

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

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12 **Running title:**

13 *Temperature shift during embryogenesis*

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35 **Abstract**

36 The study investigated effects of a temperature shift during embryogenesis on diploid and
37 triploid Atlantic salmon (*Salmo salar* L.) embryo development and juvenile skeletal
38 deformities. From fertilisation, sibling populations were incubated under one of three
39 temperatures (6, 8 or 11 °C) until 400 °days when all fish were then reared under a common
40 temperature until smolt. Survival was negatively impacted by increasing temperatures
41 irrespective of ploidy. There was no effect of incubation temperatures on growth in diploids,
42 but triploids incubated at 6 °C had improved growth rates (thermal growth coefficient; TGC 6
43 °C: 1.05, 8 °C: 0.94, 11 °C: 0.48). Fish from 11 °C in both ploidies showed increased jaw and
44 vertebral deformity prevalence. In response to the temperature change at 400 °days post-
45 fertilisation, upregulation of *bmp2*, *bmp4*, *col2a1*, *mmp13*, *opn*, *sparc*, and downregulation of
46 *ocn* further suggest that bone and cartilage formation is compromised after experiencing a
47 thermal shift. The data show that temperature profile during embryogenesis strongly influences
48 future growth and deformity prevalence. Triploids appear to require a lower incubation
49 temperature than the current industry standard of 8 °C to promote better overall performance,
50 however, a thermal shift during embryogenesis was shown to impact expression of important
51 developmental genes.

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

69 **1. Introduction**

70 Temperature regimes are routinely adjusted in aquaculture to manipulate production windows
71 and/or promote growth. The ontogeny of embryonic stages, previously described for Atlantic
72 salmon (*Salmo salar* L.) (Gorodilov, 1996), is directly influenced by environmental conditions,
73 in particular, water temperature. Higher incubation temperatures lead to accelerated
74 development (Hayes, Pelluet and Gorham, 1953; Gorodilov, 1996) in relation to an increased
75 metabolic rate (Clarke and Johnston, 1999). Diploid salmon embryos can tolerate temperatures
76 from 0-16 °C (Hayes *et al.*, 1953), however, early reports suggested an increase in mortalities
77 in diploid embryos incubated below 4 °C and above 8 °C (Peterson, Spinney and Sreedharan,
78 1977; Gunnes, 1979; Fraser, Fleming, Poppe and Fjellidal, 2014). Thus, a maximum embryo
79 incubation temperature of 8 °C has been adopted in commercial salmon hatcheries.

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

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

103 2010a) deformities. There is little known about the underlying mechanisms of skeletal
104 abnormalities as a result of elevated temperatures, however it has been suggested that increased
105 muscle mass is poorly supported by under-mineralised bone found in faster growing salmon
106 (Fjelldal *et al.*, 2006).

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

131 Thermosensitivity of triploid embryos during embryogenesis may therefore be of
132 particular importance. Fraser *et al.* (2015) showed that incubating embryos at 6 °C, rather than
133 8 or 10 °C, from fertilisation to first feeding can reduce the prevalence of skeletal deformities
134 in triploids, supporting a likely different optimal thermal range compared to diploid siblings.
135 However, no studies to date have investigated how temperature changes during embryogenesis
136 may affect survival and growth performance of triploid salmon. Further, bone regulatory

137 mechanism discussions, specific to triploid salmon, are limited to later life stages (Fjelldal *et*
138 *al.*, 2015; Amoroso *et al.*, 2016b; Smedley *et al.*, 2018; Vera *et al.*, 2019) and results during
139 embryogenesis have not yet been reported. Ytteborg *et al.* (2010b) described upregulation of
140 genes associated with bone formation (e.g. alkaline phosphatase, *alp*; collagen type 1 alpha 1
141 chain, *coll1a1*; osteocalcin; *ocn*; bone morphogenetic protein 2, *bmp2*; and bone morphogenetic
142 protein 4, *bmp4*) in diploids in response to elevated temperatures during development.
143 Furthermore, Wargelius *et al.* (2005) found that gene expression associated with vertebral
144 development was altered in salmon embryos exposed to an acute heat shock at 12 °C. With a
145 different optimal range, it is likely that triploids require lower incubation temperatures to
146 promote optimal skeletal development. This said, the adoption of lower and continuous
147 hatchery temperatures to ensure optimal performance later in production inevitably slows down
148 fish development and extends the hatchery phase.

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

155

156 **2. Materials and methods**

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

164

165 2.1. Fish stock and culture conditions

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

171 water bath at 8 °C prior to triploid induction. Triploidy was induced in one group (655 bar of
172 hydrostatic pressure for 6.25 mins. at 8 °C, 37 mins. post-fertilisation) according to Smedley
173 *et al.* (2016), while the others experienced the handling but did not receive a hydrostatic shock
174 and were maintained as diploids. After water hardening, eggs were further divided into three
175 incubation temperatures (5.8 ± 0.7 , 8.3 ± 0.1 or 10.8 ± 0.1 °C, referred to as 6, 8 and 11) and
176 incubated in triplicate in aluminium egg trays (total = 54; 3 trays⁻¹ family⁻¹ temperature⁻¹ ploidy⁻¹)
177 within temperature-specific trough systems (15 mL sec⁻¹ flow) in darkness. Temperature was
178 monitored in 30 min intervals with temperature data loggers (HOBO 64K Pendant®, Onset
179 Computer Corporation) submerged in the middle of the incubation trays, central in each trough
180 system. At 400 °days post-fertilisation, ~800 eggs from the two best performing families (400
181 eggs family⁻¹) were pooled from each treatment (2 ploidies x 3 temperatures) and transferred
182 to single 6 x 0.3 m³ recirculation system (RAS) tanks and incubated at a constant temperature
183 of 8 °C until first feeding (Fig. 1). One of the three families was removed from the study due
184 to a high mortality rate in both ploidies suspected to be due to poor egg quality. Embryos in all
185 treatments were kept in constant darkness until first feeding at which point fry were given
186 constant light (LL). Three treatments per ploidy (a history of 6, 8 and 11 °C) were created, with
187 eggs incubated at 8 °C referred to as the “control treatment” as routine in commercial practice.
188 Whilst in RAS, pH measurements were regularly checked and maintained (pH 6.8 – 7.2), with
189 the addition of sodium bicarbonate (NaHCO₃) as necessary. At 1 g, all fish were transferred to
190 the Niall Bromage Freshwater Research Unit, Stirling, UK and reared in 6 x 1.6 m³ (2 ploidies
191 x 3 temperatures) individual circular tanks under ambient temperature and simulated natural
192 photoperiod (SNP) until smolt (24th April 2016). Due to the incubation temperature differences
193 and subsequent impact on developmental speed, the number of feeding days (d) from first
194 feeding until the end of the trial differed between treatments (6, 364d; 8, 386d; 11, 395d).
195 Diploids were fed a standard commercial diet (BioMar INICIO Plus, 4.9 g kg⁻¹ available P)
196 and triploids were fed a triploid-specific diet (BioMar INICIO TRI-X, 7.7 g kg⁻¹ available P),
197 with the only difference in the formulation being P supplementation in TriX, and thus in
198 accordance with previously published triploid specific P requirements (Fjelldal *et al.*, 2015;
199 Smedley *et al.*, 2018; Sambraus *et al.*, 2020) and the manufacturer’s guidelines (BioMar Ltd,
200 UK).

