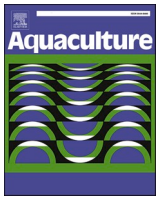
Aquaculture 582 (2024) 740305

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture



The impact of post-ovulatory ageing on the development of diploid and triploid Atlantic salmon (*Salmo salar* L.)

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ARTICLE INFO

Keywords:
Atlantic salmon
Triploid
Oocyte
Post ovulatory ageing
Egg quality
Proteomics

ABSTRACT

This study is the first to investigate the effects of post-ovulatory ageing (POA) on both diploid and triploid Atlantic salmon (*Salmo salar* L.). Following ovulation, female broodstock were partially stripped periodically (at 0, 5, 10, 15 and 20 days post-ovulation, DPO), creating five groups of ova with differing durations held in the body cavity. Survival and bone architecture were negatively impacted by increasing POA, irrespective of ploidy, and an increase in individual growth variation was observed, particularly in triploids. Several ovarian fluid and egg quality parameters were screened to determine potential prospective biomarkers that may predict the future success of an egg batch. Ovarian fluid osmolality and egg lipid composition, vitamin E concentration and TBARS emerged as good candidates. Furthermore, significant changes in abundance of 19 proteins involved in cell organisation, metabolism and reproduction were observed at different stages of POA. Also, a suite of genes involved in energy metabolism, apoptosis and cell cycle regulation showed significantly reduced expression in eggs at 20 DPO compared to 0 DPO. The data show that increasing POA negatively impacts egg survival and future growth and increases deformity prevalence. Several potential biomarkers show promise and should be investigated further. Although egg quality appears to remain stable up to 10 DPO, the data suggests that fertilising eggs within 5 DPO would reduce the variation in growth, thus improving future performance potential and further benefitting stock management of both diploid and triploid Atlantic salmon.

1. Introduction

Optimal egg quality is a fundamental biological requirement for survival and normal development of any vertebrates. Fish oocytes are provided with maternal reserves that are used during embryonic development until newly hatched larvae can acquire exogenous food sources. The process of egg degradation from prolonged over-ripening is also known as post-ovulatory ageing (POA) and can lead to decreased viability of fish oocytes (Craik and Harvey, 1984). Unlike other species, salmonid eggs are able to remain in the body cavity for a period of less than one week (rainbow trout, Lahnsteiner, 2000) to 20 days (coho salmon, Fitzpatrick et al., 1987) depending on broodstock holding temperature with little impact on egg quality and fish survival, growth and deformity (Bromage et al., 1992; Aegerter and Jalabert, 2004; Mommens et al., 2015). However, this window can be as little as <1 h to few hours in marine fish species (Migaud et al., 2013). Beyond these windows, oocytes undergo compositional changes in response to POA as previously described in several salmonid species including *Oncorhynchus mykiss* (Craik and Harvey, 1984; Springate et al., 1984; Lahnsteiner, 2000; Aegerter and Jalabert, 2004; Rime et al., 2004), lake trout (*Salmo*

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https://doi.org/10.1016/j.aquaculture.2023.740305
Received 29 April 2023; Received in revised form 27 October 2023; Accepted 30 October 2023
Available online 3 November 2023
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trutta lacustris) (Lahnsteiner et al., 1999) and Atlantic salmon (*Salmo salar*) (Mommens et al., 2015). Despite the extended window during which egg quality can remain optimal in salmonids while retained in the body cavity, POA remains operationally problematic in salmonid hatcheries and can lead to significant reductions in productivity as broodstock require stripping. Unlike in the wild, domesticated Atlantic salmon do not spawn naturally as environmental and social cues are lacking. While farmers regularly check fish for signs of ovulation, if the number of dams to assess is high, it is likely that a proportion of ovulated dams may not be stripped on a particular day. Temperature is the main factor controlling time of ovulation (Taranger and Hansen, 1993; Vikingstad et al., 2016). Delayed stripping may lead to POA of the eggs resulting in a significant reduction of egg quality, fertilisation and hatching success (Bromage et al., 1992; Aegerter and Jalabert, 2004; Aegerter et al., 2005; Taylor et al., 2011; Mommens et al., 2015), increase in deformity prevalence (Taylor et al., 2011; Mommens et al., 2015) and spontaneous occurrence of triploidy (Aegerter and Jalabert, 2004; Nomura et al., 2013; Glover et al., 2015; Jørgensen et al., 2018). Understanding the impact of POA on salmonid hatchery performance, welfare and long-term fish robustness is especially relevant to the production of sterile, triploid stocks which add an additional handling and stress to newly stripped eggs. New biomarkers of POA and egg quality would help to define ploidy-specific ripening thresholds and improve stripping protocols for both diploids and triploids and subsequently lead to reduced mortality and enhanced performance.

Historically, triploid Atlantic salmon have shown highly variable performance with low survival and poor growth in some cases (Galbreath et al., 1994; McGeachy et al., 1995; O'Flynn et al., 1997). However, with the implementation of triploid-specific rearing conditions, more recent trials have reported survival and growth rates comparable to, or better than their diploid siblings (Oppedal et al., 2003; Burke et al., 2010; Taylor et al., 2011, 2013, 2014; Fraser et al., 2013; Benhaïm et al., 2020). However, as reported recently, triploid welfare remains a concern in commercial conditions (Madaro et al., 2021). A high prevalence of skeletal deformities has also been observed in triploid fish (Sutterlin et al., 1987; Sadler et al., 2001; Fjelldal and Hansen, 2010; Taylor et al., 2011). Although not fully resolved, more recent research has shown that the prevalence of deformities can be significantly reduced, supporting the concept that culture requirements of triploids are different than their diploid siblings. For example, nutritional studies have demonstrated that triploids have a greater requirement for dietary phosphorous (Fjelldal et al., 2015; Smedley et al., 2016; Smedley et al., 2018; Vera et al., 2019) and dietary histidine (Taylor et al., 2015; Sambraus et al., 2017) to reduce the occurrence of spinal malformation and cataracts, respectively. Furthermore, the incidence of jaw and spinal deformities in triploids are positively correlated with increasing egg rearing temperature (Fraser et al., 2014, 2015; Amoroso et al., 2016; Clarkson et al., 2021) and exposure to temperature shift during embryogenesis (Clarkson et al., 2021) as also shown in diploids (Takle et al., 2005). This highlights the increased sensitivity of triploids to production stressors especially during embryogenesis which may result in increased mortality and poorer growth performance (Fraser et al., 2012). Lowering the egg incubation temperature from 8 to 6 °C was shown to reduce skeletal deformities in triploids, however prevalence still remains higher than in diploids (Fraser et al., 2015).

The lack of apparent reliability in performance of triploids raised concerns regarding their stock management (Taylor et al., 2011). Variability in survival rates of triploids during early embryogenesis and later during production (O'Flynn et al., 1997; Cotter et al., 2002) coupled with a higher variation in growth during the production cycle may be related to egg quality. The current protocol for inducing triploidy requires increased handling of the oocytes during a vulnerable developmental stage, which may increase egg drop out and reduce robustness during later development. Furthermore, if the eggs used for triploid induction are already of "sub-optimal' quality, the process of triploidisation will likely exacerbate the negative impacts on performance. Investigations in triploid Atlantic salmon have generally used egg batches collected towards the end of the stripping season when the quality of the oocytes are usually reduced, keeping eggs obtained during the optimal window for commercial supply (Taylor et al., 2011). Another important factor that has not been studied yet is the effects of POA on triploid survival, yield and long-term development. In diploids, an increase in mortality and prevalence of abnormality was correlated with POA in Atlantic salmon (Mommens et al., 2015). Furthermore, if a proportion of eggs are already spontaneously triploid as a result of POA, subjecting them to triploid induction will likely cause mortality. It is therefore important to understand the ploidy-specific thresholds of eggs exposed to POA at this early stage.

