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Dietary effects on biomarkers of growth, stress, and welfare of diploid and triploid Atlantic salmon (*Salmo salar*) during parr-smolt transformation

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ABSTRACT

Triploidy is induced in Atlantic salmon (*Salmo salar*) to produce sterile fish for genetic containment and to hinder early sexual maturation in farmed fish, but it can have unwanted negative effects on growth, health, and welfare. However, the growth and welfare of triploid fish may be improved by adjusting the rearing environment, feeding conditions and diets. This study evaluated physiological changes and used a suite of biomarkers to assess the potential impact of diet on growth and welfare of diploid and triploid salmon during the parr-smolt transformation. Diploids and triploids, held at low temperature, were fed a standard salmon feed or one with hydrolyzed fish proteins thought to be suitable for triploid Atlantic salmon. Fish muscle was collected monthly from October to December (2454–3044 degree-days post-start feeding, ddPSF) for analysis of biomarkers, and the progress of the parr-smolt transformation was monitored using a seawater challenge test. Real-Time PCR and radioimmunoassay were used to assess growth and stress response biomarkers (expression of genes of the GH-IGF axis and HSP70; cortisol concentrations), and oxidative stress biomarkers of lipids (MDA) and proteins (AOPP) were assayed. Changes in the biomarkers were related to sampling time rather than being associated with diet or ploidy, and the changes were compatible with the progression of the parr-smolt transformation. Growth and expressions of the biomarkers in triploid Atlantic salmon were similar to those of their diploid counterparts, and there was no evidence that the rearing conditions employed in the study resulted in stress responses being elicited. Overall, the physiological indicators and biomarkers employed in this study did not point to there being any dietary effects on performance and welfare of diploid and triploid salmon that were undergoing parr-smolt transformation.

1. Introduction

Sterilization of Atlantic salmon (*Salmo salar*) has been investigated as a possible option for sustainable farming to meet industrial and environmental criteria. For example, sterility, arising from triploidy, is induced to reduce reproductive interactions between farmed and wild fish following accidental escapes (Benfey, 2016) and to mitigate adverse effects of pre-harvest sexual maturation on growth and muscle (fillet) characteristics (Piferrer et al., 2009; Taranger et al., 2010). From a production perspective, sexual maturation can cause substantial economic losses in the form of decreased fish growth and reduced product quality (Good and Davidson, 2016), and escaped farmed salmon represent an 'anthropogenic' impact factor identified as a threat to wild salmon populations in Norway (Forseth et al., 2017).

Induction of triploidy may lead to poor performance and welfare of salmonids, particularly after transfer to seawater (Fraser et al., 2012; Madaro et al., 2021; Taranger et al., 2010), perhaps because of differences in environmental, physiological, and nutritional requirements of triploids in comparison to diploids. In Norway, poor welfare scoring of triploid salmon in sea cages has led the Norwegian Seafood Authority to impose a temporary moratorium on triploid salmon production and farming.

Of the stages that make up the Atlantic salmon life-cycle, parr-smolt transformation is perhaps one of the most critical. Parr-smolt transformation involves a complex suite of physiological, morphological, and behavioral changes that enable young salmon to transition from fresh

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water to seawater during their migration. In Atlantic salmon, the parr-smolt transformation is influenced by photoperiod and temperature, and is stimulated by several endocrine changes (McCormick et al., 2013). An increase in day-length activates neuroendocrine responses in the brain-pituitary axis that ultimately result in secretion of growth hormone (GH) and cortisol (McCormick, 1996). Cortisol, the key stress hormone in fish, is a corticosteroid produced by the interrenal tissue. It is acknowledged as being a seawater adapting hormone because it promotes, both directly and in synergy with the GH/IGF (Growth hormone/Insulin-like growth factor) system, salinity tolerance and mechanisms needed for hypo-osmoregulation in seawater (McCormick, 2001; Nisembaum et al., 2021; Wood et al., 2005).

Concentrations of circulating cortisol increase following exposure to stressors because of activation of the hypothalamic-pituitary-interrenal (HPI) neuroendocrine axis (Barton, 2002). The resulting mobilization of energy reserves via protein catabolism and gluco-neogenesis allows the fish to face an adverse situation and restore homeostasis (Mommsen et al., 1999). Cortisol, along with GH and IGF-I, also participates in the regulation of fish growth and metabolism (Madsen et al., 1995; Nisembaum et al., 2021). Cortisol release reduces fish growth, affects GH/IGF pathways (Breves et al., 2020) and has an influence on the expression of IGF-I and MSTN (Myostatin) (Bertotto et al., 2011; Davis and Peterson, 2006; Ellis et al., 2002).

At the cellular level, exposure to environmental stressors induces synthesis of heat shock proteins (HSPs), a family of highly conserved proteins that maintain cellular homeostasis via acting as molecular chaperones. In fish, HSP70 is expressed in several tissues and organs following exposure to thermal or osmotic shock (Bertotto et al., 2011; Iwama et al., 1998; Smith et al., 1999). In addition, exposure to stressors may trigger the production of reactive oxygen species (ROS). When ROS production exceeds the ability of the cells to eradicate them, oxidative stress arises and leads to molecular damage to nucleic acids, lipids and proteins and, eventually, to cell death (Davies, 1995; Winston and Di Giulio, 1991). Malondialdehyde (MDA), a by-product of oxidation of polyunsaturated fatty acids, is used as a biomarker to assess ROS-damage (Slaninova et al., 2009) because its concentration reflects the level of membrane lipid peroxidation and, consequently, the integrity of cells and organelles (Shao et al., 2012). In addition to MDA, other biomarkers include advanced oxidation protein products (AOPP) that are used to assess protein oxidation (Bansal and Kaushal, 2014; Witko-Sarsat et al., 1998).