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202 2.2. Verification of ploidy

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

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

212

213 2.3. Sampling procedures

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

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

235

236 2.4. Growth assessment

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

239 for body weight (BW_i and BW_f) and fork length (FL) measurements. Growth rate was
240 calculated using the thermal growth coefficient (TGC, % BW °C d⁻¹).

241

242 2.5. Myogenic morphology assessment

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

250

251 2.6. Radiological deformity analysis

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

261

262 2.7. Mineral composition

263 Mineral composition was determined from whole smolt carcass (3 pools; 3 individuals pool⁻¹
264 treatment⁻¹ ploidy⁻¹) using the nitric acid (HNO₃) digestion technique. Pools of fish were
265 homogenised, and oven dried at 75 °C for 24 hrs. and subsequently powdered using a mortar
266 and pestle. Samples were digested in Kheldal digestion tubes with 69 % nitric acid using a
267 MARS microwave digestion system (CEM MARSXpress, CEM ltd., Buckingham, UK) using
268 the following program: 10 mins. heating phase to 190 °C, maintain 190 °C for 20 mins., cooling
269 phase to 21 °C for 60 mins. Samples were then diluted with distilled water to 2 % HNO₃ and
270 analysed for mineral content via Inductively Coupled Plasma Mass Spectrometry (ICP-MS;
271 Thermo X series II; Collision cell technology). Due to a technical error during processing, no
272 data for either ploidy of 11 °C are available.

273

274 2.8. Gene expression analysis

275 2.8.1. RNA extraction and cDNA synthesis

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

286

287 2.8.2. Sequence information and primer design

288 Sequence-specific primers for genes were based on registered sequence data in Atlantic salmon
289 from the National Centre for Biotechnology Information (NCBI) website
290 (www.ncbi.nlm.nih.gov). Sequence information was then subjected to BLAST analysis against
291 an Atlantic salmon genome and transcriptome (NCBI). Primer pairs were manufactured by
292 MWG Eurofins Genomics (Ebersberg, Germany) and sequences with associated information
293 are detailed in Table 1. Each primer product was purified by GeneJET PCR Purification Kit
294 (Thermo Scientific, UK) according to manufacturer's instructions. Products were then cloned
295 using the pGEM®-T Vector System (Promega) and plasmids were harvested using a
296 GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich, UK) according to manufacturer's
297 instructions. Each resulting plasmid was subsequently sequenced via LIGHTrun™
298 sequencing (GATC, Cologne Germany) to confirm identity. Plasmids were then linearised by
299 enzymatic digest and standards for qPCR assays were generated using a serial dilution from
300 10⁸ copies to 10 copies of each gene investigated.

301

302 2.8.3. Quantitative PCR (qPCR)

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

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

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

321

322 2.9. Statistics

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

337

338 3. Results

339 3.1. Mortality

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

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

348 No ploidy difference was also shown in mortality between 400 °days to hatch in 6 or
349 11 °C treatments, however triploids had a significantly higher mortality than diploids when
350 incubated at 8 °C (Table 2). In diploids, mortality was comparable in 6 and 8 °C treatments,
351 however a significant increase was shown in embryos incubated at 11 °C. In triploids, a
352 significant increase in mortality was found in both 8 and 11 °C compared to 6 °C.

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

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

361

362 3.2. Growth performances

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

368 There was a significant effect of both ploidy ($p < 0.001$, $2N > 3N$) and temperature (p
369 < 0.001 , $8 > 6 > 11$ °C) on final smolt weight (BW_f) and a significant interaction between
370 ploidy and temperature ($p < 0.001$). BW_f was comparable between ploidies in fish at 6 °C,
371 however, diploids had significantly higher BW_f than triploids in 8 and 11 °C treatments (Fig.
372 2b). Within diploids, there was an increase in fish BW_f between 6 and 8 °C treatments, and

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

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

378

379 3.3. Muscle analysis in smolts

380 Diploids had an overall higher final fibre number (FFN) compared to triploids, however within
381 temperature treatments, differences were significant only in fish from the 6 °C treatment with
382 a higher (+23 %) FFN in diploids than triploids (Table 3). An overall temperature effect was
383 observed with FFN in fish from 8 °C being significantly greater than 11 °C, but both
384 comparable to FFN in fish at 6 °C. In diploids, there was no significant difference in FFN
385 between any temperature treatments, while in triploids, fish at 8 °C had significantly more
386 fibres per mm² compared to 6 °C, but both were comparable to 11 °C.

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

393

394 3.4. Deformity analysis

395 Triploids had a significantly increased visible external deformity prevalence in 8 °C (8
396 vs. 0 %) and 11 °C (50 vs. 18 %), however no difference was found in 6 °C (Table 4a). Within
397 diploids, visible external deformity prevalence was significantly greater in 11 °C compared to
398 8 °C but not different from 6 °C. Triploids also had increased deformity prevalence in 11 °C
399 compared to 8 °C, but also significantly higher when compared to 6 °C. These deformities were
400 mainly comprised of jaw malformation as externally visible vertebral deformities were
401 negligible. Triploids had higher jaw deformity prevalence in 8 °C and 11 °C compared to
402 diploids but no difference between ploidy was evident at 6 °C. In diploids, jaw deformity
403 prevalence was significantly higher in 11 °C compared to 8 °C, with those in 6 °C showing
404 intermediate prevalence. However, in triploids, jaw deformity prevalence was significantly
405 higher in 11 °C compared to both 6 and 8 °C.

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

410 Triploids had a significantly higher deformity prevalence than diploids in 8 °C (72 vs.
411 24 %), and 11 °C (88.2 vs. 40 %) treatments, but prevalence was comparable between ploidies
412 at 6 °C (Table 4b). Within diploids, there was no significant difference in the prevalence of
413 radiologically deformed individuals with increasing incubation temperatures. Within triploids
414 a comparable deformity prevalence was exhibited between 8 and 11 °C, however those from 6
415 °C had a significantly lower prevalence.

416 Deformed individuals were categorised by severity bands (Fig. 3; None: 0 dV; Mild: 1-
417 5 dV, Moderate: 6-9 dV or Severe: ≥ 10 dV). Within diploids, in comparison to the control
418 treatment, incubated at a constant 8 °C, the likelihood of fish from 11 °C treatments having
419 increased deformity severity was significant (OLR CoEf = -0.91, $p = 0.036$), however, no
420 differences were found when compared to fish from treatments 6 °C (OLR CoEf = -0.01, $p =$
421 0.991). With reference to triploids incubated at a constant 8 °C, there was a significantly
422 reduced likelihood of deformity occurrence in fish from 6 °C treatments (OLR CoEf = 1.15, p
423 = 0.004) and a significantly increased likelihood when originating from 11 °C treatments (OLR
424 CoEf = -1.75, $p < 0.001$).

425 The most common location for these deformed vertebrae was in the tail fin (R4)
426 irrespective of ploidy or temperature (Fig. 4a, b, c). In triploids, there is a common peak at v54
427 in all temperatures, however, in diploids there was a shift in the peak with increasing
428 temperatures (6, v54-55 < 8, v54-56 < 11 °C, v56-57). The prevalence in this region appeared
429 to be consistent in diploids in each temperature treatment (~10 %), however an increase was
430 observed in triploids in response to increasing temperature treatments (6, ~20 % < 8, ~30 % <
431 11 °C, ~50 %). Both diploids and triploids showed a similarly increased deformity prevalence
432 in the caudal trunk (R2) when incubated at 11 °C, however triploids had a much larger increase
433 in the cranial trunk (R1) compared to diploids (35 vs. 10 %).

