting that early rearing of triploids at 6 ˚C until first feeding is preferential for development and welfare (Fraser *et al*., 2015). While such a regime adds ~2 weeks to the period of embryogenesis under a production cycle, it has a major advantage on triploid Atlantic salmon health compared to the conventional 8 °C rearing regime. Despite this, the temperature switch at 400 °days did result in altered expression of important developmental genes, highlighting the sensitivity to temperature changes likely experienced during commercial embryo rearing which may have influenced mortality, growth or deformity. This should be kept in mind in aquaculture production, particularly with triploids, as both broodstock and hatchery sites regularly manipulate rearing temperatures to meet customer demands. Evidently, a balance must be met, and optimisation of triploid-specific hatchery temperature conditions further contributes to the growing

knowledge of triploid culture requirements to promote optimal performance and welfare to support potential commercial implementation.

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Declaration of Interest

None.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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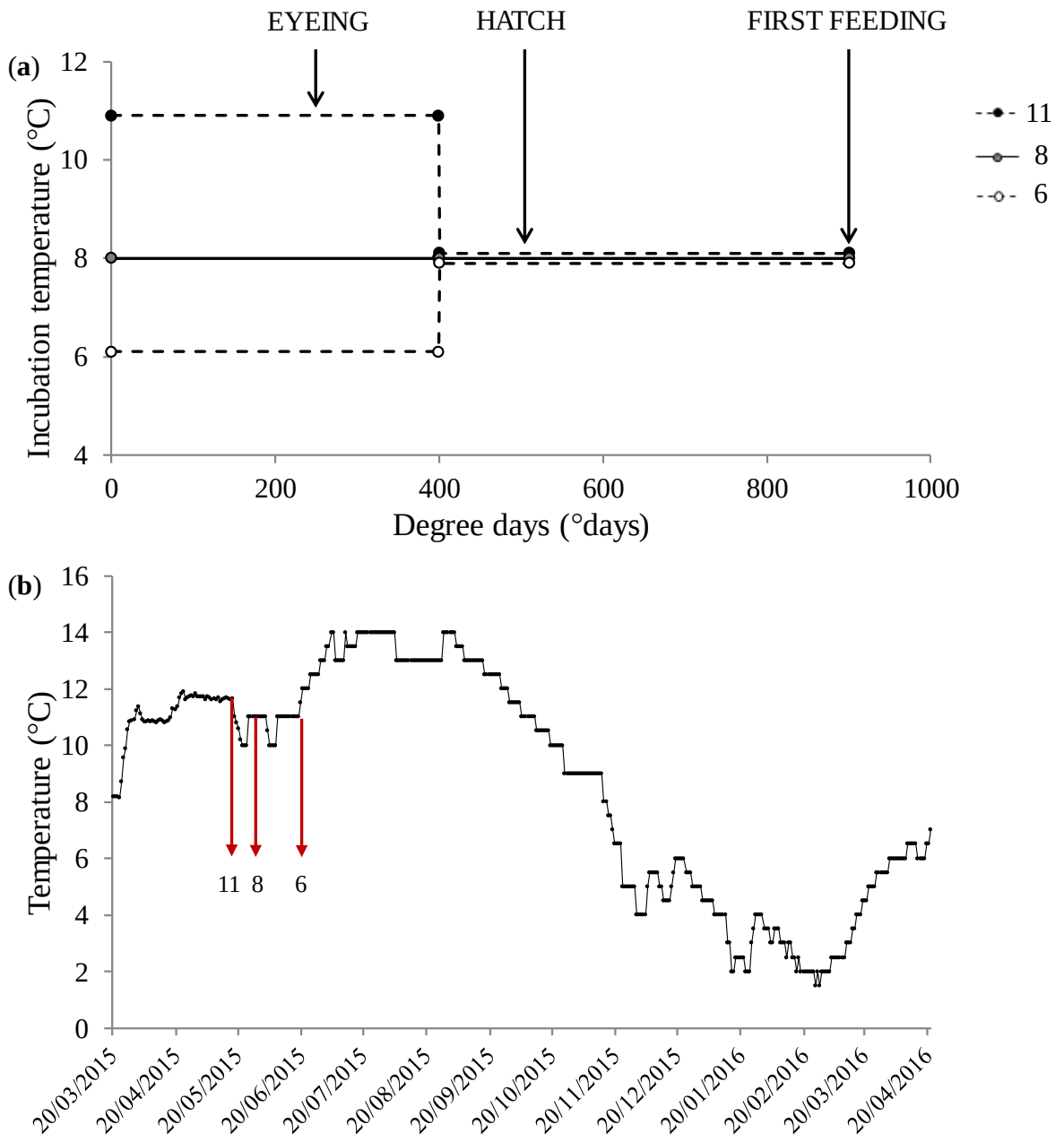

Figure 1. (**a**) Embryo incubation temperature including 6 °C (dashed line, opened circles), 8 °C (control; solid line, grey circles) and 11 °C (dashed line, black circles), and (**b**) average daily temperatures during subsequent development from first feeding until smolt. Introduction of fish (~1g) from each treatment on to ambient temperatures are shown with red arrows.