To understand whether, and how, rearing and feeding conditions might affect diploid and triploid Atlantic salmon during parr-smolt transformation we investigated a series of biomarkers associated with growth, stress, and welfare. The fish used were those described in the study of Peruzzi et al. (2018), and in our work the fish continued to be fed either a standard fish meal (STD) diet or a modified diet in which 45% of the fish meal fraction was replaced with hydrolyzed fish proteins (HFM) as they progressed through the parr-smolt transformation. We chose to investigate the expression of GH, GHrec, IGF-I and MSTN as they are genes involved in fish growth, and of HSP70 since it is involved in the stress response. MDA and AOPP were selected as they are robust oxidative stress markers. We chose to assess the GH/IGF-I axis and cortisol as they are involved in fish osmoregulation, a key aspect of the parr-smolt transformation. The biomarkers were assayed in muscle samples because muscle is less sensitive than plasma to rapid changes that may be induced by handling during sampling of fish. Muscle cortisol concentrations correlate with those in plasma because it is a lipophilic hormone that diffuses readily through cell membranes (Bertotto et al., 2010).

2. Materials and methods

2.1. Ethics statement

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Norwegian regulations for use of animals in experiments and was approved by the Norwegian Committee on Ethics in Animal Experimentation via project license (Permit ID 8180) issued by the Norwegian Food Safety Authority (Mattilsynet, FOTS). The growth trial was carried out in an approved facility (Tromsø Aquaculture Research Station, FOTS license nr. 110) by trained and licensed personnel. Terminal measurements were performed on fish euthanized with an overdose of anesthetic (Benzocaine, 120 mg L^{-1}). All efforts were made to minimize fish suffering.

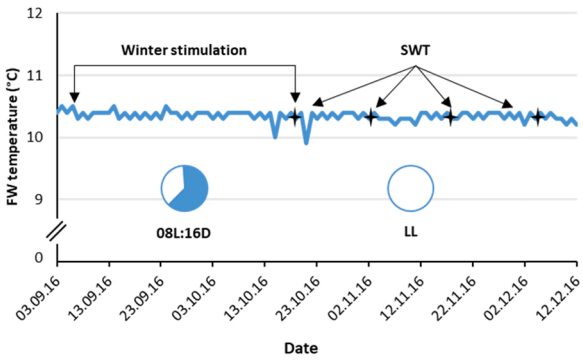

Fig. 1. Overview of rearing conditions and sampling points during the trial. Black stars (4-points) indicate sampling points for seawater challenge test (SWT). FW = fresh water; LL = constant light; 08L:16D = light regime used for `winter' stimulation of parr-smolt transformation.

2.2. Animal rearing and sampling

Details on the origin, production and rearing protocols of the experimental fish are provided by Peruzzi et al. (2018). Briefly, fertilized Atlantic salmon eggs were obtained from a commercial hatchery (Stonfiskur Salmo Breed AS, Iceland) and, at 300 degree min post-fertilization at 5 °C, half of the eggs were subjected to a hydrostatic pressure shock of 9500 psi applied for 5 min (Johnstone and Stet, 1995) to induce triploidy. Eyed-eggs (ca. 400 day-degrees) were then transferred to the Tromsø Aquaculture Research Centre (Norway) to complete hatching and ploidy status of the fish was verified by flow cytometry in newly hatched fry (Peruzzi et al., 2018). Prior to start feeding, fish from each ploidy were transferred to twelve 200 L circular indoor tanks (initially ca. 3000 fish per tank; tank biomass ca. 620 g), reared in triplicate tanks and fed either a standard fish meal (STD) diet or a modified diet in which 45% of the fish meal fraction was replaced with hydrolyzed fish proteins (HFM) (Table A1; Skretting AS, Stavanger, Norway). Therefore, the four experimental groups are: (i) diploid (2 N) fish fed STD diet, (ii) 2 N fed HFM diet, (iii) triploid (3 N) fish fed STD diet and (iv) 3 N fed HFM diet. Phosphorus inclusion in STD and HFM diets (MasterLab, Boxmeer, Netherlands) was 19 g kg^{-1} and 18 g kg^{-1}, respectively, which is considerably higher than the reported requirement of Atlantic salmon (8 g phosphorus kg^{-1} diet). Feed was delivered via electrically driven disc feeders programmed to supply 6–9 meals each day, and the amount of feed provided was always higher than that consumed. Fish were reared at low temperature (10 ± 0.5 °C) from start-feeding to the end of parr-smolt transformation, except during the summer (04 July - 02 September) when fish were exposed to ambient water temperature (range 9.5–12.5 °C). Fish were then transferred from 200 L to 500 L tanks and continuous light (LL) was used during the experiment, except for a period (06 September to 18 October) of reduced day length (08 L:16D) needed to mimic winter conditions and induce parr-smolt transformation (Fig. 1). Sampling was carried out each month from October to December (2454–3044 degree-days post-start feeding, ddPSF) covering the period of parr-smolt transformation. Data on fish mortality are given in Appendix (Table A2). At each sampling time, a portion of dorsal muscle (1 cm x 1 cm, below dorsal fin and

Table 1
Primer sequences used.

Gene	Forward Primer (5′-3′)	Reverse Primer (5′-3′)
GH	5′- CCCTAGCCAGACCCTGATCAT-3′	5′-GAGCAGGTTGAGCCCACTT-3′
GHrec	5′-GGAAGACATCGTGGAACCAGA-3′	5′-CAAACTGGCTCCCGGTTAGA-3′
IGF-I	5′-CGGTCACATAACCGTGGTATTG-3′	5′-CTGCCTTGCCAGACTTGACA-3′
MSTN	5′-GCGCTACAAGGCCAACTACTG-3′	5′-GGTGCCGCGAGGGTTAG-3′
HSP70	5′-CAGGCCAGCATTGAGATTGA-3′	5′-CCCTGAAGAGGTCGGAACAC-3′
β-ACT	5′-CAGCCCTCCTTCCTCGGTAT-3′	5′-AGCACCGTGTTGGCGTACA-3′

without skin) was collected from 12 fish from each experimental (ploidy and feed treatment) group (4 fish per tank and 3 tanks for each treatment). Muscle samples were frozen on dry ice and then stored at −80 °C.