434

435 3.5. Mineral composition

436 Overall, triploids had higher levels of calcium (Ca; 4581.8 – 4765.2 vs. 3852.1 – 3897.4 μg
437 mg^{-1} , $p = 0.002$), phosphorous (P; 4521.1 – 4548.0 vs. 4067.7 – 4078.0 μg mg^{-1} , $p = 0.008$) and
438 Ca:P (1.00 – 1.05 vs. 0.94 – 0.95, $p = 0.002$) compared to diploids. No temperature difference
439 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 *srebpl* 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 *igfbprp* (~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

474 Diploid ova at the eyeing stage showed a significant upregulation of *alp* in 11 °C (~1.7 fold)
475 compared to 8 and 6 °C (Fig. 7a). Both *coll1a1* and *col2a1* expression was significantly higher
476 in diploids of 11 °C compared to 6 °C, although were comparable to 8 °C. In triploids, there
477 was a significant downregulation of *alp*, *col2a1*, *opn* and *sparc* in 6 °C compared to both 8 and
478 11 °C (Fig. 7b). No other differences were observed.

479 At first feeding in diploids, no difference in *alp* expression was observed (Fig. 7c),
480 however a significant upregulation in first feeding triploids from 11 °C (~2 fold) compared to
481 8 °C was found (Fig. 7d). A significant upregulation of *bmp2*, *bmp4*, *col2a1*, *mmp13*, *opn* and
482 *sparc* occurred in first feeding fry from the 6 and 11 °C treatments, irrespective of ploidy (Fig.
483 6c, d). An upregulation was also found in *coll1a1* expression in diploids from 11 °C compared
484 to 8 °C (~2 fold), however no differences were found between any of the temperature
485 treatments in triploids. *ocn* was the only gene associated with bone formation to show a
486 significant downregulation in 6 and 11 °C compared to 8 °C, and this occurred in both ploidies.

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

489

490 **4. Discussion**

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

499 As reported previously in other trials, mortality rate during embryogenesis was overall
500 higher in triploids compared to diploid siblings (Sutterlin, Holder and Benfey, 1987; O'Flynn,
501 McGeachy, Friars, Benfey and Bailey, 1997; Benfey, 2001; Taylor *et al.*, 2011; Fraser *et al.*,
502 2015; Amoroso *et al.*, 2016a). However, triploids incubated at a lower temperature (~6 °C)
503 during embryogenesis showed an increased survival as previously reported (Fraser *et al.*,
504 2015). Indeed, comparable survival between ploidies were observed within each period prior
505 to first feeding, when incubated at 6 °C, supporting the theory that triploids have a lower
506 optimal thermal range than diploids. Results from Fraser *et al.* (2014) suggested that increased
507 mortality in embryos incubated at elevated temperatures may be linked with aplasia of the

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

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

542 In the present study, we observed no differential expression in genes associated with
543 lipid utilisation between incubation temperatures at the eyeing stage (~250 °days) in both
544 ploidies. However, the ~2 °C temperature change at 400 °days clearly influenced the ability to
545 utilise the yolk later during embryonic development as individuals from both the 6 and 11 °C
546 treatments showed upregulation of *fas* in diploids and *srebpl* in both ploidies at first feeding
547 (~900 °days). The expression of these genes, involved with fatty acid biosynthesis and
548 subsequent storage, appears to be significantly increased as a result of the temperature shift,
549 suggesting that acute changes in temperature has a direct influence on the energy status of a
550 developing fish. Although the gene expression pattern appeared to be the same in fish incubated
551 at 6 and 11 °C, the resulting growth was very different. Furthermore, a downregulation of *lxr*
552 was found in triploid first feeding fry previously incubated at 6 °C, whilst no differences were
553 observed in those from 11 °C or in diploids from either experimental temperature. This suggests
554 that the two ploidies show different metabolism in response to different temperatures from an
555 early life stage and is supported by the difference in first feeding weights between ploidy (2N
556 > 3N). Published studies in rainbow trout (*Oncorhynchus mykiss*) and red crucian carp
557 (*Carassius auratus*) have shown higher protein absorption in triploids compared to their diploid
558 siblings and several studies have suggested that enhanced growth may be associated to an
559 upregulation of glutamate dehydrogenase and oligopeptide transporter (Olivia-Teles and
560 Kaushik, 1990; Liu et al., 2012; 2014). Yolk proteins are metabolised and used to build muscle
561 through a combination of hyperplasia (fibre recruitment) and hypertrophy (growth of present
562 fibres).

563 This initial difference in first feeding weight would certainly impact the subsequent
564 growth trajectory of the cohorts. Due to fertilisation of all treatments occurring on the same
565 day (to control for parental effect) and each treatment experiencing different thermal regimes,
566 the first feeding dates ultimately differed. As a result, treatments experienced a different
567 number of feeding days up to the point of smoltification. This differential grow out duration in
568 part resulted in different final body weights (BWf) at smolt between temperature treatments
569 that could not be compared directly. However, when comparing ploidies, the growth rate, and
570 therefore BWf, was reduced in triploids from the 8 and 11 °C treatments relative to diploids.
571 This agrees with the theory that triploids have a lower thermal tolerance than diploids and
572 concurs with a recent study where metabolic rate and aerobic scope in triploids suffered at 10.5
573 °C but was comparable to diploids at 3 °C (Riseth, Fraser, Sambraus, Stien and Hvas, 2020).
574 Nevertheless, triploids have also been reported to have better growth rates than diploids under
575 different experimental incubation temperatures (Taylor *et al.*, 2011; Fraser *et al.*, 2015). In the

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

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

589 Previous studies in diploid Atlantic salmon have reported a preference for muscle
590 growth through an increase in hypertrophy over hyperplasia when embryos were incubated at
591 10 or 11 °C compared to 1.6 or 6 °C, respectively (Stickland *et al.*, 1988; Usher *et al.*, 1994).
592 This may be an energy preservation strategy as nuclear division (hyperplasia) requires more
593 energy expenditure than hypertrophy. This theory coincides with reports of fewer, yet larger
594 fibres and fewer nuclei present in muscle samples of fish incubated at high temperatures (Usher
595 *et al.*, 1994). In the present study, a temperature effect was observed in triploids as smolts from
596 the 6 °C treatment had a significantly lower FFN and higher fibre area compared to 8 °C. It is
597 important to note that fish from each temperature treatment were at different developmental
598 stages at this sampling point due to their earlier differences between thermal profiles, yet this
599 would be the transition to seawater in a commercial setting (i.e. end of parr-smolt
600 transformation). This means that although a significant difference was observed at smolt in
601 triploids incubated at 6 °C, they had less developmental days than the 8 °C (22 days less) and
602 11 °C (31 days less) treatments. However, comparing ploidies within a temperature treatment
603 shows that triploids had a lower FFN and higher fibre area relative to their diploid counterparts
604 in the 6 °C treatment, but comparable in 8 and 11 °C treatments. Johnston *et al.* (1999) reported
605 a lower density of satellite cells in triploids which are important precursors to muscle fibre
606 recruitment which contrasts with the current result. This may be a result of the different
607 freshwater temperatures experienced in each of the studies during embryonic development
608 (fluctuations between 10 – 3 °C vs. constant 6 °C then 8 °C) or differing temperature
609 maximums reached during the parr stage (21 vs. 14 °C). Fish with a higher proportion of newly