Table 1. Primer sequences of target genes and associated information used for real time qPCR mRNA levels investigation in diploid and triploid Atlantic salmon.

Gene name	ID	Forward sequence 5' – 3'	Reverse sequence 5' – 3'	T_A °C	Accession no.
Lipid utilisation					
Fatty acid synthase	*fas*	GTGAGCCCTGCCTCTTTTCT	AGAGCTTGCTTGCCTGTGAT	59	**CK876943**
Liver X receptor	*lxr*	GCCGCCGCTATCTGAAATCTG	ATCCGGCAACCAATCTGTAGG	58	**NM001159338**
Sterol regulatory element binding transcription factor 1	*srebp1*	CCAACATGGCTACCGTCACT	ACCGCTCGGAAAGTGTTCAA	56	**NM001195818.1**
Myogenesis					
Insulin-like growth factor 1	*igf1*	CAAAACGTGGACAGAGGCAC	TCCCTGTCCGTTAGCTTCTG	56	**M81904**
Insulin-like growth factor 1 receptor	*igf1r*	GTCGGCCAGCATGAGAGAGA	ACGGGTCTTTAGCCCGTAGT	58	**EU861008**
Insulin-like growth factor 2	*igf2*	TTGCGCCGGACTTTTAACTG	ATCTTGCATCGACCCTCACA	56	**AY049955**
Insulin-like growth factor binding protein related protein	*igfbprp*	GTGCGTTAAGAGCGACAAGA	CAATGACAGGTGTTGGG	56	**EF432866.1**
Myogenic factor 5	*myf5*	GCCTAAGGTGGAGATCCTGC	AGTCAACCATGCTGTCGGAG	57	**DQ452070**
Myogenic differentiation 1	*myod*	ACTCCAAATGCTGATGCCAGA	CTACCCTCCTGAACTGATAAC	56	**NM001123601.1**
Bone formation and mineralisation					
Alkaline phosphatase	*alp*	ATCCTGCTCATCTGCTCCTGC	AGTATTCGTGCTGCCGTCACT	56	**FJ195609**
Bone morphogenic protein 2	*bmp2*	TCTCATATCGCTGCTGG	TCCGAACATATTGAGCAGCC	56	**BTO59611**
Bone morphogenic protein 4	*bmp4*	GAACTCTACCAACCACGCCA	CGCACCCTTCCACTACCATT	56	**FJ195610**
Collagen type 1 alpha 1 chain	*col1a1*	TGGTGAGCGTGGTGAGTCTG	TAGCTCCGGTGTTTCCAGCG	56	**FJ195608**
Collagen type 2 alpha 1 chain	*col2a1*	TGGTCGTTCTGGAGAGACT	CCTCATGTACCTCAAGGGAT	56	**FJ195613**
Matrix metallopeptidase 13	*mmp13*	CCAACCCAGACAAGCCAGAT	GCTCTGAGAGTGGATACGCC	56	**DW539943**
Osteocalcin	*ocn*	GACTCCTCTACCTCCACTGC	AATGATCCCAGCTGTGTCCA	60	**FJ195611**
Osteopontin	*opn*	CTTACTGAGGTGGCCCCTGT	GCTGTCCGATGTTGGGTCTG	57	**AF223388.1**
Osteonectin	*sparc*	TCCTGCCACTTCTTTGCCCA	CAGCCAGTCCCTCATACGCA	56	**FJ195614**

T_A: annealing temperature.

Table 2. Mortality (%) during specific developmental windows in diploid and triploid Atlantic salmon incubated as embryos under different temperature treatments. Where there was replication (between fertilisation and 400 °days), data are expressed as means ± SD ($p < 0.05$, Two-Way ANOVA). Where there were single groups thereafter, data are expressed as tank value ± SD ($p < 0.05$; Analysis of CLT). Superscripts denote significant differences between treatments and ploidy within specific developmental stages.

		Developmental windows				
Temperature regime	**Ploidy**	**Fertilisation to 400 °days**	**400 °days to Hatch**	**Hatch to First feeding**	**First feeding to Smolt**	**Cumulative mortality**
6	Diploid	16.4 ± 19.2	3.0 ± 1.2^{b}	10.6 ± 2.2^{bc}	13.8 ± 2.6^{c}	37.5
	Triploid	22.3 ± 21.9	2.8 ± 1.1^{b}	8.5 ± 2.0^{c}	26.7 ± 3.3^{b}	49.3
8	Diploid	18.7 ± 20.5	2.1 ± 0.5^{b}	14.5 ± 1.2^{a}	27.4 ± 1.7^{b}	50.6
	Triploid	26.4 ± 23.1	7.1 ± 1.1^{a}	14.4 ± 1.5^{a}	46.6 ± 2.5^{a}	68.7
11	Diploid	27.0 ± 19.9	6.4 ± 1.7^{a}	14.1 ± 2.5^{ab}	32.0 ± 3.6^{b}	60.1
	Triploid	34.1 ± 24.0	7.8 ± 1.9^{a}	16.6 ± 2.7^{a}	46.5 ± 4.1^{a}	72.9