Egg quality indicators have been investigated for several fish species (Migaud et al., 2013; Samarin et al., 2015), however, reliable biomarkers that can be applied early during embryogenesis are still lacking in fish and the impact of POA remains poorly understood. Reliable indicators of egg quality are likely species-specific as the incubation periods and ovulatory processes differ between species (Shields et al., 1997). Reading et al. (2018) and Samarin et al. (2019a) reviewed gene transcripts and proteins identified in fish in relation to egg quality. More recently, a study analysed maternal transcript profiles associated with rainbow trout egg viability and showed maternal transcripts to be differentially expressed between high and low quality eggs. However, variability between eggs of similar quality prevents transcript abundance to be used as a reliable predictor of egg quality (Weber et al., 2021). In rainbow trout, POA eggs showed differences in expression of transcripts associated with cell cycling, chromatin remodelling and IGF1 (Aegerter et al., 2005). Other studies compared the proteomic profiles between good and poor quality eggs and suggested a number of potential protein egg quality markers. In zebrafish, poor eggs were shown to be deficient in proteins involved in protein synthesis, energy and lipid metabolism, as well as vitellogenin and lectins, while showing an overabundance of proteins involved in *endo*-lysosomal activities, autophagy, and apoptosis, and some egg envelope proteins (Yilmaz et al., 2017). When studying the effect of POA on egg quality in rainbow trout, Rime et al. (2004) showed accumulation of proteins in the coelomic fluid during POA which could also be used as biomarkers of egg quality in relation to POA. Despite these studies, there is no consensus on the use of reliable markers of POA which are most likely species specific.

The main aims of the present study were (i) to investigate the effects of POA on survival, growth and deformity prevalence in diploid and triploid Atlantic salmon during embryogenesis and development to parr stage, (ii) to correlate these physiological traits with biochemical, proteomic and molecular changes in the eggs (and ovarian fluid) and (iii) to identify potential prospective biomarkers, at early developmental stages, of egg quality and future performance of diploids and triploids.

2. Materials and methods

2.1. Fish stock and culture conditions

Broodstock stripping and subsequent incubation in the hatchery was carried out at Landcatch Natural Selection (Ormsary, Scotland). Female broodstock (2-sea winter, 10.9 ± 1.4 kg, $n = 10$) reared under ambient conditions, that all ovulated within 24 h, were isolated on 22nd November 2017 and held in separate tanks. After sedation (Tricaine, Pharmaq; 50 ppm), eggs were partially stripped (~300 ml newly stripped eggs (~2500 eggs) known as "green eggs") from five 'experimental' dams on increasing days post-ovulation held in the body cavity (0, 5, 10, 15 and 20 days post ovulation; DPO). Five additional 'control' dams were fully stripped after 20 DPO to assess the impact of the partial stripping in the experimental dams. Milt from one sire (to minimise paternal influence on development) was initially collected when the dams first ovulated (0 DPO), aliquoted into 1 ml portions and stored at 4 °C with Ringer solution (0.5 ml Ringer 1 ml milt^{-1}) until use on subsequent stripping dates (Ginsburg, 1963). Milt motility was

determined under microscope before each fertilisation event. A *second* sire was stripped at 20 DPO and used to fertilise subgroups of the control eggs to assess any potential impact of milt quality deterioration over 20 days storage in the original sire. Eggs from the control dams were not triploidised.

Following stripping of the dams at respective time points, every egg group (~300 ml) was fertilised (30 s. mixing 1 ml milt, 60 s. rinse with 8 °C freshwater) using the same non-related sire, creating half-sib families. After initial fertilisation, each egg batch was divided volumetrically in two (~150 ml) and placed into a water bath at 8 °C prior to triploid induction. Triploidy was induced in one group (655 bar of hydrostatic pressure for 6.25 mins. at 8 °C, 37.5 mins. Post-fertilisation) according to Fjelldal and Hansen (2010), while the others were handled in the same way but did not receive a hydrostatic shock and were maintained as diploid controls. The procedure was repeated for each dam and at each stripping date. Following water hardening (~1 h), individual egg groups were disinfected (Buffodine, Europharma, 1:100) for 10 mins. Before laying down. Egg batches were then incubated separately in 6 L silos (15 ml sec^{-1} flow) in darkness under ambient conditions (3.5 ± 1.4 °C).

Eggs were transferred to the Temperate Aquarium Facilities (IoA, Stirling, UK) at 320 degree days (°days) where the second phase and remainder of the experiment took place. Egg groups from 0, 5, 10 and 15 DPO were used for continuing investigations, while, groups stripped after 20 DPO were not kept for further monitoring due to insufficient numbers resulting from very low survival rates (<10% at egg shocking, data not shown). However, egg and ovarian fluid samples to assess quality parameters were still collected for all five DPO groups as described later. Eggs from the five families (dams) were pooled for each DPO (except for 20 DPO) and triplicated creating 24 experimental tanks (0.3 m^2) with 250 eggs each ($n = 3$; 50 $family^{-1}$ DPO^{-1} $ploidy^{-1}$). Again, resulting from a higher mortality, fewer eggs were present in the 15 DPO tanks ($n = 3$, 210 and 110 for diploidsand triploids, respectively). Tanks were kept under 24 h light at 6.0 ± 0.2 °C according to Clarkson et al. (2021) until hatch in a recirculating tank system. The temperature was increased gradually to 7.7 ± 1.6 °C after hatch (~ 500°days post fertilisation) and then to 13.0 ± 0.9 °C after first feeding (~900°days post fertilisation) and maintained until the end of the experiment at ~1850°days post fertilisation (~5 g) for final assessment. Fish were fed to satiation ploidy-specific diets (Inicio Plus and Tri-X for diploids and triploids, respectively) according to manufacturer's guidelines (BioMar Ltd., Grangemouth, Scotland).

2.2. Sampling procedures

The experiment is split into two phases, from newly stripped eggs to eyed eggs (320°days post fertilisation, 5 dams × 5 DPO × 2 ploidy) and then transfer of eyed eggs to parr (1850°days post fertilisation, 3 replicates × 4 DPO × 2 ploidy).

Ovarian fluid was collected from each dam (x5) at each DPO (x5) (25 samples) into 1 ml aliquots and immediately snap frozen on liquid nitrogen and stored at −70 °C for later analyses of osmolality, mineral composition and proteomic analysis. Newly stripped eggs were also collected from each dam (x5) at each DPO (x5) (total of 25) for proximate analyses (30 ml, ~250 eggs) and aliquots of 7 ml for analyses of carotenoid composition, vitamin E content, TBARS, fatty acid and lipid class composition. Samples were again immediately snap frozen on liquid nitrogen, and stored at −70 °C until later processing. Six stripped eggs from each dam (x5) at each DPO (x5) (total of 150 eggs) were collected in RNAlater® for gene expression analysis. After 20 DPO, the five dams were sacrificed (Tricaine, Pharmaq; 1000 ppm) and body weight (BW) and fork length (FL) were recorded.

A random sample of eggs (50 $silo^{-1}$, 5 dams from each of the 5 DPO and 2 ploidy) was assessed for fertilisation success at 120°days post-fertilisation by immersing eggs in clearing solution (methanol: acetic acid: water; 1:1:1, v:v) for 2 mins. Eggs which presented neural tube formation were classified as fertilised. Viable eggs were determined after mechanical shock at 300°days. Dead eggs were counted and removed, and the rest used to determine survival rates. Egg diameter was determined at the point of transfer to the experimental tanks (320°days). Photographs were taken and measurements were made using Fiji image analysis software (ImageJ).

2.3. Verification of ploidy

To confirm ploidy status of triploidised groups and potential spontaneous triploidy in diploid groups, red blood smears were prepared from samples taken from the caudal peduncle of euthanised fish (20 fish DPO^{-1} $ploidy^{-1}$; 6.5 ± 1.4 g). Air dried slides were fixed in 100% methanol and then placed into Giemsa stain for 10 mins. Slides were digitised using a slide scanner at 20× magnification (Axio Scan.Z1, Zeiss) and erythrocyte length and diameter was determined by Fiji image analysis software (ImageJ). A total of 30 randomly chosen nuclei per slide were measured to the nearest 0.01 μm and a mean taken for presumed diploid and triploid fish.