2.3. Parr-smolt transformation

To monitor parr-smolt transformation a standardized seawater challenge test (SWT) (Blackburn and Clarke, 1987) was conducted on 18 October, 1 November, 16 November and 02 December (Fig. 1). At the start of each test, 18 (3 × 6) fish from each ploidy group (STD diet only) were randomly netted from each tank and transferred directly from fresh water to a test tank supplied with seawater (10 °C, 33‰ salinity). After 24 h of seawater exposure the numbers of dead fish were recorded and the remaining fish euthanized with an overdose (120 mg L^{-1}) of anesthetic (Benzocaine, Sigma-Aldrich Company Ltd., UK). Blood samples were drawn from the caudal vessels with 1 mL Li-heparinized vacutainers, centrifuged at 2780 × *g* and 1 °C for 8 min. Plasma was then removed and stored at −80 °C until analyzed. The hypo-osmoregulatory ability of the sampled fish was assessed by measuring plasma chloride concentration (Corning 925 chloride titrator, CIBA Corning Diagnostics, Essex, UK) and plasma osmolality (FiskeOne-Ten Osmometer, Fiske Associates, MA, USA). In addition, at all sampling dates, 18 (3 × 6) fish were sampled directly from the tanks (fresh water), sacrificed as above, and fish condition factor (K) was calculated as:

$$K = 100 \quad (WL\text{-}3)$$

where W is body weight (g) and L is the fork length (cm).

2.4. Fish growth

Biomass increase of fish in each tank was monitored during two periods (October-November and November-December, respectively) covering 2454–3044 degree-days post-start feeding (ddPSF).

In addition, fish growth ($n = 30$ fish per experimental group, 3 tanks per experimental group, 10 fish per tank) was assessed using specific growth rate (SGR) calculated as:

$$\text{SGR} = (\ln(\text{final weight in grams}) - \ln(\text{initial weight in grams}) \div \text{t(in days)}) \times 100$$

2.5. RNA extraction and Real-Time PCR

Quantitative Real-Time PCR was carried out using the methods described by Bertotto et al. (2011).

Total RNA was extracted from dorsal muscle dissected from fish at the three different sampling times: (i) October (2454 ddPSF), (ii) November (2745 ddPSF), (iii) December (3044 ddPSF; for each sampling time, $n = 6$ fish per experimental group: 3 tanks per experimental group, 2 fish per tank). RNA was extracted using TRIZOL reagent (Life Technologies, Carlsbad, CA, USA) following the manufacturer's protocol.

PCRs were performed in triplicate in a Real-Time PCR 7500 thermal cycler (Applied Biosystems, Life Technologies) and analyzed with SDS Software (Applied Biosystems, Life Technologies). Relative quantification of the expression of genes involved in fish growth and seawater adaptation (GH, GHrec, IGF-I, and MSTN) and stress response (HSP70), was performed using β-actin as the housekeeping gene to standardize results. Specific primer sequences for the studied genes and for β-actin (β-ACT), used as reference gene, were designed using Primer Express software version 3.0 (Applied Biosystems, Life Technologies) and are reported in Table 1.

2.6. Radioimmunoassay (RIA)

For cortisol analysis, a specific microtiter radioimmunoassay (RIA) was used to measure muscle cortisol levels as described in Bertotto et al. (2010). Muscle samples ($n = 12$ fish per experimental group: 3 tanks per experimental group, 4 fish per tank) were thawed and then pulverized in liquid nitrogen, and 100 mg of the resulting powders were suspended in 500 μL RIA buffer (Na_2HPO_4-$12H_20$ 61 mM, NaH_2PO_4-H_20 40 mM, NaCl 154 mM, pH 7.2) and extracted with 8 mL of diethyl ether. The dry extracts were dissolved in PBS 10 mM (Na_2HPO_4-$12H_2O$ 0.2 M, NaH_2PO_4-H_2O 0.2 M, pH 7.2 by adding NaCl) and used for RIAs. Briefly, a 96-well microtiter plate (Optiplate, Perkin Elmer Life Sciences) was coated with anti-rabbit c-globulin serum raised in a goat (dilution 1:1000 in 0.15 mM sodium acetate buffer, pH 9, at 4 °C) and, after PBS double washing, incubated overnight at 4 °C with the specific antiserum solution. Standards, quality controls, unknown extracts and 3H tracers were then added and, after overnight incubation at 4 °C, the plate was washed with PBS, 200 μL scintillation cocktail (Microscint 20, Perkin Elmer Life Sciences) added and then counted on a beta-counter (Top--Count, Perkin Elmer Life Sciences).

The anti-cortisol serum showed the following cross-reactions: cortisol 100%, prednisolone 44.3%, 11-deoxycortisol 13.9%, cortisone 4.95%, corticosterone 3.5%, prednisone 2.7%, 17-hydroxyprogesterone 1.0%, 11-deoxycorticosterone 0.3%, dexamethasone 0.1%, progesterone < 0.01%, 17-hydroxypregnenolone < 0.01%, pregnenolone < 0.01%.

2.7. Oxidative stress

Muscle ($n = 12$ fish per experimental group: 3 tanks per experimental group, 4 fish per tank) was used to assess lipid peroxidation. Two hundred milligrams of tissue were homogenized with Tris HCL 0.125 M pH 6.8, centrifuged at 415 ×*g* at 4 °C for 15 min, and the supernatant was used for the assays. Muscle lipid peroxidation was assayed in muscle by measuring thiobarbituric acid-reactive substances (TBARS) according to Yoshida et al. (2005). Briefly, thiobarbituric acid reaction was carried out by mixing 0.2 mL sodium dodecyl sulfate solution (8.1%, w/v), 1.5 mL acetic acid buffer (20%, v/v, pH 3.5), 1.5 mL thiobarbituric acid (1%, v/v), 0.6 mL water, and 0.05 mL ethanol containing butylated hydroxytoluene (0.8 wt%, w/v) with 0.02 mL of supernatant. The reaction mixture was incubated at 100 °C for 60 min and then cooled on ice followed by mixing vigorously with 1 mL water and 5 mL of n-butyl alcohol and pyridine (15/1, by volume). Then, the mixture was centrifuged (0 °C, 1400 ×*g*) for 10 min and the supernatant was measured spectrophotometrically at 535 nm. Tetramethoxypropane was used as a standard to estimate TBARS formation as nanomoles of