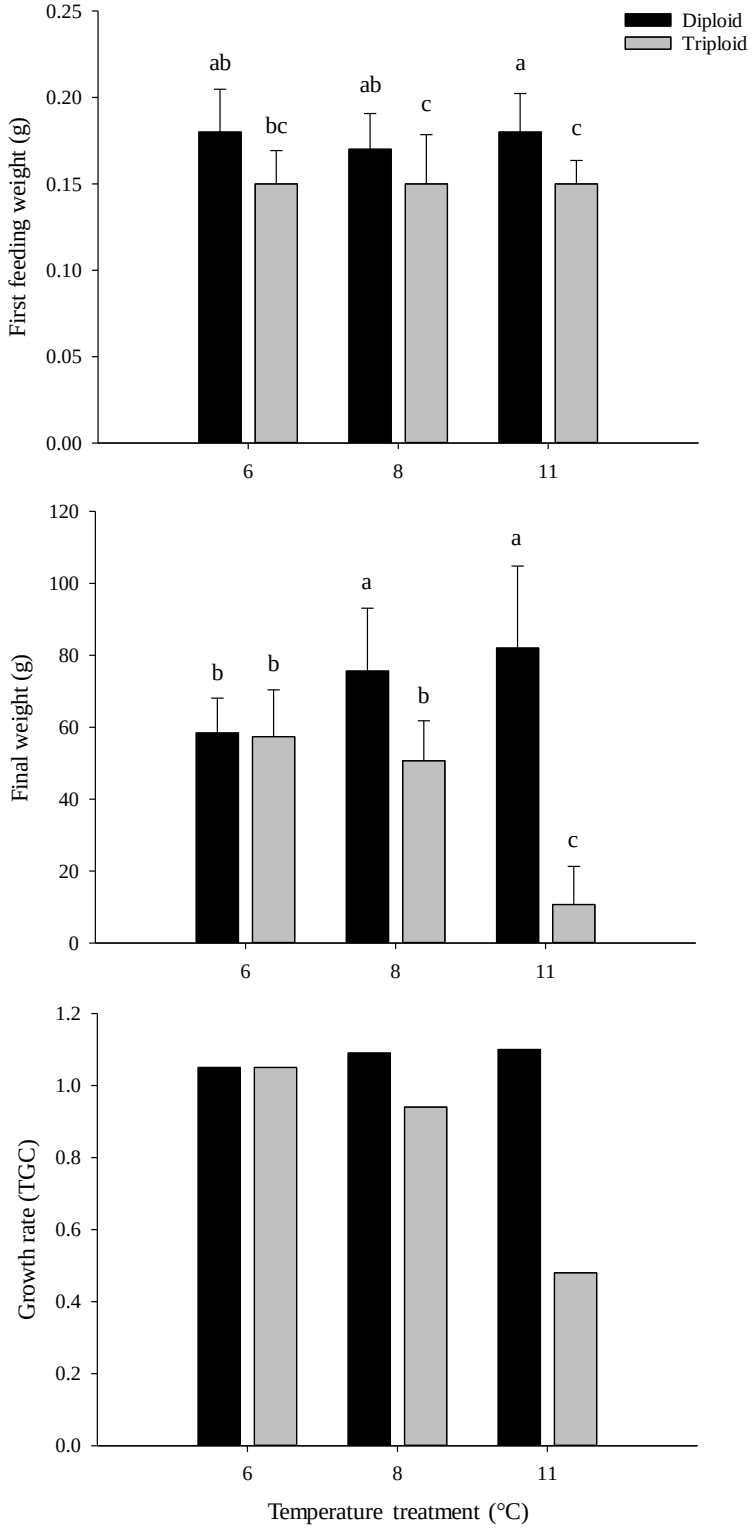

Figure 2. Growth summary of diploid (black) and triploid (grey) Atlantic salmon incubated under different temperature treatments including (**a**) body weight in first feeding alevins; BWi (g), (**b**) final body weight in smolts; BWf (g), and (**c**) thermal growth coefficient (TGC) between first feeding and smolt. Data are expressed as mean ± SD (n = 34 – 50 fish treatment^{-1} ploidy^{-1}). Superscripts denote significant differences between treatments ($p < 0.05$, Two-Way ANOVA).

Table 3. White muscle fibre morphometrics of diploid and triploid Atlantic salmon at smolt including final fibre number per mm^2 (FFN, mm^{-2}) and fibre cross-sectional surface area (μm^2). Data are expressed as means ± SD (6 fish $treatment^{-1}$ $ploidy^{-1}$) and significant differences between ploidies and temperature are denoted by different superscripts ($p < 0.05$, Two-Way ANOVA).