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

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

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

644 in fish that experienced a temperature switch at 400 °days compared to the constant 8 °C
645 control. Furthermore, there was an increase in prevalence of jaw malformation in fish exposed
646 to 11 °C for both ploidies. In accordance with previous studies (O'Flynn *et al.*, 1997; Benfey,
647 2001; Fraser *et al.*, 2015), there was a higher occurrence of LJD in triploids compared to
648 diploids, however, this was only evident in 8 and 11 °C treatments whereas no difference was
649 observed at 6 °C. This further supports the suggested lower optimal thermal range of triploids
650 relative to their diploid counterparts, and that triploids can perform similarly to diploids when
651 incubated at 6 °C or below. It should be noted that results reported from Amoroso *et al.* (2016b)
652 were obtained from dissected jaw tissue from smolts with known LJD whereas in the current
653 study, vertebral tissue was analysed, and deformity status was unknown at the point of
654 sampling which may in part explain the contrasting results between both studies. Nonetheless,
655 *col2a1*, associated with cartilage formation, is likely associated with LJD and a temperature
656 change during embryogenesis may alter normal cartilage development. Further investigations
657 into these biomarkers for specific malformation aetiology using individuals that possess
658 vertebral deformities should be explored, in addition to histological verification of differences
659 in the ECM and collagen development.

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

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

694

695 **5. Conclusions**

696 The present study confirmed that an incubation temperature of 6 °C until 400 °days is
697 favourable to maintain good development of diploid and triploid Atlantic salmon embryo. This
698 lower temperature during embryogenesis reduced mortality rates and severity of deformity
699 historically associated with triploids and matched the growth performance of their diploid
700 siblings. This investigation agrees with previous results, suggesting that early rearing of
701 triploids at 6 °C until first feeding is preferential for development and welfare (Fraser *et al.*,
702 2015). While such a regime adds ~2 weeks to the period of embryogenesis under a production
703 cycle, it has a major advantage on triploid Atlantic salmon health compared to the conventional
704 8 °C rearing regime. Despite this, the temperature switch at 400 °days did result in altered
705 expression of important developmental genes, highlighting the sensitivity to temperature
706 changes likely experienced during commercial embryo rearing which may have influenced
707 mortality, growth or deformity. This should be kept in mind in aquaculture production,
708 particularly with triploids, as both broodstock and hatchery sites regularly manipulate rearing
709 temperatures to meet customer demands. Evidently, a balance must be met, and optimisation
710 of triploid-specific hatchery temperature conditions further contributes to the growing

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

713

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719

720 **Declaration of Interest**

721 None.

722

723 **Data Availability**

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

726

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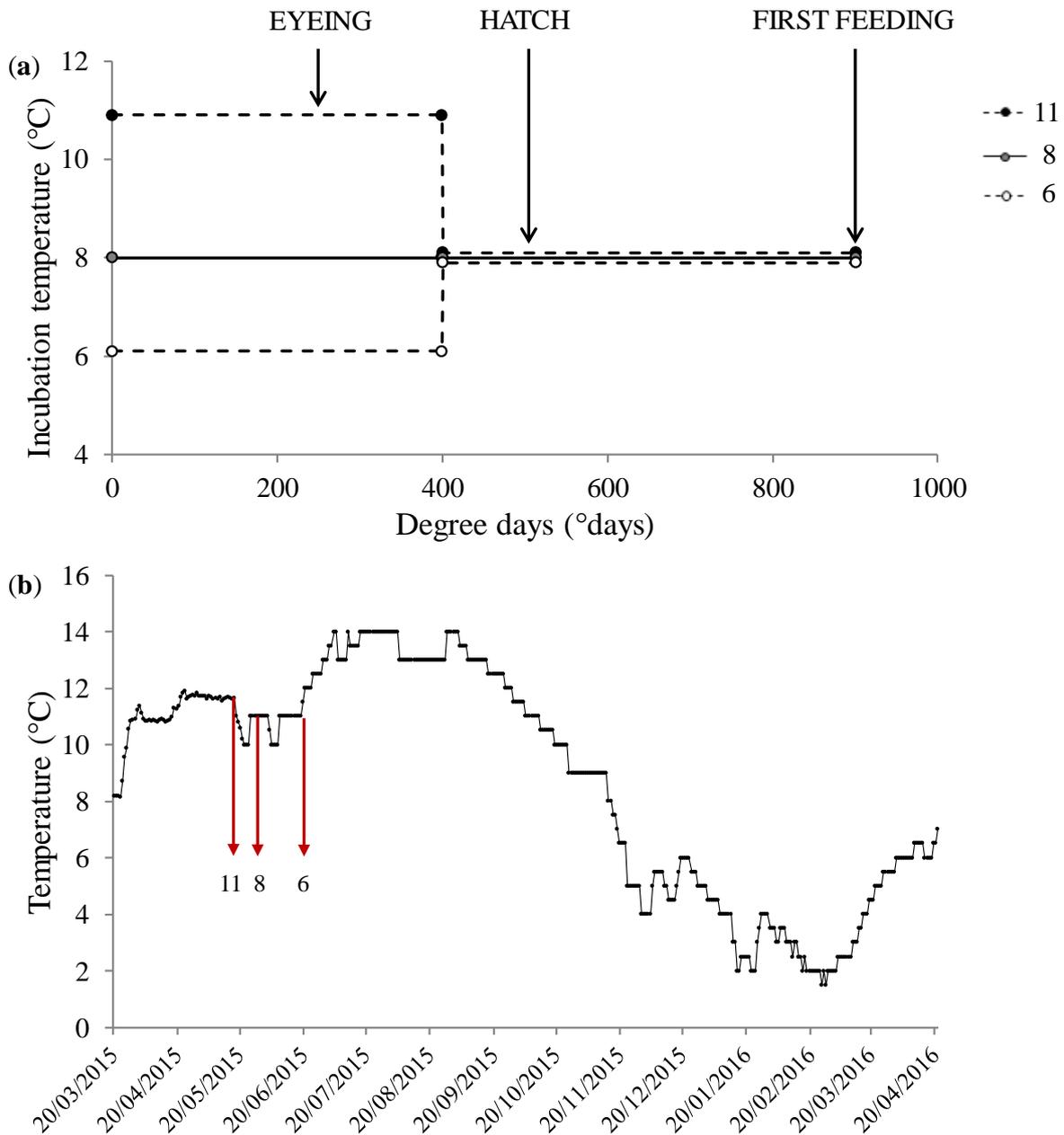