2.4. Ovarian fluid pH, conductivity and osmolality

Ovarian fluid pH (FiveGo F2, Mettler Toledo, Leicester, UK) and electric conductivity (EC; pHOX 52) were measured in situ immediately after the dams were stripped (5 dams × 5 DPO, 25 samples). One aliquot of ovarian fluid from each dam at each DPO was defrosted and osmolality was determined from an average of 3 × 200 μl samples according to manufacturer's guidelines (3250 Single Sample Osmometer; Thermo Fisher).

2.5. Newly stripped egg and ovarian fluid biochemical analyses

2.5.1. Mineral composition

Mineral compositions were determined on eggs and ovarian fluid from all dams (x5) at each DPO (0, 5, 10, 15 and 20 DPO) using the nitric acid (HNO_3) digestion technique (Clarkson et al., 2021). Briefly, egg samples were oven dried at 75 °C for 24 h and subsequently powdered using a mortar and pestle. Ovarian fluid samples were analysed wet. Three runs of 0.1 g samples of eggs and ovarian fluid from each dam at all DPO were digested in Kheldal digestion tubes with 69% nitric acid using a MARS microwave digestion system (CEM MARSXpress, CEM ltd., Buckingham, UK) using the following program; 10 mins heating phase to 190 °C, maintain 190 °C for 20 mins, cooling phase to 21 °C for 60 mins. Samples were then diluted with distilled water to 2% HNO_3 and analysed for mineral content via an Inductively Coupled Plasma Mass Spectrometry (ICP-MS; iCAP RQ; Collision cell technology).

2.5.2. Proximate composition

Proximate composition of newly stripped eggs was determined according to standard procedures (AOAC, 2000). Moisture content was determined after drying in an oven at 110 °C for 24 h and ash content determined after incineration at 600 °C for 16 h. For protein analysis, eggs were homogenised in a blender (Waring Laboratory Science, Winsted, CT, USA) and crude protein content was measured by determining nitrogen content (N × 6.25) using automated Kjeldahl analysis (Tecator Kjeltec Auto 1030 analyser, Foss, Warrington, U.K). Energy content was measured using bomb calorimetry calibrated with benzoic acid (Gallenkamp Autobomb, Gallenkamp & Co. Ltd., London, UK). Total lipid was extracted from eggs according to Folch et al. (1957). Approximately 0.25 g of eggs were homogenised in 10 ml chloroform: methanol (2:1, v:v) using an Ultra-Turrax tissue disrupter (Fisher Scientific, Loughborough). Lipids were separated from contents by adding 5 ml of potassium chloride (KCl; 0.88% w:v) and left on ice for 1 h. The upper layer was aspirated, and the lower layer was dried under nitrogen. Total lipid content of each sample was determined gravimetrically after 12 h in a vacuum desiccator.

2.5.3. Fatty acid composition

Fatty acid methyl esters (FAME) were prepared from total lipid by acid-catalysed transesterification at 50 °C for 16 h (Christie, 2003), and FAME extracted and purified as described previously (Tocher and Harvie, 1988). FAME were separated and quantified by gas-liquid chromatography using a Fisons GC-8160 (Thermo Scientific, Milan, Italy) equipped with a 30 m × 0.32 mm i.d. × 0.25 μm ZB-wax column (Phenomenex, Cheshire, UK), on-column injector and a flame ionisation detector. Data were collected and processed using Chromcard for Windows (version 2.01; Thermoquest Italia S.p.A., Milan, Italy). Individual FAME were identified by comparison to known standards and published data (Tocher and Harvie, 1988).

2.5.4. Lipid classes

Lipid class composition was determined by high-performance thin-layer chromatography (HPTLC) using 20 × 10 cm plates (VWR, Lutterworth, England). Approximately 2 μL of total lipid alongside polar lipid and neutral lipid standards were applied as single spots. Plates were first left in a polar solvent; methyl acetate: isopropanol: chloroform: methanol: 0.25% aqueous KCl (25:25:25:10:9, by vol) until solvent had reached halfway up the plate. After drying for 15 mins the plate was fully developed in a neutral solvent; isohexane: diethyl ether: acetic acid (85:15:1.5, by vol.). The lipid classes were visualised by charring at 160 °C for 25 mins after spraying with 3% (w:v) aqueous cupric acetate containing 8% (v:v) phosphoric acid and quantified by densitometry using a CAMAG-3 TLC Scanner (VWR International, Dorset, UK) (Henderson and Tocher, 1992). Scanned images were recorded automatically and analysed by computer using winCATS Planar Chromatography Manager (version 1.2.3).

2.5.5. Carotenoid contents

To assess total carotenoid content and composition of the samples, 1 g of eggs was homogenised and carotenoids were extracted in three-phases using ethyl acetate: ethanol (1:1, v:v), ethyl acetate, and isohexane. After evaporation, samples were re-suspended in isohexane and the absorbance measured on a spectrophotometer (CE 2021 2000 series, Cecil Instruments Ltd., Cambridge, UK) at 470 nm against an isohexane blank. All samples were subsequently transferred to auto-sampler vials and processed through high performance liquid chromatography (HPLC; Waters Alliance System, Waters Corporation, MA, USA). Identification of carotenoids present was determined using retention time against a known standard of astaxanthin and canthaxanthin. To quantify the outputs, the area of each peak was measured using Empower 2 chromatographic processing software (Waters Corporation, MA, USA).

2.5.6. Vitamin E contents

Vitamin E (α-tocopherol) was extracted and determined by reverse phase HPLC (Waters Alliance System, Waters Corporation, MA, USA) with UV detection at 293 nm according to Cowey et al. (1981). 1 g of eggs was homogenised in 5 ml of 2% ethanolic pyrogallol. The homogenates were incubated in a water bath of 70 °C for 5 mins. and then 1 ml of 60% KOH was added and mixed. Samples were incubated at 70 °C for a further 20 mins. With regular mixing in 5 min intervals. On removal, samples were cooled on ice and 4 ml distilled water and 6 ml isohexane (+ BHT) was added. Each tube was vortexed for exactly 1 min to allow for extraction of the vitamin E into the isohexane layer. Layer separation was encouraged by incubating the samples at −20 °C for 45 mins 4 ml of the isohexane layer was transferred to fresh vials and completely evaporated under nitrogen. Samples were re-dissolved in 1 ml methanol and analysed using HPLC. Vitamin E content of each sample was determined against a known standard using Empower 2 chromatographic processing software (Waters Corporation, MA, USA).

2.5.7. Peroxidative stress (TBARS)

The measurement of thiobarbituric acid reactive substances (TBARS) was carried out using a method adapted from Sorensen and Jorgensen (1996). Briefly, 1 g of eggs was homogenised in 15 ml of 7.5% trichloroacetic acid (TCA). The homogenate was filtered to remove protein precipitates and 2 ml transferred to a fresh vial. 2 ml of thiobarbituric acid (TBA) was added and mixed before heated at 100 °C for 35 mins. Samples were analysed with a spectrophotometer (CE 2021 2000 series, Cecil Instruments Ltd., Cambridge, UK) at 532 nm against a positive and a negative control.

2.6. Gene expression

A suite of genes were selected to study the effects of DPO on energy metabolism (atp5f1, atp5f1c, and coxII), oxidative stress (sod1, sod2, cata, hsp70), cell cycle regulation (calm2, mkk6a, tbb5, and ccnb3) and apoptosis (bclx). These gene targets were selected based on available literature (Xu et al., 1997; Gordo et al., 2002; Takle et al., 2005; Lord and Aitken, 2013; Chapman et al., 2014; Aegerter et al., 2005; Samarin et al., 2019a).