Temperature regime	**Ploidy**	**FFN (mm^{-2})**	**Fibre area (μm^2)**
6	Diploid	474.4 ± 32.0^{a}	2115.0 ± 137.9^{b}
	Triploid	386.3 ± 36.7^{b}	2608.1 ± 269.0^{a}
8	Diploid	466.7 ± 44.5^{a}	2157.4 ± 205.9^{b}
	Triploid	476.3 ± 37.4^{a}	2109.2 ± 167.1^{b}
11	Diploid	427.0 ± 11.1^{ab}	2343.2 ± 60.1^{ab}
	Triploid	398.9 ± 38.6^{ab}	2522.3 ± 241.5^{ab}
***p* value**	ploidy	**0.026**	**0.018**
	temp	**0.013**	**0.018**
	ploidy*temp	**0.038**	**0.035**

Table 4. (**a**) Prevalence (%) of external jaw and vertebral deformities and (**b**) radiologically assessed vertebral deformities in diploid and triploid Atlantic salmon smolts incubated as embryos under different temperature treatments (34 - 50 fish treatment^{-1} ploidy^{-1}). Data are expressed as means ± SD and significant differences are denoted by different superscripts ($p < 0.05$, Analysis of CLT).

	6		**8**		**11**	
	Diploid	**Triploid**	**Diploid**	**Triploid**	**Diploid**	**Triploid**
(a) Externally visible						
Deformed (%)	6.0 ± 6.6^{bcd}	2.0 ± 3.9^{cd}	0.0 ± 0.0^{d}	8.0 ± 7.6^{bc}	18.0 ± 10.8^{b}	50.0 ± 17.1^{a}
Jaw (%)	4.0 ± 5.5^{bc}	2.0 ± 3.9^{bc}	0.0 ± 0.0^{c}	8.0 ± 7.6^{b}	14.0 ± 9.7^{b}	47.1 ± 17.0^{a}
Vertebral (%)	2.0 ± 3.9	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	4.0 ± 5.5	2.9 ± 5.8
(b) Radiologically assessed						
Mean vertebrae number	58.4 ± 0.7	58.2 ± 0.5	58.5 ± 0.6	58.2 ± 0.4	58.6 ± 0.7	58.4 ± 0.7
Mean number of dV	2.8 ± 3.3	2.5 ± 1.8	2.0 ± 1.5	2.8 ± 2.4	6.2 ± 5.2	5.9 ± 3.5
Deformed (%)	24.0 ± 12.0^{b}	42.0 ± 13.8^{b}	24.0 ± 12.0^{b}	72.0 ± 12.6^{a}	40.0 ± 13.7^{b}	88.2 ± 11.0^{a}

dV: deformed vertebrae.

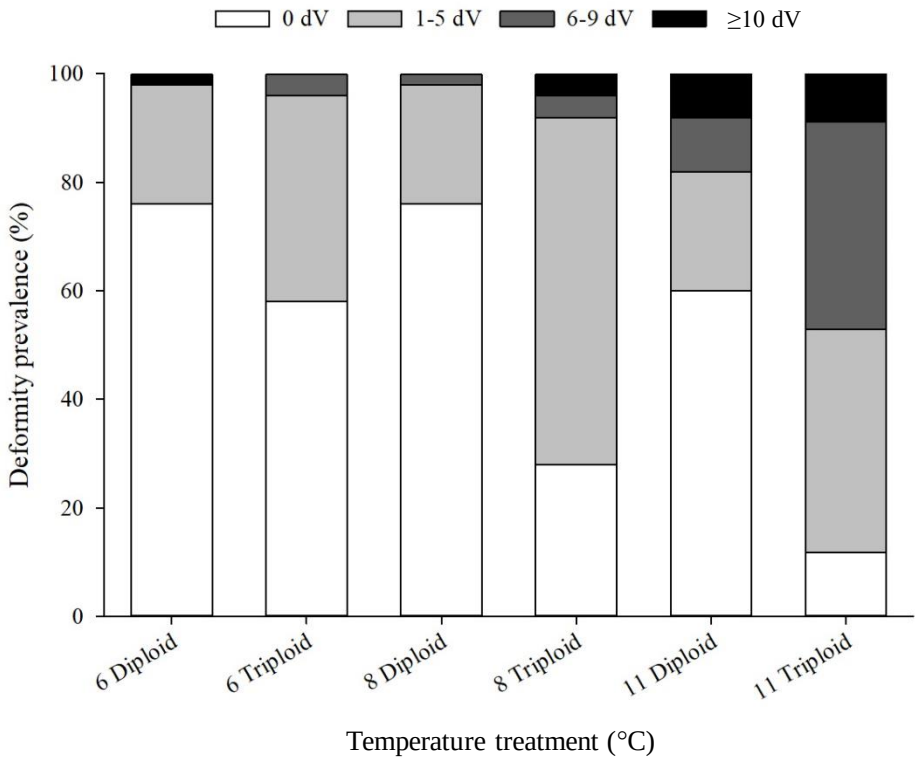

Figure 3. Prevalence (%) of X-ray radiologically assessed deformities presented according to severity index (Mild, 1-5 dV; Moderate, 6-9 dV; Severe, ≥10 dV) in diploid and triploid Atlantic salmon smolts incubated as embryos under different temperature treatments (34 - 50 fish treatment^{-1} ploidy^{-1}).