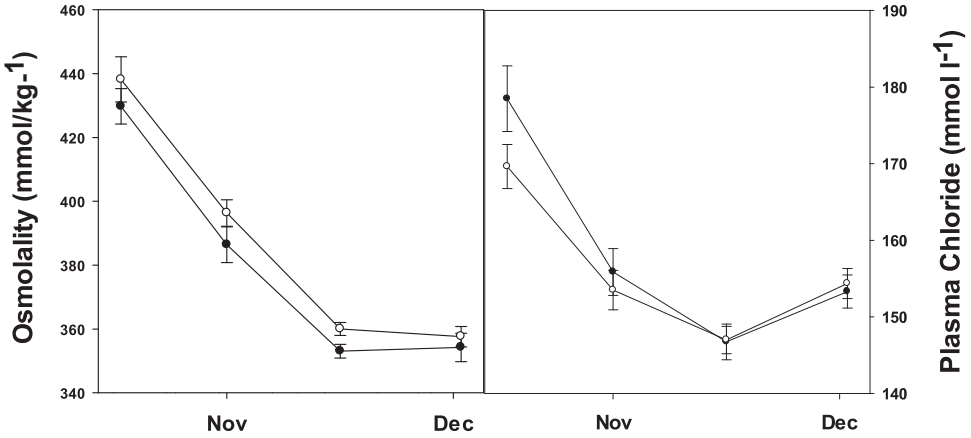

Fig. 2. Plasma osmolality (mean ± standard error, SE) and plasma chloride (mean ± SE) in diploid (·) and triploid (o) salmon sampled after a 24-h SW challenge test (SWT) ($n = 18$).

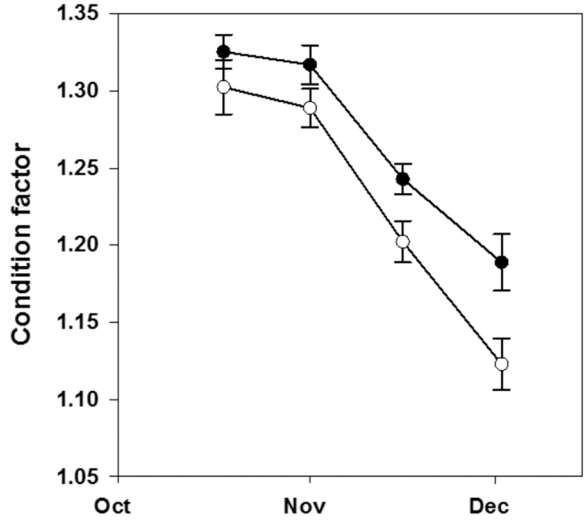

Fig. 3. Condition factor (mean ± SE) of diploid (·) and triploid (o) Atlantic salmon sampled in fresh water ($n = 18$).

malondialdehyde (MDA) equivalents per mL of muscle.

Advanced oxidation protein products (AOPP) in muscle were measured ($n = 12$ fish per experimental group: 3 tanks per experimental group, 4 fish per tank) following the Witko-Sarsat spectrophotometric method (Witko-Sarsat et al., 1996, 1998). Calibration involved use of chloramine-T (10 mM; Fluka, St. Louis, MO) solutions which in the presence of potassium iodide absorb at 340 nm Briefly, two hundred microliters of muscle extract diluted 1:4 in PBS (20 mM, pH 7.4) or chloramine-T standard solutions (0–100 μmol/L), were placed in each well of a 96-well microtiter plate (Perkin Elmer Life and Analytical Sciences, Shelton, CT, USA), followed by 20 μL of acetic acid. Ten microliters of 1.16 M potassium iodide (Sigma) were then added, followed by 20 μL of acetic acid. The absorbance of the reaction mixture was read at 340 nm using a microplate reader (Packard Instrument, Meriden, CT, USA) against a blank containing PBS, potassium iodide and acetic acid. The chloramine-T absorbance at 340 nm was linear within the range of 0–100 μmol/L. AOPP concentrations were corrected according to the total protein concentration, determined using the BCA kit (Thermo Fisher), and expressed in μmol/L of chloramine-T equivalents.

2.8. Statistical analysis

Prior to statistical analysis, carried out using R software (R Core Team, 2020), all the data were evaluated for normality and transformed logarithmically if necessary.

Parr-smolt transformation data (plasma osmolality, plasma chloride and condition factor) were analyzed using a two-way ANOVA with time and ploidy as main factors.

Fish growth metrics, and data from Real-Time PCR, RIA, TBARS and Witko-Sarsat analyses were analyzed using a linear mixed model (the R package *lme4*) with sampling time, diet and ploidy as main factors and with tank as random factor.

All data were expressed as mean ± standard error (SE). Differences among means with $p < 0.05$ were accepted as being statistically significant.

3. Results

3.1. Parr-smolt transformation: SW-challenge tests (SWT)

Dead fish were observed in the triploids on two SWT dates (16th of October - 1 dead after SWT and 1st of November - 3 dead after SWT). For both diploids and triploids there was a significant effect of time on plasma osmolality ($p < 0.01$) and chloride concentration ($p < 0.001$). Post-SWT plasma osmolality and plasma chloride decreased significantly, from 16th of October (end of winter period) to mid-November (Fig. 2). No significant differences between diploids and triploids were found at any of the SWT times.

There was a significant decrease in condition factor ($p < 0.05$) for both diploids and triploids over time, but with no significant differences between ploidies at any sampling time (Fig. 3).

3.2. Fish growth and stress response biomarkers

There were no clear effects of either ploidy or diet on the specific growth rate (SGR) of the fish. However, fish growth changed over time (Fig. A1), with growth being reduced during the second period (November-December, 2745–3044 ddPSF). Neither diet nor ploidy were found to have significant effects on the expression of GH, GHrec, IGF-I, or MSTN genes. However, gene expression changed over time for GH, GHRec, and IGF-I, with the trends being clearest for the triploids. The mRNA levels for GH increased from October to November ($p < 0.01$) and then decreased in December ($p < 0.01$; Fig. 4a). Similar trends were seen in mRNA levels for GHrec and IGF-I (Fig. 4b and c). Sampling time did not have a significant effect on MSTN mRNA levels (Fig. 4d).