2.6.1. RNA extraction and cDNA synthesis

Freshly stripped eggs (6 eggs × 5 dam × 5 DPO) from each experimental group (0, 5, 10, 15, and 20 DPO) were added to TRIzol® (Invitrogen, UK) at a ratio of 100 mg/ml reagent according to the manufacturer's protocol. Total RNA (totRNA) concentration was determined using a Nanodrop spectrophotometer (ND-1000; Labtech Int., East Sussex, UK) and quality of samples was confirmed by assessing the integrity of 28S and 18S ribosomal RNA (rRNA) with agarose gel electrophoresis (1%). cDNA was reverse transcribed from 1 μg of total RNA using QuantiTect Reverse Transcription kit (Qiagen Ltd., Manchester, UK). The resulting cDNA was diluted 20-fold with nuclease-free water.

2.6.2. Sequence information and primer design

Sequence-specific primers for genes were designed using the software PRIMER3 (Untergasser et al., 2012) and then subjected to BLAST analysis against an Atlantic salmon genome and transcriptome (National Centre for Biotechnology Information; www.ncbi.nlm.nih.gov) (Table 1). Only primer pairs with no unintended targets were selected and then manufactured by MWG Eurofins Genomics (Ebersberg, Germany). The efficiency of the primers was verified and validated by performing standard curves for all genes investigated.

2.6.3. Quantitative PCR (qPCR)

Relative quantification (to 0 DPO) qPCR assays were designed and performed in accordance with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Stephen et al., 2009). qPCR was performed in duplicate on individual samples using a Lightcycler® 480 II (Roche Diagnostics, West Sussex, UK) with relevant primer pairs, cDNA template, nuclease-free water, and Luminaris™ Color HiGreen qPCR Master Mix (Applied Biosystems, UK) in a total reaction volume of 10 μL. Amplification was achieved in 384-well plates and conducted in a thermo cycling programme as follows; 50 °C for 2 mins., 95 °C for 10 mins. Followed by 40 cycles of; 95 °C for 15 s., TA °C for 30 s., and 72 °C for 30 s. This was followed by a temperature ramp from 70 to 90 °C for melt-curve analysis to verify that no primer–dimer artefacts were present and only one product was generated from each qPCR assay. Relative expression of target genes was calculated by the ΔΔCt method (Pfaffl, 2001) using *b2m*, *ef1a* and *rpl1* as the reference genes, which were chosen as the most stable according to RefFinder (Xie et al., 2012).

2.7. Proteomic analysis of ovarian fluid

2.7.1. Sample preparation

Ovarian fluid samples from the five dams at each DPO (0, 5, 10, 15 and 20 DPO) were thawed on ice and protein content was determined by BCA assay (Interchim Uptima, France) using Bovine Serum Albumin (BSA) (stock 2 mg ml^{-1}) as standard. To check the quality and integrity

Table 1
Primer sequences of target genes and associated information used for real time qPCR mRNA expression.

Gene	Primer sequence 5′ – 3′	T_A °C	Accession no.	Reference
cata	F: GTTCTAACGTGTTCCCTGCCCAT R: TGGCATGGACAATTCAGACAGCA	60 °C	NM_001140302.1	New design
hsp70	F: GGTCCTGGTGAAGATGAGGGAGA R: GTGGCCTGTCTCTGTGAATCGTT	58 °C	AJ632154.1	New design
sod1	F: GTCGGAAGACTGGCAACGCT R: TCGGGGGTAAGCTACGGTGG	60 °C	NM_001123587.1	New design
sod2	F: GTGCGTGATGACAGTGCACATA R: CAAGACTGGGCACACTCGGA	60 °C	XM_014145196.1	New design
ccnb3	F: TCTGGGTACGGTGTGTCAGA R: CGGAATTGTCGCGACTTCAA	56 °C	NM_001140296.1	New design
calm2	F: GGGGTTGGAGAGCGCTTAAT R: CATAGCTGCCCCAAGTGACA	57 °C	NM_001139713.1	New design
mkk6a	F: ATGAGACATGTGCCCAGTGG R: TGAAGAAGCAGTCCACGGTC	57 °C	NM_001123709.1	New design
tbb5	F: AGGGAGAGGTCTCGGAATTGA R: ACACACAAATTTGCTGAGGTTCC	57 °C	NM_001139793.1	New design
bclx	F: TTGCCGTTGACTAGGGCCTG R: TCGTGACACACCCCTTCGGA	58 °C	NM_001141086.1	New design
coxII	F: ACGCCCGGTCAATTTCGTCT R: AGGGACGGCTCAGGAGTGAA	57 °C	L04501.1	New design
atp5f1c	F: TGGTGTTTGAGCATAAAGCTAGGC R: ACAGCCTATATTTGGCAGTGCAT	57 °C	NM_001139678.1	New design
atp5f1	F: CCCCAGCAGGAGAAAGCGAG R: GGGTTCAGTGGCGACGGTCTA	60 °C	NM_001139691.1	New design
b2m	F: TCCCAGACGCCAAGCAG R: TGTAGGTCTTCAGATTCTTCAGG	60 °C	BT046451.2	Carmona-Antoñanzas et al. (2015)
ef1a	F: CACCACCGGCCATCTGATCTACAA R: TCAGCAGCCTCCTTCTCGAACTTC	60 °C	DQ834870	Ytteborg et al. (2010)
rpl1	F: ACTATGGCTGTCGAGAAGGTGCT R: TGTACTCGAACAGTCGTGGGTCA	60 °C	NM_001140826.1	Carmona-Antoñanzas et al. (2015)

TA: annealing temperature.

of the fluid proteins, 10 μg of protein from each sample were visualised by 1-Dimensional Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (1D SDS-PAGE gel) (data not shown). Protein samples (100 μg) were then processed for protein digestion. The following steps, protein reduction, alkylation, precipitation, trypsin digestion and TMT labelling of peptides were performed following manufacturer instructions (TMT10plex™ Isobaric Label Reagent Set, ThermoFisher Scientific). Each labelling group consisted of ten study samples and one pooled internal standard (^{131}C label), which was generated by pooling the same amount of protein from all the samples included in the study. After digestion, peptides were cleaned-up using Hypersep SpinTip (ThermoFisher Scientific), according to manufacturer instructions. Finally, samples were dried using a vacuum drier (Savant DNA SpeedVac 110, Thermo Scientific).

2.7.2. LC MS/MS analysis of TMT

Multiplexed samples were injected into a Dionex Ultimate 3000 RSLS nanoflow system (Dionex, Camberly, UK) set up with a Dionex 0.1 × 20 mm 5 μm C18 nano trap column. Mobile phase was 2% acetronitrile (ACN), 0.1% formic acid (FA) and the analytical column was an Acclaim PepMap C18 nanocolumn (75 μm × 50 cm, 2 μm, 100 Å). Peptides were eluted at a flow rate of 300 nl min^{-1} using a 1% B (80% ACN:01% FA) for 5 min rising to 5% B at 10 min then to 25% B at 360 and 65% B at 480 min. The column was then washed and re-equilibrated prior to each sample injection. Ionisation voltage was set at 2.6 kV and the capillary temperature was 275 °C. Precursor resolution was 60,000 and 7500 for higher-energy collisional dissociation (HCD) fragments. Mass spectrometry analysis was performed in an Orbitrap LTQ Velos (Thermo Finnigan, Bremen, Germany) scanning from 380 to 2000 *m/z*. The top 20 multiply charged ions were selected from each scan for MS/MS analysis using HCD at 35% collision energy. Dynamic exclusion was enabled with a repeat count of 1, exclusion of 30 s.

2.7.3. LC MS/MS data analysis and sequence annotation for TMT

Raw files were analysed using Proteome Discoverer 1.4 software package (Thermo Finnigan, San Jose, CA) using the Sequest search algorithm and the Uniprot Atlantic salmon (*Salmo salar*) database downloaded in March 2019. The following search parameters were applied: (i) precursor mass tolerance: 10 ppm and fragment mass tolerance: 0.05 Da; (ii) full tryptic digestion; (iii) max missed cleavage sites: 2; (iv) static modifications: carbamidomethylation of cysteine and TMT tags on lysine residues and peptide N termini; (v) dynamic modifications: oxidation of methionine. Only unique peptides identified with high-confidence were used for quantification ($q < 0.01$). Data across samples was normalised based on protein median. Data is reported as fold changes. For protein assignment, peptides were processed using a clustering approach that allows combining the data sets acquired in the course of multiple MS runs. This approach groups features that are defined by calibrated elution time and mass into clusters, followed by sequence assignment (Lygirou et al., 2018).