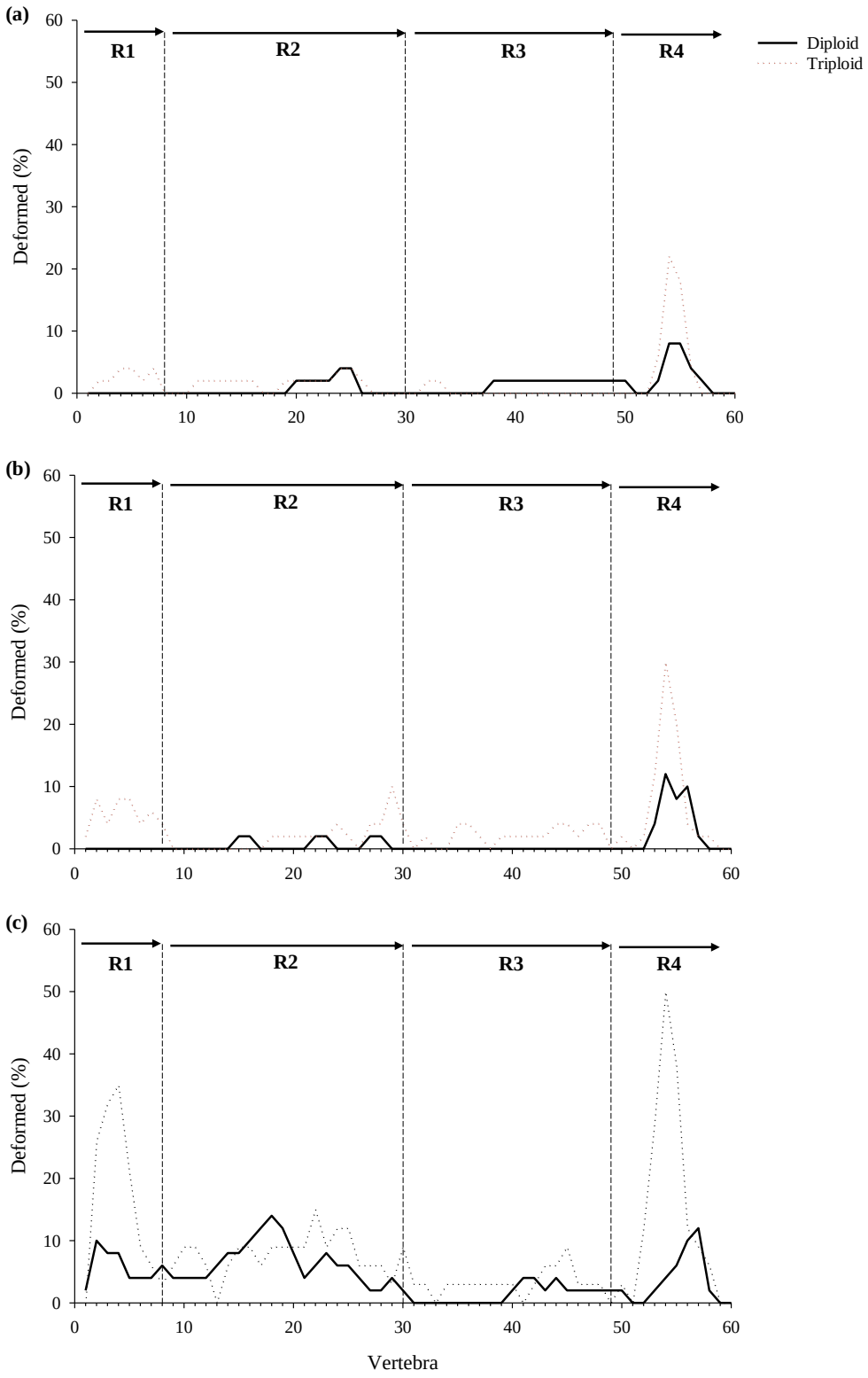

Figure 4. Prevalence (%) of deformed vertebrae observed in regions (R) according to Kacem *et al*. (1998) in diploid (solid line) and triploid (dotted line) Atlantic salmon smolts incubated as embryos under different temperature treatments; (**a**) 6 °C, (**b**) 8 °C, (**c**) 11 °C (34 - 50 fish treatment^{-1} ploidy^{-1}).