Figure 1. (a) Embryo incubation temperature including 6 °C (dashed line, opened circles), 8 °C (control; solid line, grey circles) and 11 °C (dashed line, black circles), and (b) average daily temperatures during subsequent development from first feeding until smolt. Introduction of fish (~1g) from each 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	<i>fas</i>	GTGAGCCCTGCCTCTTTTCT	AGAGCTTGCTGCCTGTGAT	59	CK876943
Liver X receptor	<i>lxr</i>	GCCGCCGCTATCTGAAATCTG	ATCCGGCAACCAATCTGTAGG	58	NM001159338
Sterol regulatory element binding transcription factor 1	<i>srebp1</i>	CCAACATGGCTACCGTCACT	ACCGCTCGAAAGTGTTCAA	56	NM001195818.1
Myogenesis					
Insulin-like growth factor 1	<i>igf1</i>	CAAAACGTGGACAGAGGCAC	TCCCTGTCCGTTAGCTTCTG	56	M81904
Insulin-like growth factor 1 receptor	<i>igf1r</i>	GTCGGCCAGCATGAGAGAGA	ACGGGTCTTTAGCCCGTAGT	58	EU861008
Insulin-like growth factor 2	<i>igf2</i>	TTGCGCCGGACTTTTAACTG	ATCTTGCATCGACCCTCACA	56	AY049955
Insulin-like growth factor binding protein related protein	<i>igfbprp</i>	GTGCGTTAAGAGCGACAAGA	CAATGACAGGTGTTGGG	56	EF432866.1
Myogenic factor 5	<i>myf5</i>	GCCTAAGGTGGAGATCCTGC	AGTCAACCATGCTGTCCGAG	57	DQ452070
Myogenic differentiation 1	<i>myod</i>	ACTCCAAATGCTGATGCCAGA	CTACCCTCCTGAACTGATAAC	56	NM001123601.1
Bone formation and mineralisation					
Alkaline phosphatase	<i>alp</i>	ATCCTGCTCATCTGCTCCTGC	AGTATTCGTGCTGCCGTCCT	56	FJ195609
Bone morphogenic protein 2	<i>bmp2</i>	TTCATATCGCTGCTGG	TCCGAACATATTGAGCAGCC	56	BT059611
Bone morphogenic protein 4	<i>bmp4</i>	GAACTCTACCAACCACGCCA	CGCACCCCTTCCACTACCATT	56	FJ195610
Collagen type 1 alpha 1 chain	<i>colla1</i>	TGGTGAGCGTGGTGAGTCTG	TAGCTCCGGTGTTTCCAGCG	56	FJ195608
Collagen type 2 alpha 1 chain	<i>col2a1</i>	TGGTCGTTCTGGAGAGACT	CCTCATGTACCTCAAGGGAT	56	FJ195613
Matrix metalloproteinase 13	<i>mmp13</i>	CCAACCCAGACAAGCCAGAT	GCTCTGAGAGTGATACGCC	56	DW539943
Osteocalcin	<i>ocn</i>	GACTCCTCTACCTCCACTGC	AATGATCCCAGCTGTGTCCA	60	FJ195611
Osteopontin	<i>opn</i>	CTTACTGAGGTGGCCCTGT	GCTGTCCGATGTTGGGTCTG	57	AF223388.1
Osteonectin	<i>sparc</i>	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 \pm SD ($p < 0.05$, Two-Way ANOVA). Where there were single groups thereafter, data are expressed as tank value \pm SD ($p < 0.05$; Analysis of CLT). Superscripts denote significant differences between treatments and ploidy within specific developmental stages.

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

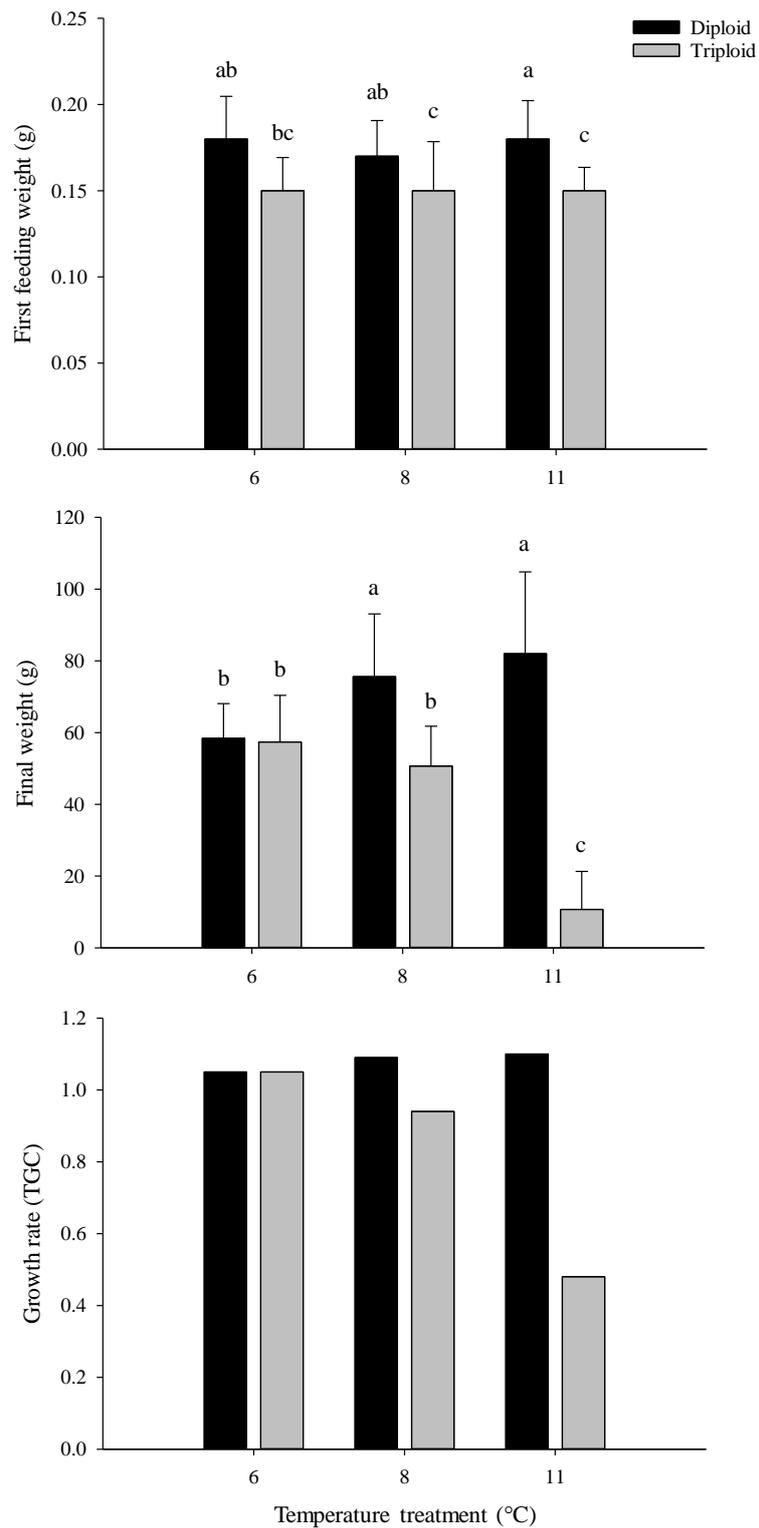


Figure 2. Growth summary of diploid (black) and triploid (grey) Atlantic salmon incubated under different temperature treatments including (a) body weight in first feeding alevins; BWi (g), (b) final body weight in smolts; BWf (g), and (c) thermal growth coefficient (TGC) between first feeding and smolt. Data are expressed as mean \pm 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 mm² (FFN, mm⁻²) and fibre cross-sectional surface area (µm²). 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 (µm²)
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}
<i>p</i> 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 \pm SD and significant differences are denoted by different superscripts ($p < 0.05$, Analysis of CLT).

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

dV: deformed vertebrae.