2.8. Fish performance and deformity

Assessment of first feeding fry was conducted at ~850°days post-fertilisation for each DPO. Fish (n = 3, 30 individuals replicate^{-1} DPO^{-1} ploidy^{-1}) were sedated (Tricaine, Pharmaq; 50 ppm) and measured for body weight (BW) and fork length (FL). At 1850°days post-fertilisation (end of trial), all fish (n = 3, 80 to 217 fish per tank) were sacrificed following a 24 h fast using an overdose of anaesthetic (Tricaine, Pharmaq; 1000 ppm) and a subsequent blow to the head. BW and FL were measured, and growth rate was calculated using the thermal growth coefficient (TGC, % BW °C d^{-1}). Random samples (n = 3, 30 fish replicate^{-1} DPO^{-1} ploidy^{-1}) were frozen flat at −20 °C until later radiological deformity analysis. Right lateral radiographs were taken of randomly selected parr (30 replicate^{-1} DPO^{-1} ploidy^{-1}) using a Faxitron UltraFocus Digital Radiography System (Faxitron Bioptics LLC., Arizona, USA) exposing individuals for 1.8 mA at 26 kV. Radiographs were digitalised (AGFA CR35-X) and subsequently examined using ClearCanvas Workstation (Personal Edition, Synaptive Medical, Toronto, Canada) by two independent evaluations.

2.9. Statistical analysis

All data were analysed using Minitab statistical analysis software (Version 18.0, Minitab Inc., Pennsylvania, USA). Percentage data were arcsine transformed to meet model requirements. A One-Way ANOVA was used to compare the difference in egg viability and fertilisation rate from both experimental and control broodstock. Linear mixed effect (LME) models were used to assess the impact of both ploidy and POA on egg performances, and POA on ovarian fluid, egg quality parameters and gene expression data, using the stripped dam as the random effect, accounting for any genetic influence on parameters investigated. Grow-out performance data on pooled families (mortality, growth, deformity prevalence) were analysed with a Two-Way ANOVA incorporating POA and ploidy and the interaction between them. Normality and homogeneity of variance in the data were confirmed using Kolmogorov-Smirnov and Levene's tests, respectively. Post-hoc tests were determined by Tukey's multiple comparisons. Differentially expressed proteins were identified using the following criteria: (1) fold change value >1.2 or < −1.2 with a (2) a p value <0.05 following t-test. Correlations were determined by Pearson's correlation coefficient and expressed as r^2. All significance was accepted at $p < 0.05$.

3. Results

3.1. Triploid induction success and spontaneous triploidy

Diploid groups at 0, 5 and 10 DPO had significantly smaller erythrocyte nuclear lengths, with no overlaps with the pressure shocked triploid groups (2 N, 6.5 ± 0.6 μm; 3 N, 9.6 ± 0.7 μm) confirming that all analysed eggs subjected to hydrostatic pressure shock developed as triploid fish (data not shown). No diploid groups at 0, 5 and 10 DPO had any occurrence of spontaneous triploidy however at 15 DPO, 6.7% of 'diploids' experienced spontaneous triploidy (no assessment could be done in 20 DPO fish due to high egg drop out).

3.2. Impact of partial stripping and milt motility on oocyte viability and fertilisation

No difference was found in fertilisation rates and survival of eggs between the experimental and control dams that had been crossed with the original sire (Control A) (Fig. 1). A significantly higher fertilisation rate was observed in the control groups when using the fresh milt from the second sire (Control B). This coincides with a reduction in milt motility from 100% at 0 DPO to 25% at 20 DPO (data not shown). However, this did not affect the survival as all eggs that had spent 20 days in the body cavity had comparable survival rates.

3.3. Impact of POA on oocyte survival, fertilisation and size

There was an overall higher survival rate in diploid eggs compared to triploid eggs during the periods between fertilisation and mechanical shock ($p = 0.002$) and between transfer and hatch ($p = 0.034$) (Table 2a). There was also an overall POA effect during both periods as survival significantly decreased between 0 and 20 DPO and 0 to 15 DPO ($p < 0.001$). Both ploidy responded the same to increasing POA during the period between fertilisation and mechanical shock, however a ploidy and POA interaction was observed between transfer and hatch ($p = 0.020$). In diploids, survival was not affected by increasing POA, however, triploids had a greater survival in 0 and 5 DPO groups compared to 10 DPO. Also, triploid egg survival during this period was significantly greater at 0 DPO compared to 15 DPO. No dam effect was observed in survival during either period.

Triploids had an overall smaller green egg diameter compared to diploid siblings ($p = 0.005$) and a significant increase in egg diameter was observed in response to POA ($p < 0.001$) (Table 2b). Fertilisation success was also significantly reduced in triploids compared to diploids ($p = 0.008$) and a significant decrease was observed in response to POA ($p < 0.001$). No dam effect was observed for both egg diameter and fertilisation.

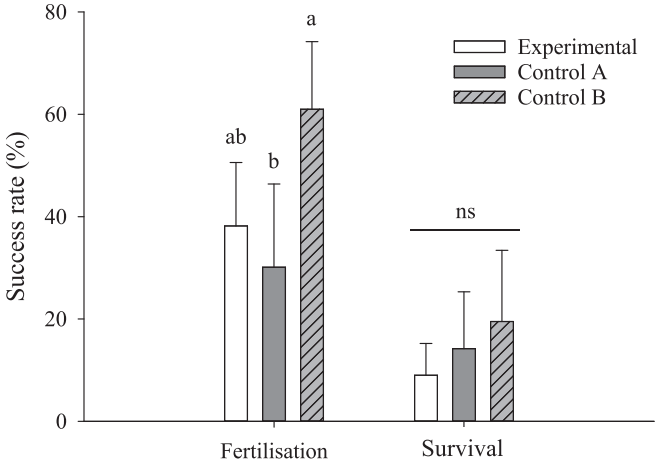

Fig. 1. Impact of partial stripping done in the main study on 5 dams and milt motility from stored milt for 20 DPO on fertilisation success and egg survival after 20 DPO in diploid egg groups from experimental (5 dams from the main study partially stripped at 0, 5, 10, 15 and 20 DPO) and control (5 additional dams only stripped at 20 DPO). Data are presented as means ± SD (5 dams) and significant differences are denoted by different superscripts ($p < 0.05$; One-Way ANOVA). Control A = eggs from control dams (x5) stripped only at 20 DPO and fertilised with milt stored during the 20 DPO from original sire used in the main study. Control B = same eggs fertilised with fresh milt from second sire stripped at 20 DPO.

3.4. Impact of POA on fish performance

3.4.1. Survival, performance and size variation during grow out

Survival between hatch and first feeding was not impacted by ploidy. However, a significantly lower survival was observed in 0 and 10 DPO compared to 5 and 15 DPO and also in 0 DPO compared to 10 DPO ($p < 0.001$) (Table 3a). Between first feeding and the end of the trial, triploids showed a lower survival than diploids ($p = 0.001$), however, no POA effect or interaction was observed.

Diploids had a larger first feeding body weight than triploids ($p = 0.012$), however, no POA effect was observed in either ploidy (Table 3b). Diploids also had an overall greater TGC than triploids ($p < 0.001$) and a lower TGC was found in 5 DPO compared to other DPO groups ($p < 0.001$). This was reflected in the final body weight with diploids having significantly greater weights ($p < 0.001$) and a significant DPO effect was observed (0 DPO = 5 DPO < 10 DPO = 15 DPO) ($p < 0.001$). No ploidy or DPO effects were observed in weight variation expressed by the interquartile range (IQR) (Table 3b) however, an overall ploidy effect on length was observed with diploids significantly longer than triploids ($p < 0.001$)(Table 3b, Fig. 2). Both ploidies showed significantly smaller lengths in 0 and 5 DPO groups compared to 10 and 15 DPO ($p < 0.001$). A greater length IQR was observed in triploids compared to diploids ($p = 0.041$) however no DPO effect was apparent (Fig. 2). Condition factor was significantly greater in diploids compared to triploids ($p < 0.001$) and an overall POA effect was observed with condition factor in 0 DPO fish significantly greater than in 15 DPO ($p = 0.046$)(Table 3b).