Regarding the stress response biomarkers, there were no significant effects of diet, ploidy, or sampling time on heat shock protein 70 (HSP70) gene expression (Fig. 5a).

On the other hand, cortisol levels were significantly affected by both sampling time and ploidy. Higher values were recorded in November and December than in October, and triploids had higher muscle cortisol than diploids in December ($p < 0.001$; Fig. 5b).

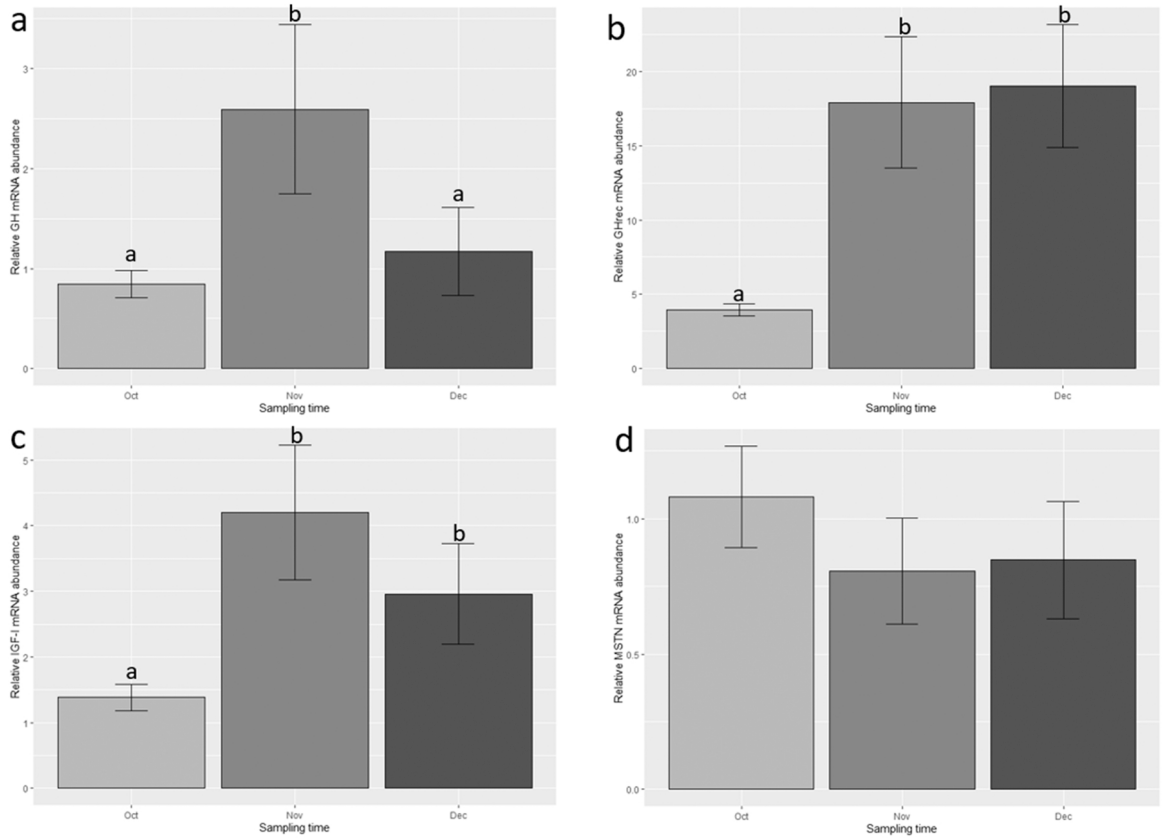

Fig. 4. Relative expression of: (a) growth hormone (GH); (b) growth hormone receptor (GHrec); (c) insulin-like growth factor I (IGF-I) and (d) myostatin (MSTN) mRNA detected in Atlantic salmon muscle according to sampling time during parr-smolt transformation. Values are expressed as mean ± SE ($n = 6$). Different letters indicate significant differences between means in relation to sampling time ($p < 0.01$). STD: phosphorus-rich fish meal based standard diet; HFM: phosphorus-rich hydrolyzed fish proteins-based diet.

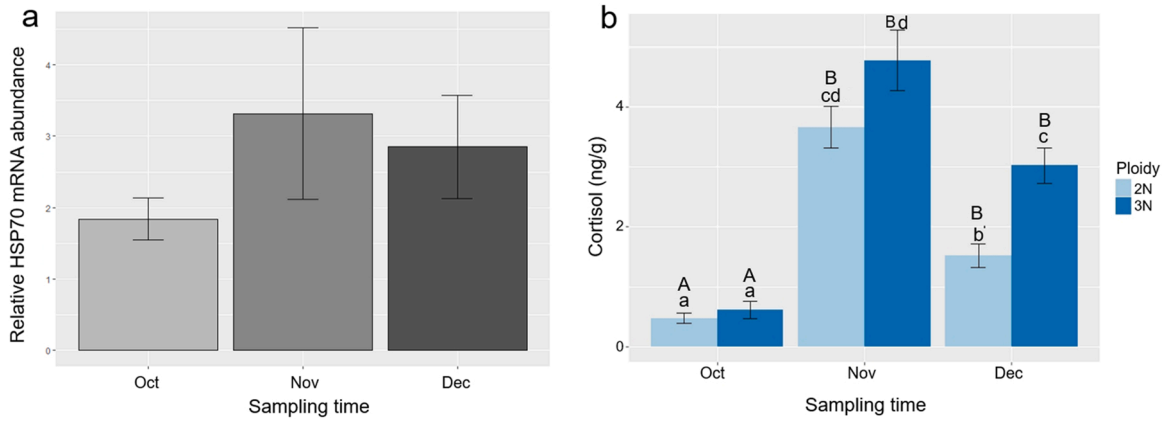

Fig. 5. Relative expression of heat shock protein 70 (HSP70) mRNA according to sampling time (a) and cortisol concentration according to sampling time and ploidy (b) detected in Atlantic salmon muscle during parr-smolt transformation. Data are expressed as mean ± SE ($n = 6$ for gene expression and $n = 12$ for cortisol analysis). Different uppercase and lowercase letters indicate significant differences between means in relation to sampling time and to ploidy, respectively ($p < 0.001$). STD: phosphorus-rich fish meal based standard diet; HFM: phosphorus-rich hydrolyzed fish proteins-based diet. 2 N: diploid salmons; 3 N triploid salmons.