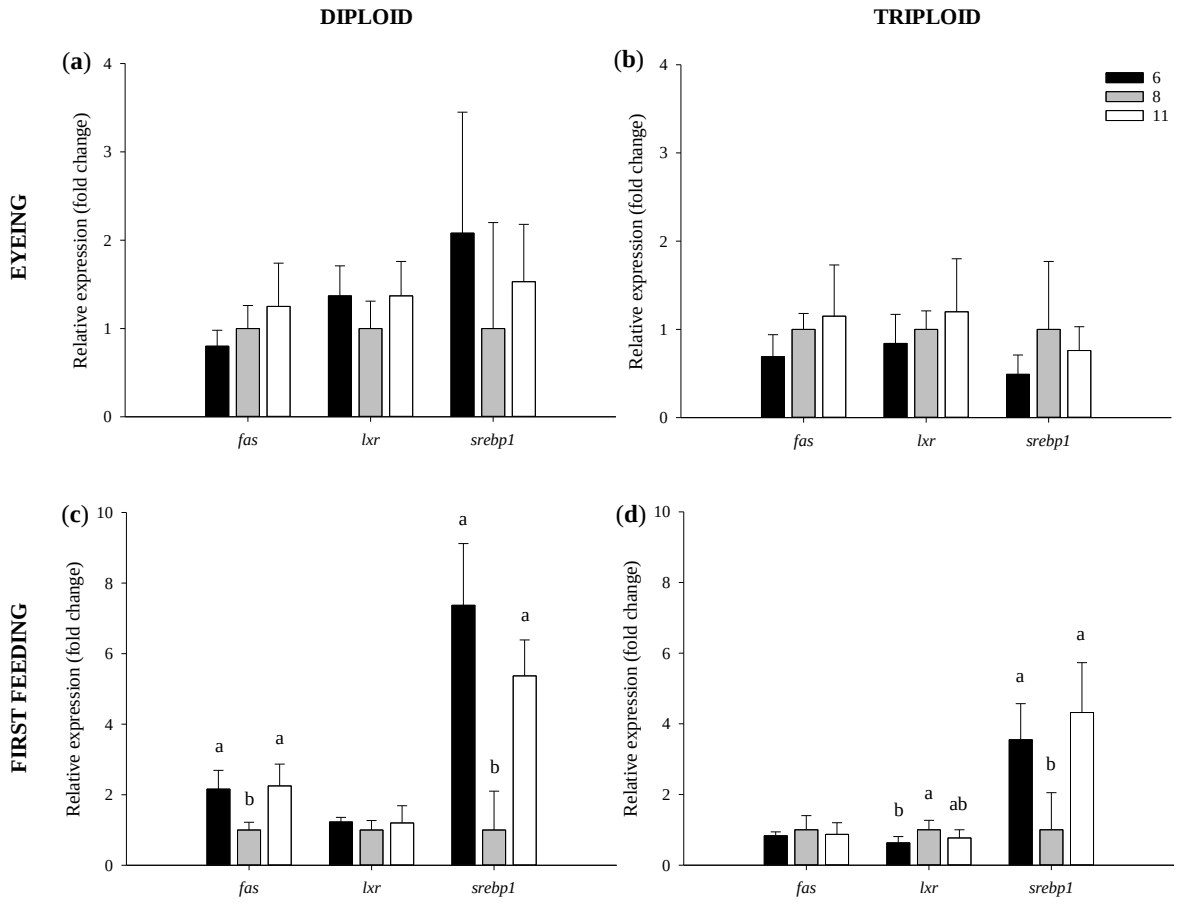

Figure 5. mRNA levels of genes involved in lipid metabolism (*fas*, *lxr* and *srebp1*) expressed as fold change relative to 8 °C (control) within each ploidy at respective developmental stage. Data show expression at eyeing in (**a**) diploids and (**b**) triploids and at first feeding in (**c**) diploids and (**d**) triploids. Data are expressed as means ± SD (6 fish treatment^{-1} ploidy^{-1} sampling point^{-1}) and different superscripts denote significant differences ($p < 0.05$).