3.4.2. Skeletal deformities

The percentage of deformed (ranging from 0.4 and 2.9%) or pin (ranging from 0.1 to 2.2%) alevins were significantly greater in triploids compared to diploids ($p = 0.007$ and $p = 0.001$, respectively). No POA effect was observed in either deformed or pin alevins (Table 4a).

On a final visual assessment of parr, there was no overall difference

Table 2
(a) Survival between egg fertilisation (120°days post fertilisation) and mechanical shock (300°days), and between transfer to tanks (320°days) and hatch (500°days, 5 dams × 5 DPO × 2 ploidy). (b) Performances including egg diameter (at 320°days) and fertilisation success (at 120°days) of Atlantic salmon diploid and triploid eggs (5 dams × 5 DPO × 2 ploidy) exposed to increasing days of post-ovulatory ageing.

	Days post-ovulation										*p* value		
	0 DPO		5 DPO		10 DPO		15 DPO		20 DPO				
	Diploid	Triploid	Diploid	Triploid	Diploid	Triploid	Diploid	Triploid	Diploid	Triploid	Ploidy	DPO	Ploidy*DPO
(a) Survival (%)													
Fertilisation to shock	57.1 ± 18.8	47.1 ± 18.4	36.5 ± 22.6	32.3 ± 11.8	60.3 ± 8.8	36.1 ± 17.3	31.7 ± 13.1	13.5 ± 11.5	9.0 ± 6.2	5.6 ± 2.4	**0.002**	< **0.001**	ns
Transfer to hatch	92.4 ± 1.4[a]	91.5 ± 1.4[a]	86.8 ± 2.5[abc]	90.2 ± 1.1[ab]	87.9 ± 3.6[ab]	78.5 ± 1.2[c]	87.3 ± 2.2[abc]	83.9 ± 4.1[bc]	·	·	**0.034**	< **0.001**	**0.020**
(b) Performances													
Egg diameter (mm)	6.6 ± 0.2	6.5 ± 0.2	6.7 ± 0.2	6.5 ± 0.3	6.8 ± 0.3	6.7 ± 0.2	6.8 ± 0.3	6.8 ± 0.2	7.0 ± 0.1	6.9 ± 0.1	**0.005**	< **0.001**	ns
Fertilisation success (%)	91.6 ± 7.9	90.5 ± 5.6	55.2 ± 21.4	52.1 ± 5.6	75.8 ± 5.6	54.0 ± 13.5	49.0 ± 9.7	40.7 ± 15.9	38.2 ± 12.4	12.4 ± 12.3	**0.008**	< **0.001**	ns

Data are presented as means ± SD and superscripts (bold) denote significant differences between treatments when a significant ploidy*DPO interaction was observed ($p < 0.05$; Linear Mixed Effects model). ns = not significant.

Table 3
(a) Survival between hatch (500°days post fertilisation) - first feeding (900°days) and first feeding - end of the trial (1850°days), and (b) performance summary including weight, interquartile range (IQR) of final weight, growth rate, length and condition factor of diploid and triploid Atlantic salmon juveniles exposed to increasing days of post-ovulatory ageing (DPO).

	Days post-ovulation								*p* value		
	0 DPO		5 DPO		10 DPO		15 DPO				
	Diploid	Triploid	Diploid	Triploid	Diploid	Triploid	Diploid	Triploid	Ploidy	DPO	Ploidy*DPO
(a) Survival (%)											
Hatch to first feeding	91.8 ± 3.4	92.9 ± 2.0	96.0 ± 1.6	95.8 ± 0.9	87.1 ± 0.9	86.5 ± 3.1	98.1 ± 1.3	95.5 ± 1.1	ns	< **0.001**	ns
First feeding to end	98.4 ± 1.0	95.3 ± 3.3	97.6 ± 0.4	92.6 ± 2.2	98.8 ± 0.7	95.4 ± 0.9	97.8 ± 1.9	96.1 ± 2.4	**0.001**	ns	ns
(b) Performances											
First feeding weight (g)	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	**0.012**	ns	ns
Growth rate (TGC)	1.2 ± 0.0	1.1 ± 0.0	1.1 ± 0.1	1.0 ± 0.0	1.2 ± 0.0	1.1 ± 0.0	1.2 ± 0.0	1.1 ± 0.0	< **0.001**	< **0.001**	ns
Final weight (g)	6.1 ± 0.1	5.0 ± 0.1	5.6 ± 0.3	4.5 ± 0.2	6.6 ± 0.1	5.2 ± 0.2	6.5 ± 0.1	5.8 ± 0.2	< **0.001**	< **0.001**	ns
Final weight IQR	1.9 ± 0.3	1.8 ± 0.2	2.2 ± 0.4	2.0 ± 0.2	2.1 ± 0.2	2.3 ± 0.3	2.1 ± 0.2	2.5 ± 0.7	ns	ns	ns
Length (mm)	80.2 ± 0.3	76.5 ± 0.5	78.5 ± 1.2	75.3 ± 0.6	82.6 ± 0.5	78.3 ± 0.9	83.1 ± 0.4	81.2 ± 0.5	< **0.001**	< **0.001**	ns
Length IQR	8.7 ± 0.9	8.3 ± 0.9	9.3 ± 0.9	10.5 ± 0.5	8.4 ± 0.5	10.3 ± 0.9	8.3 ± 0.9	11.3 ± 1.4	**0.041**	ns	ns
Condition (K_f)	1.16 ± 0.01	1.08 ± 0.01	1.13 ± 0.02	1.04 ± 0.02	1.14 ± 0.00	1.05 ± 0.01	1.11 ± 0.01	1.05 ± 0.02	< **0.001**	**0.046**	ns

Data are presented as means ± SD ($n = 3$, 4 dams × 2 ploidy). No superscripts are included as ploidy*DPO interactions were not significant ($p < 0.05$; Two-Way ANOVA). Significant differences between ploidy or DPO are indicated in bold, ns = not significant.

between ploidy in the prevalence of externally visible deformities (Table 4b). The prevalence of visibly deformed diploid parr significantly increased with increasing DPO (0 > 5 > 10 = 15 DPO), however no DPO effect was observed in triploids. In both ploidy, the visible deformities were largely comprised of opercula shortening (ranging from 0.8 to 11.5%) while both jaw and vertebral deformities (data not presented) were negligible. Ploidy did not affect prevalence of opercula shortening, however, ploidy responded differently as diploids showed a significant increase with increasing DPO (0 > 5 > 10 = 15 DPO) while triploids were not impacted by DPO.

Radiological assessment of the vertebral column showed no ploidy effect on the number of deformed vertebrae (dV) present in deformed individuals (Table 4c). Overall, the occurrence of dV was affected by POA with 5 and 10 DPO having a significantly greater number of dV than 0 DPO ($p = 0.015$). There was no impact of ploidy or POA on the prevalence of radiologically deformed individuals (≥1 dV).

3.5. Ovarian fluid pH, conductivity (EC) and osmolality

Ovarian fluid pH was significantly lower in 0 DPO compared to all other DPO groups (Table 5). No POA effect was observed on EC. Finally, osmolality increased significantly from 0 DPO to 10, 15 and 20 DPO groups. Further, osmolality in 20 DPO was significantly greater than in 5 DPO. No dam effect was found in any of the three parameters.