3.3. Cellular oxidative stress

Malondialdehyde (MDA) concentrations were not significantly affected by any of the three factors studied (Fig. 6a). Advanced oxidation protein products (AOPP) levels were significantly influenced by sampling time, with the highest values being recorded in November ($p < 0.001$; Fig. 6b).

4. Discussion

The use of triploid Atlantic salmon (*Salmo salar*) for commercial production is a controversial, and much-debated, issue. On one hand, the induction of triploidy, leading to the production of sterile fish, is effective for genetic containment and the control of early sexual maturation, but it also gives rise to fish that differ from diploids in environmental and nutritional requirements, and these differences can have negative effects on the growth, welfare, and health of triploids in

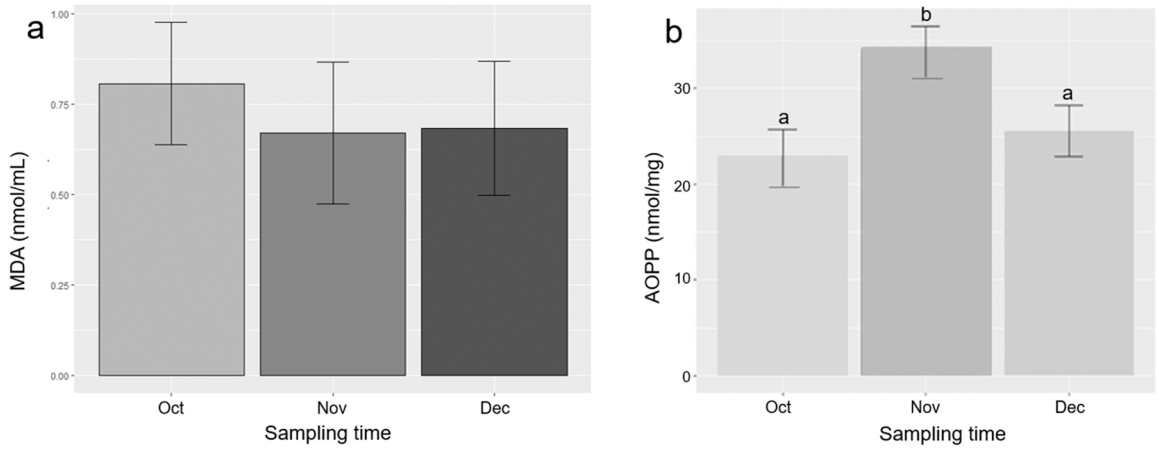

Fig. 6. Concentration levels of (a) malondialdehyde (MDA) and (b) advanced oxidation protein products (AOPP) according to sampling time detected in Atlantic salmon muscle during parr-smolt transformation. Data are expressed as mean ± SE ($n = 12$). Different letters indicate significant differences between means in relation to sampling time ($p < 0.001$). STD: phosphorus-rich fish meal based standard diet; HFM: phosphorus-rich hydrolyzed fish proteins-based diet.

comparison with diploids. Atlantic salmon are anadromous, and the life cycle includes a parr-smolt transformation that is critical for the transition from life in fresh water to one in which survival and growth in seawater is possible. There is some evidence that there may be differences in the parr-smolt transformation between diploid and triploid salmon, that result in differences between the ploidies in survival and growth following transfer from fresh water to seawater, but the findings do not allow firm conclusions to be drawn (De Fonseka et al., 2022; Leclercq et al., 2011; Smedley et al., 2016).

In the present work, which is a continuation of a published study (Peruzzi et al., 2018), we used a suite of biomarkers related to growth, osmoregulation, stress, and welfare to assess the possible effects of ploidy and diet on Atlantic salmon during the parr-smolt transformation. During our study both the diploid and triploid Atlantic salmon appeared to complete a successful parr-smolt transformation since we observed the expected fall in condition factor (Fig. 3) and reduced plasma Cl^- and osmolality following a SWT (Fig. 2), which is a confirmation of the ability of the fish to hypo-osmoregulate in seawater.

We did not record any clear and consistent differences in growth between the diploid and triploid fish in our study (Fig. A1), and when we analyzed the gene expression of myostatin (MSTN), an important regulator of muscle growth (Garikipati and Rodgers, 2012) we did not find any treatment effects (Fig. 4d). This indicates that myogenesis was not affected by any of the three factors studied (sampling time, diet, ploidy). In some previous studies, the growth of triploids in fresh water was found to be comparable with that of diploids (Peruzzi et al., 2018; Taylor et al., 2011), particularly at low rearing temperatures where any differences related to ploidy are reduced (Benhaïm et al., 2020). Given that triploids showed comparable growth and expression of growth-related biomarkers to diploids during parr-smolt transformation in our study, the evidence points to the suitability of our feeding and rearing conditions for promoting growth and parr-smolt transformation of the triploid salmon. Therefore, the water temperature and photoperiod applied in this study meet the requirements for rearing triploid salmon and the inclusion of phosphorus and hydrolyzed fish proteins in the feeds may reduce any differences between animals of different ploidy.

Many hormones have been implicated in the parr-smolt transformation, including GH, IGF-I, cortisol, and their receptors (McCormick, 2001; Nilsen et al., 2008). GH and IGF-I have an influence on the number and size of gill chloride cells (Sakamoto and Hirano, 1993), and IGF-I also has a direct effect on osmoregulation via eliciting activity of gill ion transporter enzymes (McCormick et al., 2013). In our study, the expression of GH, GHrec and IGF-I in muscle did not differ with diet or ploidy, but there were significant changes with sampling time (Fig. 4). Gene expression increased from October to November (2454–2745 ddPSF) (Fig. 4), coinciding with the time at which the most pronounced responses in the SWT were observed (Fig. 2). This result appears to confirm that both diploid and triploid salmon successfully underwent parr-smolt transformation, and that elevated levels of gene expression encoding for the hormones is a pre-adaption to prepare the fish for life in seawater.