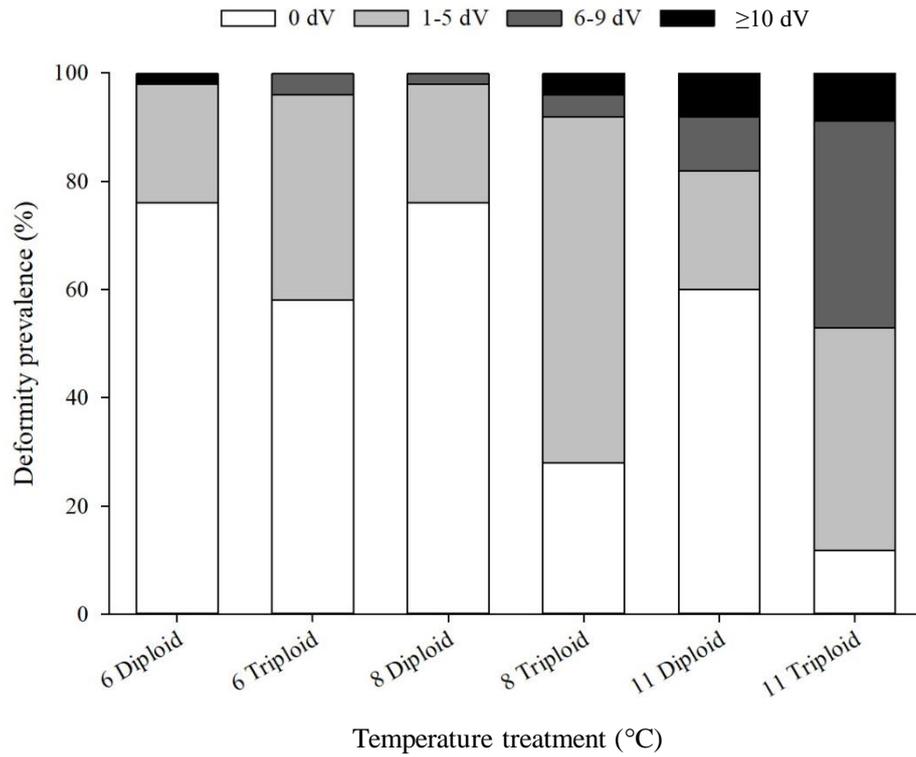


Figure 3. Prevalence (%) of X-ray radiologically assessed deformities presented according to severity index (Mild, 1-5 dV; Moderate, 6-9 dV; Severe, ≥ 10 dV) in diploid and triploid Atlantic salmon smolts incubated as embryos under different temperature treatments (34 - 50 fish treatment⁻¹ ploidy⁻¹).

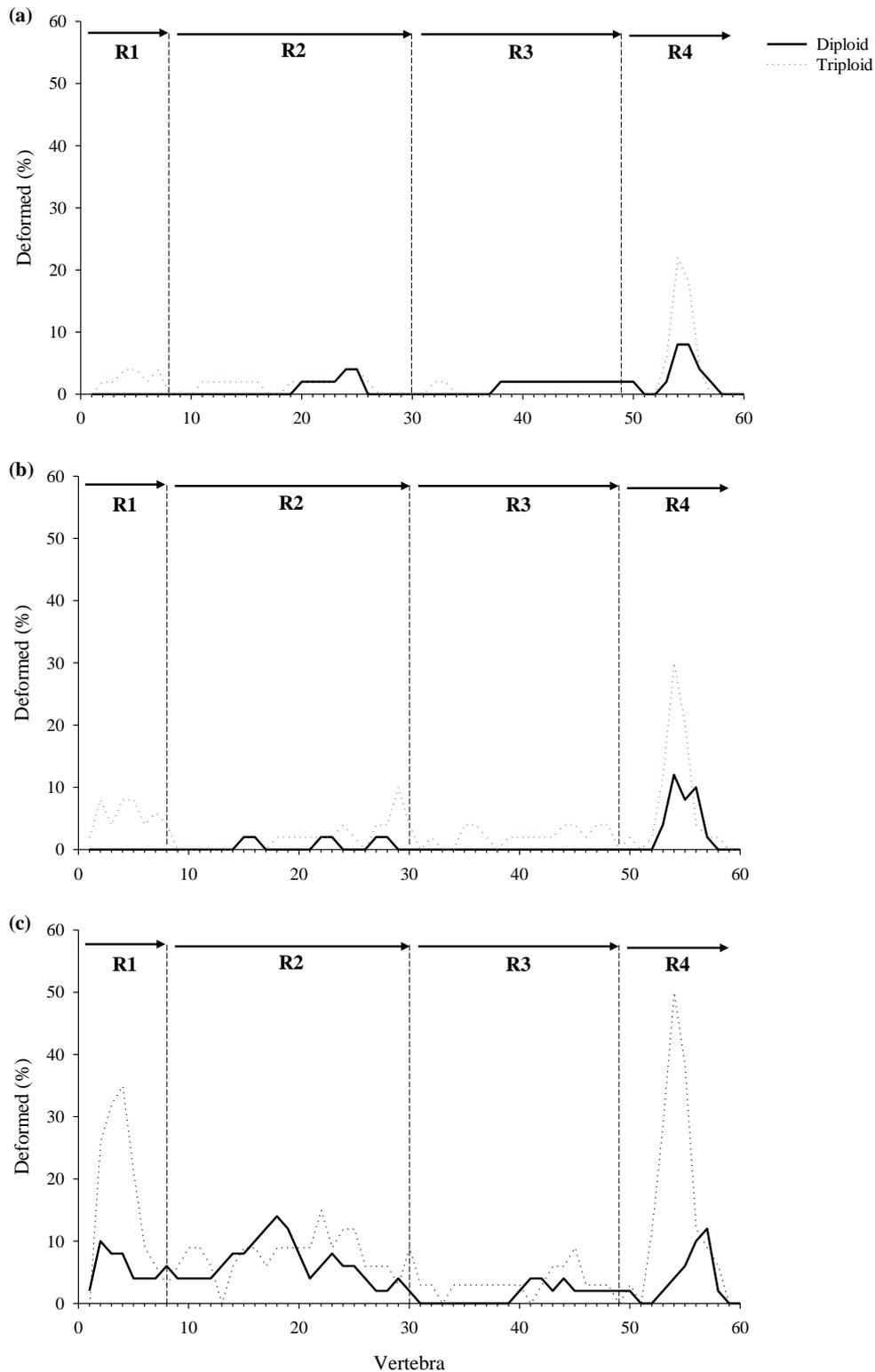


Figure 4. Prevalence (%) of deformed vertebrae observed in regions (R) according to Kacem *et al.* (1998) in diploid (solid line) and triploid (dotted line) Atlantic salmon smolts incubated as embryos under different temperature treatments; (a) 6 °C, (b) 8 °C, (c) 11 °C (34 - 50 fish treatment⁻¹ ploidy⁻¹).