3.6. Ovarian fluid proteomic analysis

No significant differences were found in protein levels in response to DPO with values of protein ranging from 1.1 to 1.3 mg/ml. Similarly, no dam effect was observed. Overall, a total of 177 proteins were identified. Statistical analysis was only performed on proteins detected in at least 3 biological replicates/DPO and detected in all DPO groups. This dataset, which contained 52 proteins is shown in Supplementary Table 1. From these proteins, 19 proteins were significantly different at some DPO stages (Table 11). In general terms, the number of significant proteins in

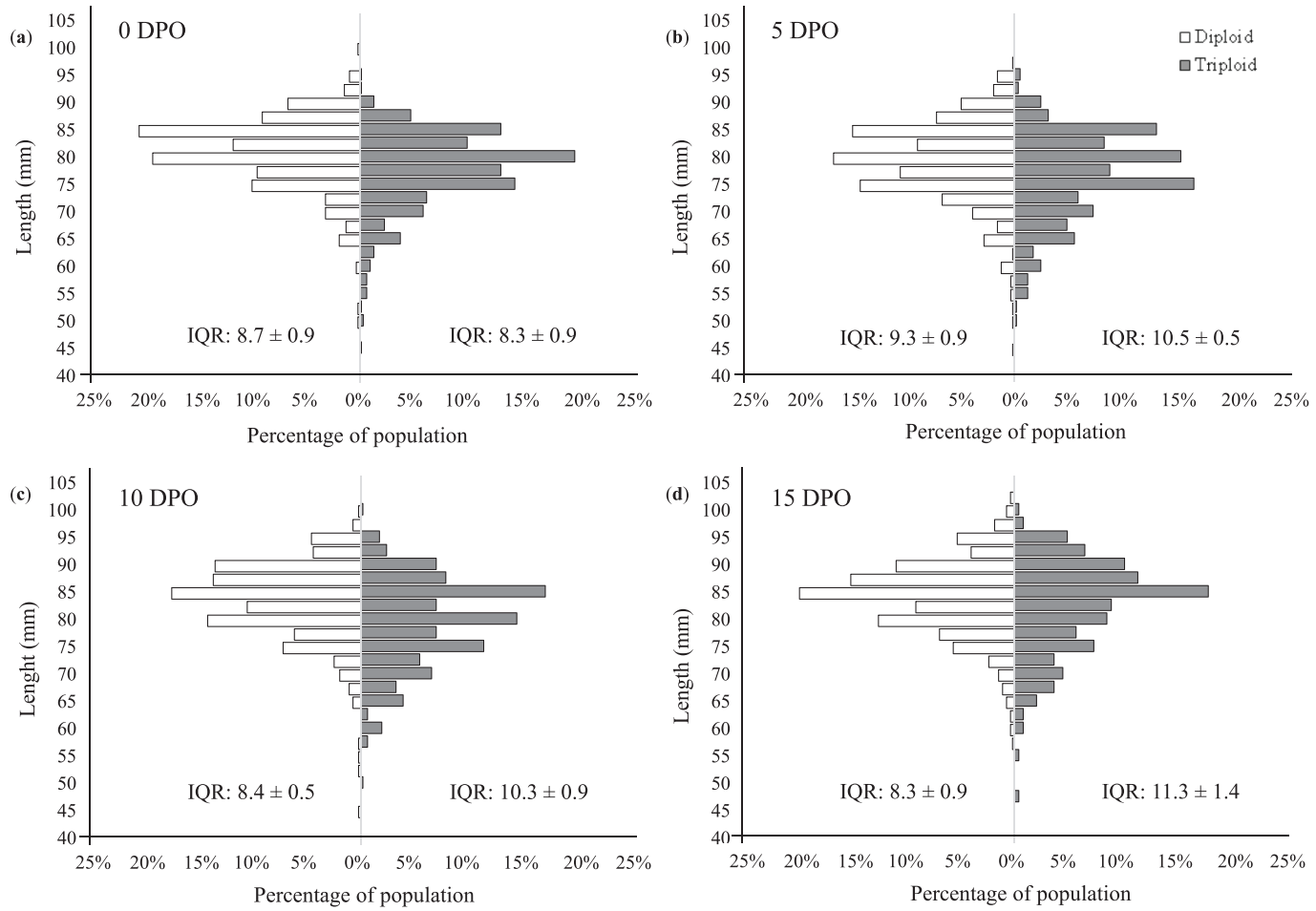

Fig. 2. Variance of final length (at 1850°days) in diploid and triploid Atlantic salmon from (a) 0 DPO, (b) 5 DPO, (c) 10 DPO and (d) 15 DPO treatments ($n = 3$, 145 to 217 fish per tank except for triploid 15 DPO tanks, 80 to 83 fish). IQR = interquartile range.

Table 4
Visible deformity assessment during (a) alevin (850°days) and (b) parr stage (1850°days) and (c) radiological assessment of diploid and triploid Atlantic salmon parr at the end of the trial exposed to increasing days of post-ovulatory ageing (0, 5, 10 and 15 DPO). Data are presented as means ± SEM ($n = 3$, 30 fish DPO^{-1} ploidy^{-1}).

	0 DPO		5 DPO		10 DPO		15 DPO		*p* value		
	Diploid	Triploid	Diploid	Triploid	Diploid	Triploid	Diploid	Triploid	Ploidy	DPO	Ploidy*DPO
(a) Alevin abnormalities											
Deformed individuals (%)	0.4 ± 0.2	1.3 ± 0.4	0.7 ± 0.2	1.1 ± 0.3	1.0 ± 0.6	2.9 ± 0.5	1.4 ± 0.5	1.9 ± 0.3	**0.007**	ns	ns
Pin head individuals (%)	0.1 ± 0.1	0.7 ± 0.4	0.7 ± 0.5	1.9 ± 0.5	0.5 ± 0.3	2.2 ± 0.3	0.4 ± 0.2	2.2 ± 1.1	**0.001**	ns	ns
(b) Parr external visible deformity											
Deformed individuals (%)	0.8 ± 0.8^{d}	4.9 ± 1.5^{bc}	3.7 ± 0.5^{c}	4.4 ± 0.8^{bc}	12.6 ± 1.1^{a}	8.8 ± 1.2^{abc}	10.5 ± 1.0^{ab}	4.5 ± 0.5^{bc}	ns	< **0.001**	**< 0.001**
Opercula shortening (%)	0.8 ± 0.8^{d}	4.1 ± 1.1^{c}	3.0 ± 0.1^{c}	4.0 ± 0.9^{c}	11.5 ± 0.7^{a}	7.3 ± 1.1^{abc}	10.3 ± 1.1^{ab}	4.5 ± 0.5^{bc}	ns	< **0.001**	**< 0.001**
(c) Parr radiological vertebral deformity											
Deformed vertebrae (dV) (No.)	2.2 ± 0.1	2.5 ± 0.2	5.0 ± 1.0	4.4 ± 0.8	4.5 ± 1.0	5.2 ± 1.0	5.0 ± 0.5	3.9 ± 0.9	ns	**0.015**	ns
Deformed individuals (≥1 dV) (%)	31.1 ± 4.8	35.6 ± 2.9	30.0 ± 5.1	35.6 ± 4.0	32.2 ± 2.9	38.9 ± 4.0	27.8 ± 4.0	27.8 ± 5.9	ns	ns	ns

Superscripts (bold) denote significant differences between treatments when a significant ploidy*DPO interaction was observed ($p < 0.05$; Two-Way ANOVA). dV: number of deformed vertebrae present in deformed individuals; ns = not significant.

the ovarian fluid increased with ageing time (1 significant protein between 0 and 5 DPO; 9 significant proteins between 0 and 10 DPO; 10 significant proteins between 0 and 15 DPO and 16 significant protein between 0 and 20 DPO). Most of these significant changes were associated with reduced abundance with DPO (10 proteins significantly lower versus 6 proteins significantly higher at 20 DPO compared to 0 DPO). However, the time point at which reductions in abundance were observed was protein specific. For instance, a lower abundance for proteins such as versican core protein, bromodomain-containing protein 4-like isoform X5, inhibin alpha chain, dnaJ homolog subfamily C

Table 5
Ovarian fluid parameters in female broodstock during post-ovulatory ageing.