In anadromous fish, cortisol plays a role in salinity adaptation, by influencing the number and size of gill chloride cells and stimulating the activity and expression of their ion transporters, i.e. Na^+, K^+-ATPases (McCormick, 2001; Zadunaisky, 1996), and it acts in synergy with the GH/IGF system to increase salinity tolerance and seawater adaptation (McCormick, 1996). In our study, muscle cortisol levels changed significantly over time, with higher concentrations being recorded in November and December than in October (Fig. 5b), thereby reflecting and confirming its role in the parr-smolt transformation and pre-adaptation for life in seawater (Chalmers et al., 2018). Our findings are in partial agreement with those reported by De Fonseka et al. (2022). In their study, highest concentrations of plasma cortisol coincided with the parr-smolt transformation and then fell, with triploids having significantly higher values than diploids irrespective of time. In our study, triploids had muscle cortisol values that were similar to those of diploids, except in December when higher values were recorded in triploids (Fig. 5b).

In this study, we were interested in assessing the stress and welfare status of the fish. Cortisol is the main stress hormone in teleost fish and can be a reliable welfare indicator (Sadoul and Geffroy, 2019). During the stress response metabolism adapts to meet the energy demands and to restore homeostasis (Tort and Teles, 2012). There are links between stress and expression of growth-related genes (Reinecke et al., 2005), with increased cortisol being associated with suppression of the somatotropic axis (Link et al., 2010; Mommsen et al., 1999; Pujante et al., 2015). For example, the influence of acute stress on IGF gene expression has been investigated in several fish species and in most cases, downregulation has been recorded in fish exposed to stressors (Breves et al., 2010; Cao et al., 2009; Cheng et al., 2015; Link et al., 2010; Sadoul and Vijayan, 2016; Saera-Vila et al., 2009). Moreover, glucocorticoids, such as cortisol, may be involved in the regulation of myostatin transcript levels via glucocorticoid response elements (GREs) in the myostatin promoter, which suggests an involvement of cortisol in regulation of muscle growth (De Santis and Jerry, 2011; Funkenstein et al., 2009; Galt et al., 2016; Garikipati et al., 2006). In our study, IGF-I gene expression was not downregulated at the time when muscle cortisol was high and MSTN gene expression did not change over time (Fig. 4), seeming to confirm that the changes in muscle cortisol we observed (Fig. 5b) were

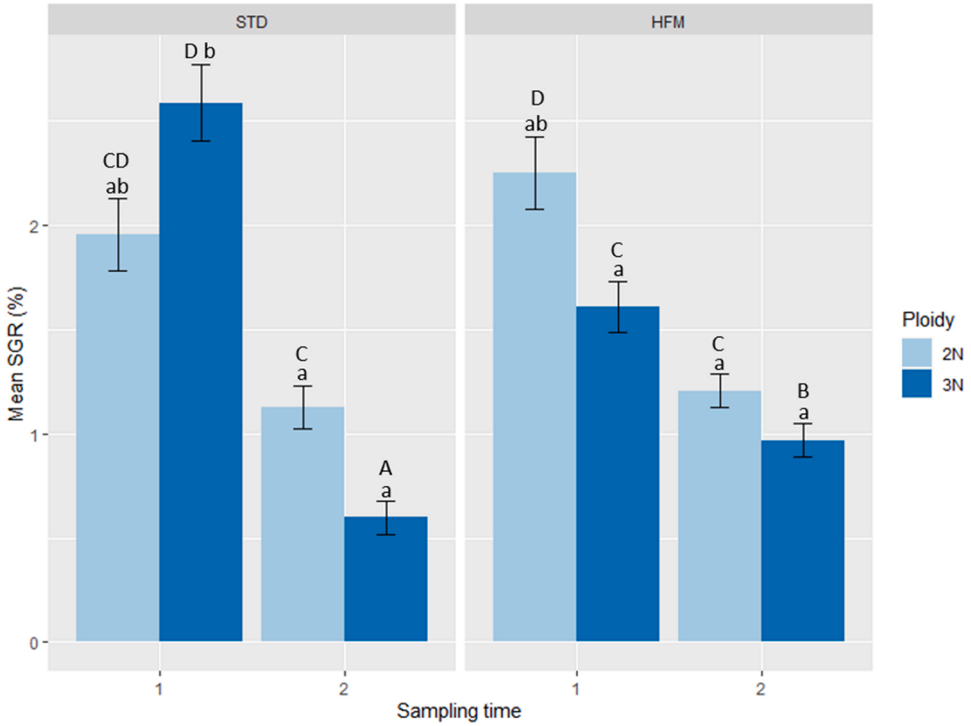

Fig. A1. Specific growth rate (SGR) of diploid (2 N) and triploid (3 N) fish fed fish meal (STD) and hydrolyzed fish protein (HFM) diets (G. Reisen, Skretting AS, Stavanger, Norway) during the two periods: 1: October-November (2454–2745 ddPSF); 2: November-December (2745–3044 ddPSF). Data are expressed as mean ± SE ($n = 30$). Different upper-case letters denote significant differences between sampling times, whereas lower-case letters within sampling times ($p < 0.05$).

related primarily to the parr-smolt transformation rather than being a response to the exposure of the fish to environmental stressors.

Heat Shock Proteins 70 (HSP70s) are highly conserved proteins that are used as stress biomarkers because of the rapid change that follows exposure of animals to stressors (Bertotto et al., 2011; Smith et al., 1999). In our study, HSP70 gene expression was not influenced by any of the factors considered (Fig. 5a), supporting the conclusions that the fish were not stressed by the rearing conditions applied in the study, and that the diets and environmental conditions were adequate for the promotion of growth and welfare of the triploid salmon.