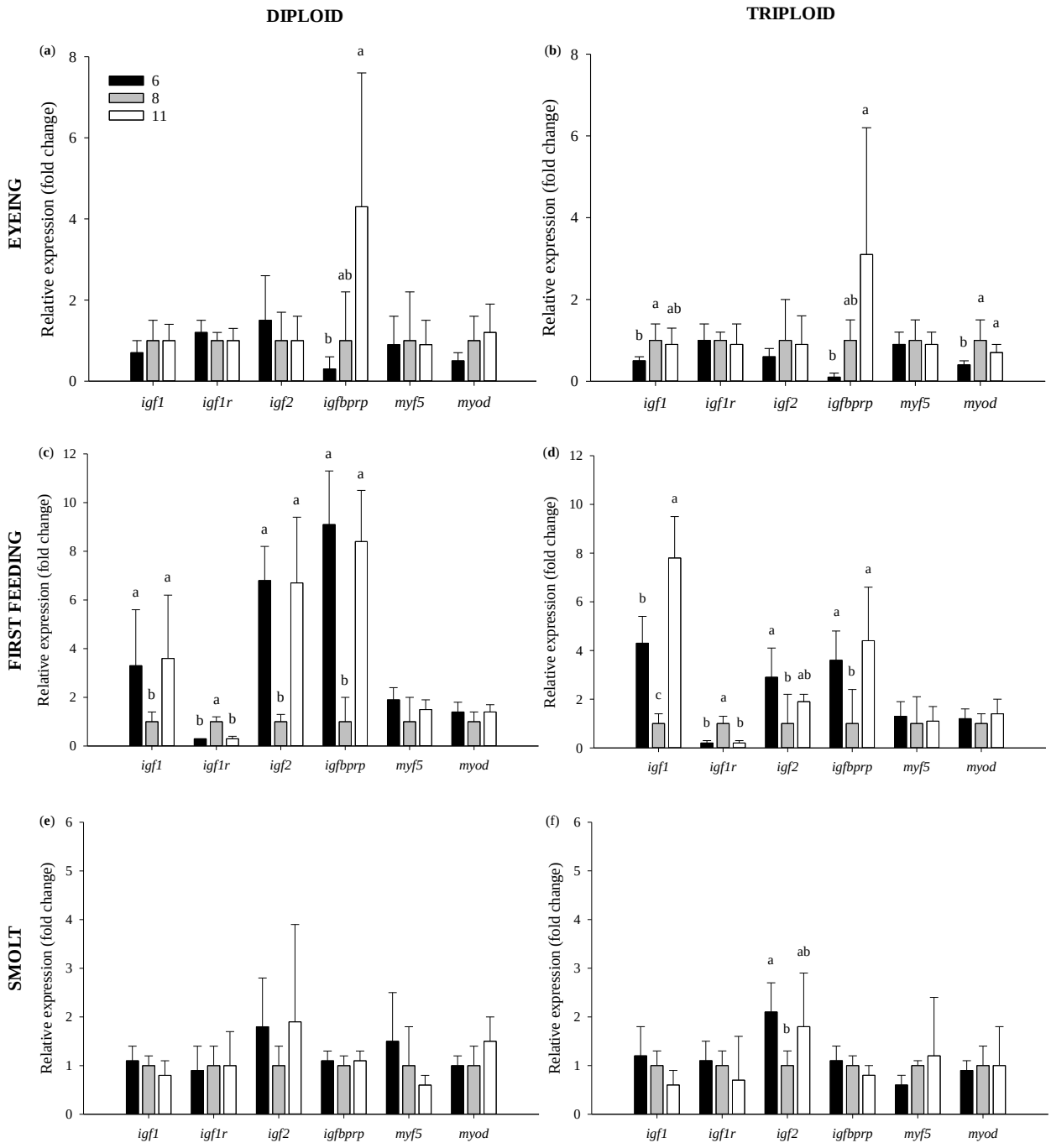

Figure 6. mRNA levels of genes involved in myogenesis (*igf1*, *igf1r*, *igf2*, *igfbprp*, *myf5* and *myod*) expressed as fold change relative to 8 °C (control) within each ploidy at respective developmental stage. Data show expression at eyeing stage in (**a**) diploids and (**b**) triploids, at first feeding in (**c**) diploids and (**d**) triploids, and at smolt in (**e**) diploids and (**f**) triploids. Data are expressed as means ± SD (6 fish treatment^{-1} ploidy^{-1} sampling point^{-1}) and different superscripts denote significant differences ($p < 0.05$).