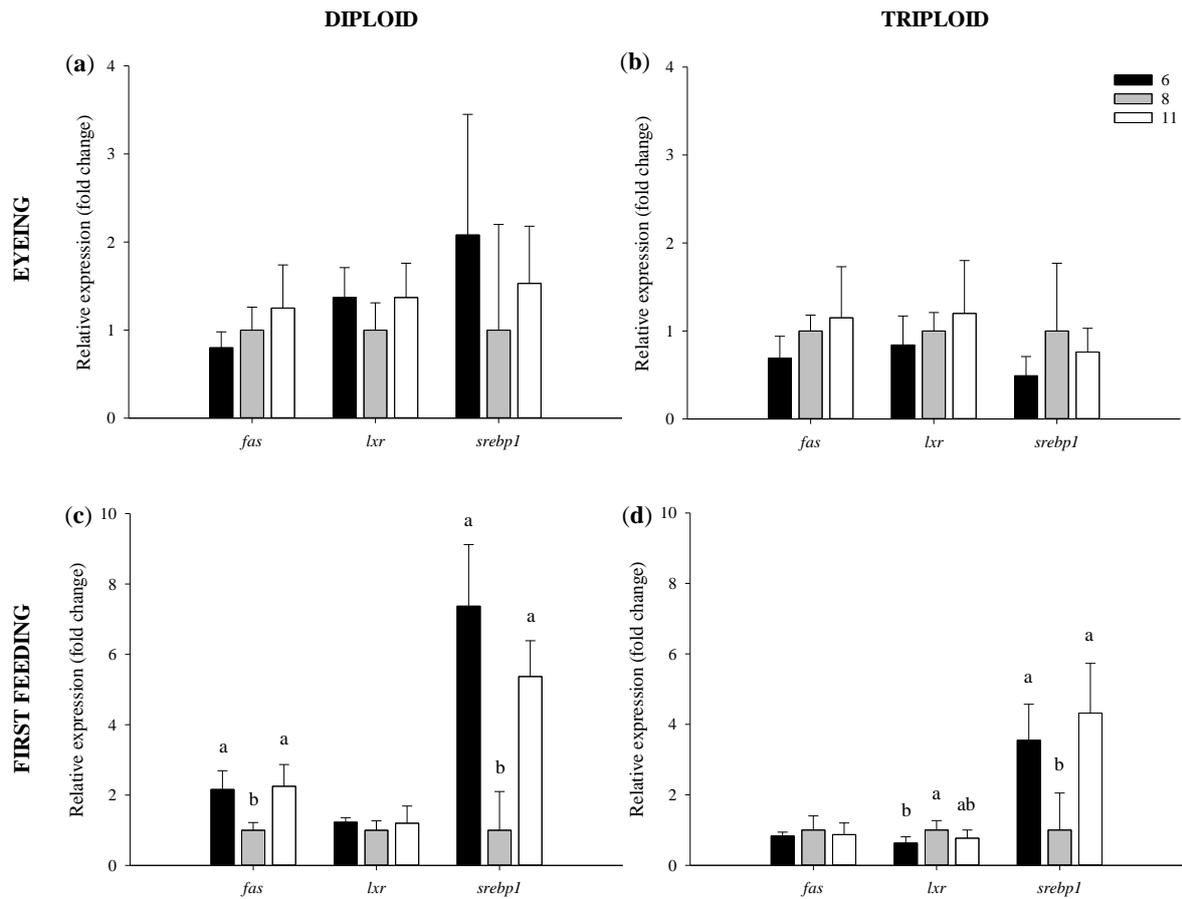


Figure 5. mRNA levels of genes involved in lipid metabolism (*fas*, *lxr* and *srebp1*) expressed as fold change relative to 8 °C (control) within each ploidy at respective developmental stage. Data show expression at eyeing in (a) diploids and (b) triploids and at first feeding in (c) diploids and (d)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

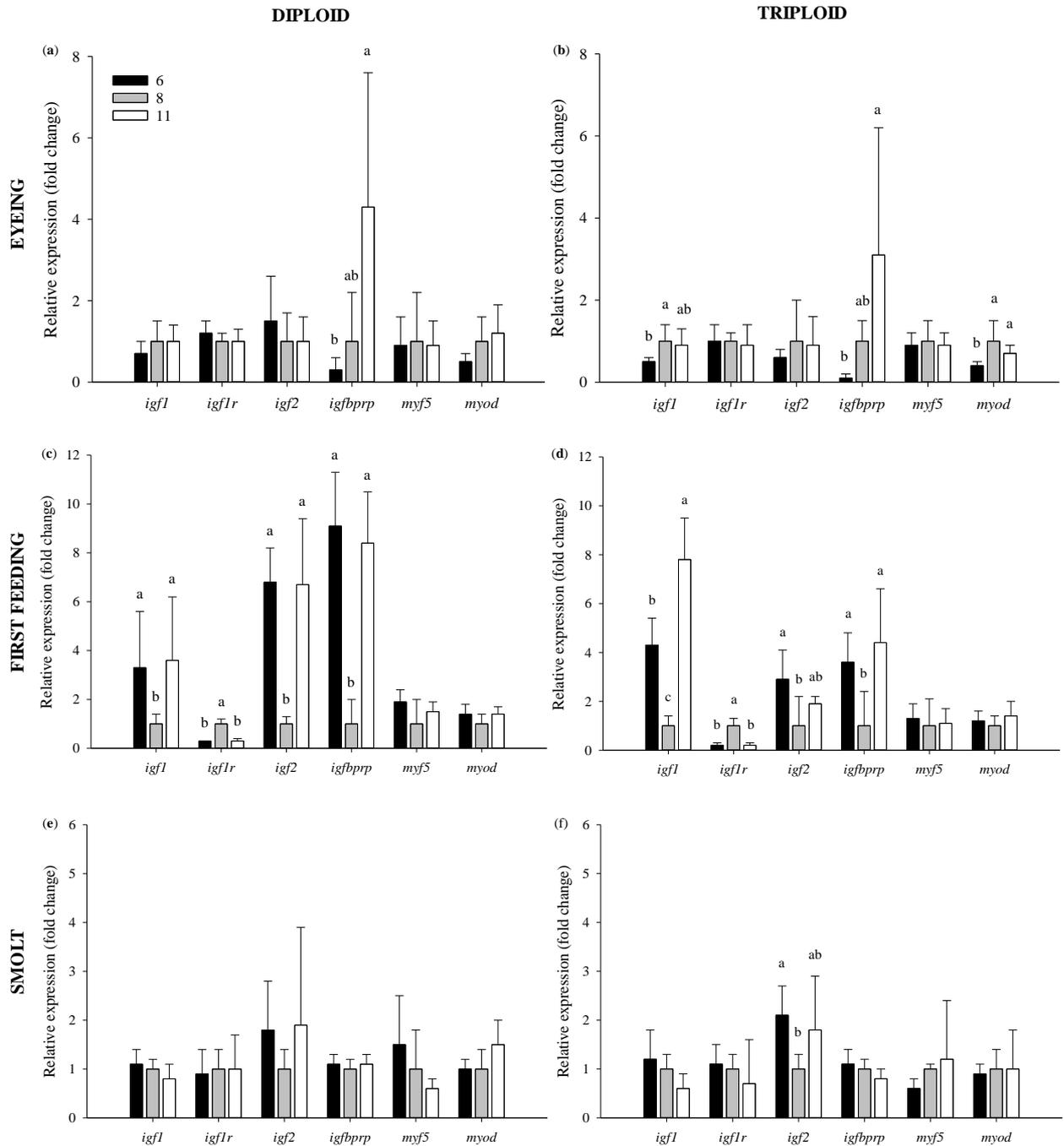


Figure 6. mRNA levels of genes involved in myogenesis (*igf1*, *igf1r*, *igf2*, *igfbprp*, *myf5* and *myod*) expressed as fold change relative to 8 °C (control) within each ploidy at respective developmental stage. Data show expression at eyeing stage in (a) diploids and (b) triploids, at first feeding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