Similarly, with regard to oxidative stress, muscle MDA was not affected by any of the three factors studied (sampling time, ploidy, diet) (Fig. 6a). In previous studies on Atlantic salmon, the oxidative stress response has been found to differ with development stage: in particular, juveniles and post-smolts seem to be susceptible, especially triploids in comparison to diploids (Sambraus et al., 2017). Formation of MDA may also be influenced by diet, with muscle MDA concentrations being higher in salmon fed fish oil-based feeds (209 nmol/g MDA) than those fed soybean oil (65 nmol/g) (Rørå et al., 2005), confirming that lipid oxidation increases in relation to muscle n-3 polyunsaturated fatty acid levels (Skonberg et al., 1994). The two diets tested in our study did not affect muscle lipid oxidation (i.e. MDA levels), possibly because they were formulated as 'low-lipid' feeds (ca 18–20% crude fat) and the same fish oil was used as the primary lipid source in the feeds (Table A1). In addition to malondialdehyde, we analyzed advanced oxidation protein products (AOPP) as a biomarker for protein oxidation. AOPP levels were significantly higher in November (2745 ddPSF) than in October (2454 ddPSF) and December (3044 ddPSF) (Fig. 6b). Few other studies have used AOPP as a biomarker in studies on fish (Florescu et al., 2021; Hermenean et al., 2015), and none have examined AOPPs in relation to feed type or ploidy. Therefore, it is difficult to discuss our results in a comparative context. However, Odei et al. (2020), compared the liver transcriptomes of three ontogenetic stages, i.e. fry, parr and smolt, of diploid and triploid fish sampled from some of the groups (STD diet only) we used. These authors examined a gene involved in oxidative stress (alcohol dehydrogenase 1-like, *adh 1*). In their comparison between parr and smolts, stages which would equate with our October and November samples, they observed an up-regulation of *adh 1* in triploid smolts. In our study of biomarkers of oxidative stress, we did not detect alterations based on ploidy, but rather on sampling time. Thus, our results are consistent for the biomarkers and physiological indicators we assessed: all data point to November being the critical month for the peak of the parr-smolt transformation of both the triploid and diploid salmon used in our study.

5. Conclusions

In our study, diploid and triploid salmon were reared at low temperature and fed on one of two high-protein phosphorus-rich diets. Assessment of physiological indicators following SWT and monitoring of a suite of biomarkers gave us good grounds to conclude that parr-smolt transformation was successfully completed by both the diploid and triploid salmon. Moreover, the triploid salmon showed similar growth and expression of biomarkers to their diploid counterparts, and there was no evidence that the feeding and rearing conditions applied triggered stress responses. In conclusion, our results on growth, stress and welfare biomarkers provide evidence that the triploid salmon were robust and rustic up to the smolt stage under the rearing conditions to which they were subjected.

Uncertainties regarding the performance and welfare of triploid salmon in seawater have hindered the widespread use of triploid salmon by the Norwegian salmon-farming industry, and there has been a temporary moratorium imposed on their production due to poorer survival, higher incidence of fish with skin damage and overall lower welfare scoring in triploids than in diploids under commercial settings (Madaro et al., 2021). In line with this, and other results on triploid salmon, future research should therefore focus on methods to improve the robustness of triploid fish aimed at safeguarding their performance and welfare following seawater transfer.

CRediT authorship contribution statement

Martina Bortoletti: Software, Formal analysis, Investigation, Data curation, Writing – original draft, Visualization. **Lisa Maccatrozzo**: Methodology, Software, Validation, Formal analysis, Resources, Data curation, Writing – review & editing, Visualization, Supervision. **Stefano Peruzzi**: Conceptualization, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Jo Espen Tau Strand**: Methodology, Validation, Formal analysis, Data curation, Writing – review & editing, Visualization. **Malcolm Jobling:** Writing – review & editing, Visualization. **Giuseppe Radaelli**: Conceptualization, Methodology, Resources, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition. **Daniela Bertotto**: Conceptualization, Methodology, Validation, Formal analysis, Resources, Data curation, Writing – review & editing, Visualization, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A

See Fig. A1, Tables A1 and A2.

Table A1
Formulation and chemical composition of the experimental (fish meal (STD) and hydrolyzed fish protein (HFM) diets (G. Reisen, Skretting AS, Stavanger, Norway).

	STD diet			HFM diet		
Pellet size (mm)	0.5/ 0.7/1.0	1.2	1.5/ 2.0/3.0	0.5/ 0.7/1.0	1.2	1.5/ 2.0/3.0
Ingredients						
Wheat	7.2	6.1	6.9	5.4	5.5	6.9
Wheat gluten	10	10	10	10	10	10
Soy Protein concentrate (SPC)	14.4	16.7	17.9	14	16.2	16.7
NA Fishmeal	55	55	50	30	30	27.5
CPSP[a]	0	0	0	25	25	22.5
Fish oil Nordic	11	10.8	11.6	9.4	9.2	10.2
Water/Moisture	0	0	0.4	1.6	0.9	1.5
Yttrium premix[b]	0	0	0.1	0	0	0.1
Premix (Min, Vit, AA)	2.4	1.4	3	4.7	3.3	4.6
Total	100	100	100	100	100	100
Chemical composition (%)						
Dry matter	7.9	7.5	7.2	7.9	7.5	7.1
Crude protein	55.9	56.8	56.0	60.3	59.0	56.9
Crude fat	17.7	18.3	19.1	17.3	18.8	19.6

[a] CPSP: Fish meal hydrolysate Special-G® (SoproPêche, Boulogne-sur-Mer, France).

[b] Marker Yttrium used in 3.00 mm diets only. Buffer capacity (mEq/ g needed to reach pH 3.0) was 1.3 and 0.9 in STD and HFM diets, the initial pH of these diets being 6.2 and 6.0, respectively.

Table A2
Mortality of diploid (2 N) and triploid (3 N) fish fed fish meal (STD) and hydrolyzed fish protein (HFM) diets (G. Reisen, Skretting AS, Stavanger, Norway) during the experimental trial (October-December).

Tank	Ploidy	Diet	Total Fish (Nr.)	Dead fish (Nr.)	Dead fish (%)	Dead fish (Mean %)
T1	2 N	STD	358	0	0	0
T2	2 N	STD	390	0	0	
T3	2 N	STD	351	0	0	
T4	2 N	HFM	368	0	0	0
T5	2 N	HFM	380	0	0	
T6	2 N	HFM	400	1	0	
T7	3 N	STD	370	6	2	1
T8	3 N	STD	353	0	0	
T9	3 N	STD	351	3	1	
T10	3 N	HFM	460	6	1	1
T11	3 N	HFM	421	4	1	
T12	3 N	HFM	363	2	1	

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