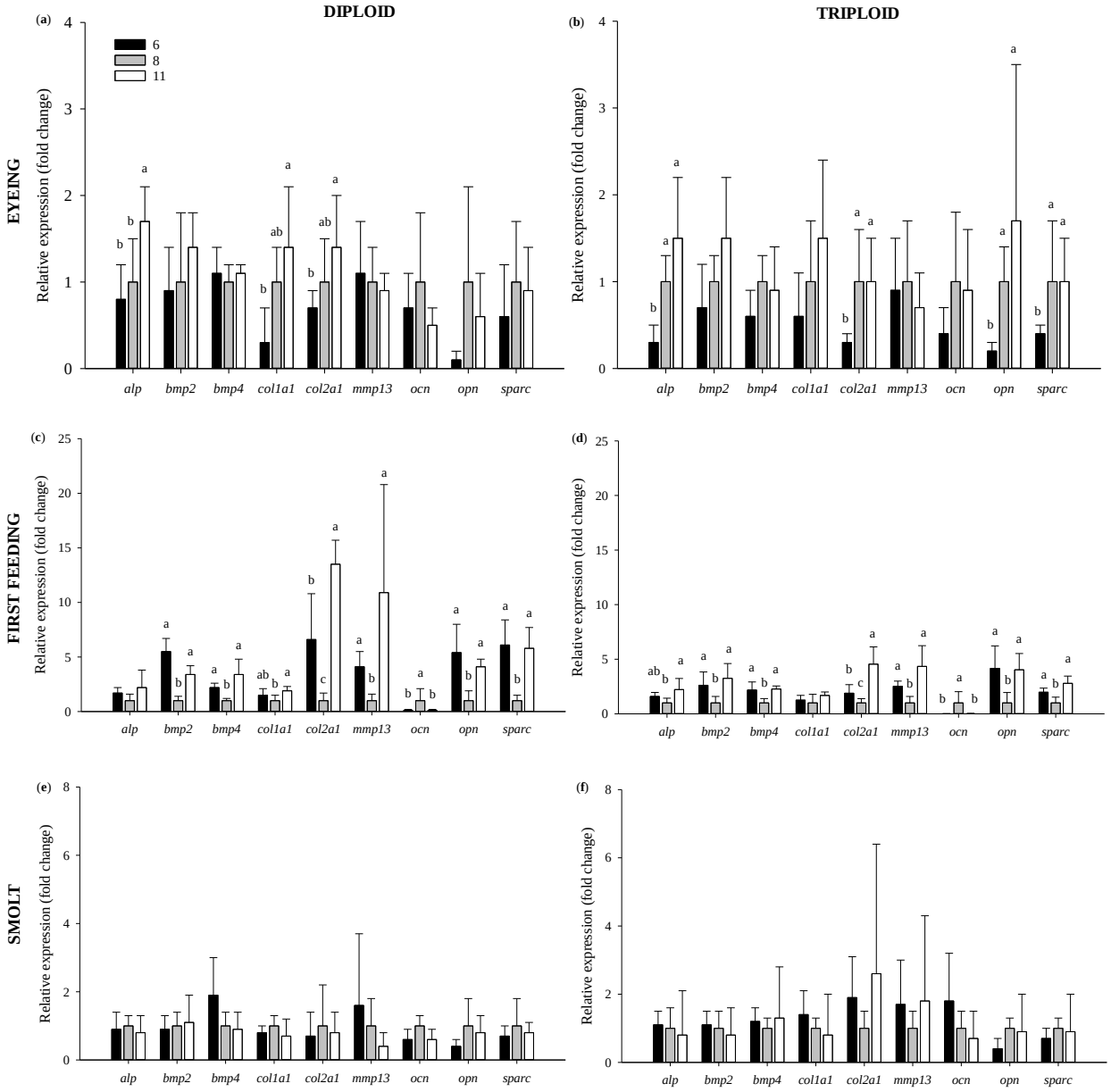

Figure 7. mRNA levels of genes involved in bone formation and mineralisation (*alp, bmp2, bmp4, col1a1, col2a1, mmp13, ocn, opn and sparc*) expressed as fold change relative to 8 °C (control) within each ploidy at respective developmental stage. Data show expression at eyeing stage in (**a**) diploids and (**b**) triploids, at first feeding in (**c**) diploids and (**d**) triploids, and at smolt in (**e**) diploids and (**f**) triploids. Data are expressed as means ± SD (6 fish treatment^{-1} ploidy^{-1} sampling point^{-1}) and different superscripts denote significant differences ($p < 0.05$).

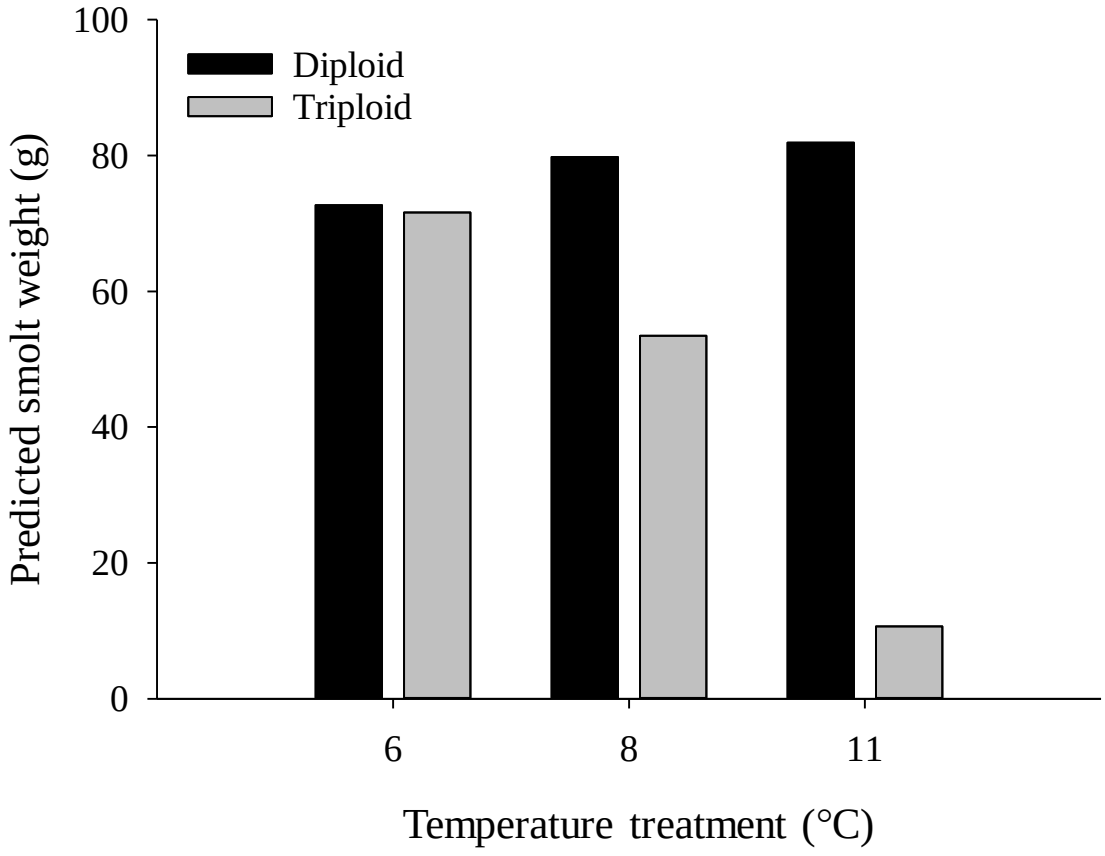

Figure 8. Predicted smolt weight of diploid and triploid Atlantic salmon incubated as embryos under different temperature treatments. Predicted weights were estimated using the respective TGC data and applied if all treatments experienced the same number of feeding days (395 days) under the same thermal regime.