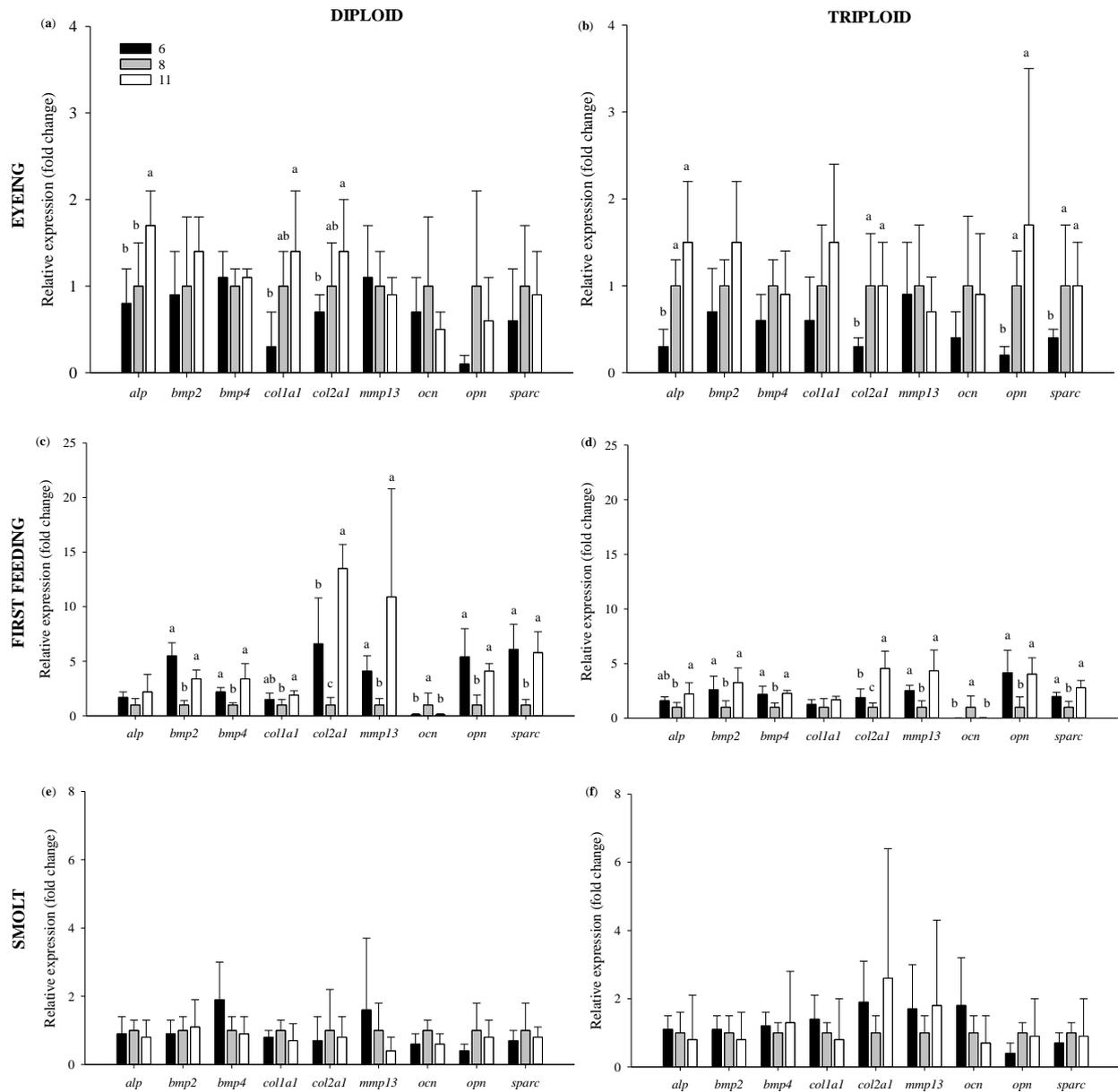


Figure 7. mRNA levels of genes involved in bone formation and mineralisation (*alp*, *bmp2*, *bmp4*, *colla1*, *col2a1*, *mmp13*, *ocn*, *opn* and *sparc*) expressed as fold change relative to 8 °C (control) within each ploidy at respective developmental stage. Data show expression at eyeing stage in (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 \pm SD (6 fish treatment⁻¹ ploidy⁻¹ sampling point⁻¹) and different superscripts denote significant differences ($p < 0.05$).

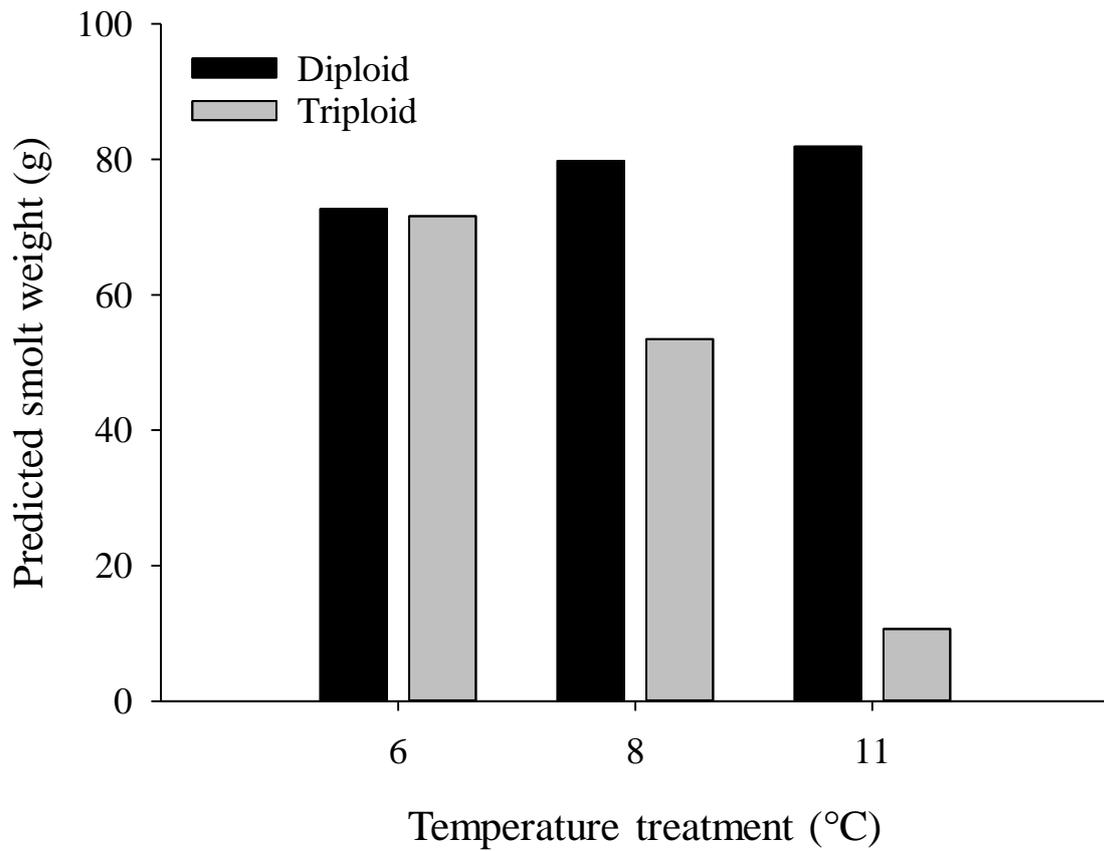


Figure 8. Predicted smolt weight of diploid and triploid Atlantic salmon incubated as embryos under different temperature treatments. Predicted weights were estimated using the respective TGC data and applied if all treatments experienced the same number of feeding days (395 days) under the same thermal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