	Days post-ovulation					Dam effect (*p* value)
	0 DPO	5 DPO	10 DPO	15 DPO	20 DPO	
pH	8.3 ± 0.0^{b}	8.5 ± 0.1^{a}	8.4 ± 0.1^{a}	8.5 ± 0.0^{a}	8.4 ± 0.0^{a}	ns
EC (μS; x10^{-3})	7.2 ± 0.1	7.1 ± 0.3	7.4 ± 0.5	7.6 ± 0.4	7.0 ± 0.3	ns
Osmolality (mOsm L^{-1})	268.1 ± 13.8^{c}	277.1 ± 14.3^{bc}	295.2 ± 11.9^{ab}	291.4 ± 11.0^{ab}	301.1 ± 4.3^{a}	ns

Data are presented as means ± SD (5 dams × 5 DPO) and superscripts denote significant differences between treatments ($p < 0.05$; Linear Mixed Effects model). EC = electrical conductivity; ns = not significant.

member 10-like, Glyceraldehyde-3-phosphate dehydrogenase and zinc finger protein 239-like was detected earlier on (10 DPO) compared to other proteins such as plasminogen, hyaluronidase, fructose-bisphosphate aldolase and zona pellucida sperm-binding protein 4-like which only decreased significantly at later stages. On the other hand, vitellogenin content in the ovarian fluid increased significantly from 5 DPO while other proteins increased in the ovarian fluid later in the ageing process (hemopexin, histidine-rich glycoprotein-like, apolipoprotein C-I-like and serum albumin 1).

3.7. Stripped egg and ovarian fluid biochemical analyses

3.7.1. Mineral composition

In eggs, no significant differences were found in mineral composition in response to DPO apart from phosphorous (P) and magnesium (Mg) having higher levels in 15 DPO compared to 10 DPO (Table 6a). In ovarian fluid samples, levels of calcium (Ca), sodium (Na), Mg and zinc (Zn) were not impacted by POA (Table 6b). However, ovarian fluid P was significantly greater in 5, 10 and 20 DPO compared to 0 DPO and also levels in 20 DPO were significantly higher than 15 DPO. The increase in P gave a corresponding higher Ca:P at 0 DPO compared to all other DPO groups. Potassium (K) and vanadium (V) significantly increased with increasing POA. Manganese (Mn) was significantly higher in 5 DPO compared to 15 DPO with no other differences observed. No dam effect was observed in any of the minerals analysed in both eggs and ovarian fluid.

3.7.2. Proximate composition

No significant differences were found in dry matter (DM), lipid, ash or energy content of the eggs in response to DPO (Table 7). However, egg protein content in 10 DPO were significantly reduced compared to 5 and 20 DPO and energy content was significantly reduced in 15 DPO compared to 5 DPO. No dam effect was observed in proximate composition analysis.

3.7.3. Fatty acid and lipid class composition

There were no significant differences in any of the fatty acids in response to increasing DPO and no dam effect was observed (Table 8). Most lipid groups were impacted by increasing DPO (Table 9). The percentage of diacylglycerol (DAG) was significantly greater in 0 DPO compared to 10, 15 and 20 DPO. Cholesterol was significantly highest in

Table 7
Proximate composition of newly stripped eggs from female broodstock during post-ovulatory ageing.

	Days post-ovulation					Dam effect (*p* value)
	0 DPO	5 DPO	10 DPO	15 DPO	20 DPO	
Dry matter (%)	38.6 ± 0.8	39.1 ± 1.2	38.1 ± 2.4	37.8 ± 1.0	38.9 ± 0.7	ns
Lipid – crude (% DM)	26.2 ± 1.2	26.3 ± 2.4	27.0 ± 0.9	28.0 ± 1.2	26.8 ± 1.6	ns
Protein – crude (% DM)	59.9 ± 1.3^{ab}	61.4 ± 1.7^{a}	58.1 ± 3.1^{b}	59.9 ± 1.2^{ab}	61.8 ± 2.9^{a}	ns
Ash (%DM)	5.5 ± 0.9	5.6 ± 0.9	5.7 ± 0.7	4.5 ± 0.9	5.1 ± 0.8	ns
Energy (kJ g^{-1})	27.0 ± 0.1^{ab}	27.1 ± 0.1^{a}	27.0 ± 0.2^{ab}	26.9 ± 0.1^{b}	27.0 ± 0.2^{ab}	ns

Lipid, protein and ash results are presented as percentage of sample dry matter (DM). Data are presented as means ± SD (5 dams × 5 DPO) and superscripts denote significant differences between treatments ($p < 0.05$; Linear Mixed Effects model). ns = not significant.

Table 6
Mineral composition of both (a) newly stripped eggs and (b) ovarian fluid from female broodstock during post-ovulatory ageing.

	Days post-ovulation					Dam effect (*p* value)
	0 DPO	5 DPO	10 DPO	15 DPO	20 DPO	
a) Eggs						
Calcium (Ca)	1638.1 ± 153.1	1680.6 ± 128.6	1637.1 ± 116.3	1700.8 ± 169.9	1649.5 ± 136.2	ns
Phosphorous (P)	10,059.7 ± 440.5^{ab}	10,295.4 ± 262.6^{ab}	9871.9 ± 216.3^{b}	10,388.0 ± 291.5^{a}	10,159.6 ± 356.1^{ab}	ns
Ca:P	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	ns
Sodium (Na)	2010.2 ± 99.4	2025.7 ± 186.6	2214.2 ± 568.6	2355.3 ± 285.9	2012.2 ± 264.6	ns
Magnesium (Mg)	736.9 ± 86.3^{ab}	754.2 ± 62.2^{ab}	734.5 ± 63.7^{b}	776.8 ± 73.7^{a}	761.0 ± 58.3^{ab}	ns
Potassium (K)	3796.9 ± 473.3	3910.2 ± 150.2	3560.9 ± 82.1	3918.3 ± 237.4	3993.8 ± 262.3	ns
Vanadium (V)	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	ns
Manganese (Mn)	9.1 ± 1.1	9.2 ± 1.6	9.0 ± 1.4	9.2 ± 1.3	9.4 ± 1.0	ns
Zinc (Zn)	74.6 ± 3.8	76.4 ± 3.7	76.2 ± 7.4	77.0 ± 3.4	74.4 ± 4.8	ns
b) Ovarian fluid						
Calcium (Ca)	86.5 ± 4.4	88.9 ± 9.1	89.2 ± 3.3	83.5 ± 9.2	86.0 ± 9.5	ns
Phosphorous (P)	49.6 ± 4.6^{c}	63.3 ± 8.5^{ab}	62.2 ± 7.0^{ab}	58.0 ± 4.2^{bc}	71.8 ± 3.5^{a}	ns
Ca:P	1.8 ± 0.2^{a}	1.4 ± 0.1^{b}	1.4 ± 0.1^{b}	1.4 ± 0.1^{b}	1.2 ± 0.1^{b}	ns
Sodium (Na)	1890.6 ± 105.3	2148.2 ± 220.2	2075.6 ± 205.7	2015.4 ± 48.3	2173.9 ± 173.4	ns
Magnesium (Mg)	9.8 ± 1.0	10.5 ± 1.5	9.9 ± 0.6	9.7 ± 1.1	9.6 ± 0.9	ns
Potassium (K)	55.9 ± 7.0^{b}	53.7 ± 7.6^{b}	60.6 ± 5.4^{ab}	58.0 ± 5.7^{b}	78.4 ± 19.8^{a}	ns
Vanadium (V)	0.002 ± 0.001^{d}	0.003 ± 0.001^{cd}	0.004 ± 0.001^{bc}	0.004 ± 0.001^{b}	0.006 ± 0.001^{a}	ns
Manganese (Mn)	0.102 ± 0.015^{ab}	0.119 ± 0.022^{a}	0.099 ± 0.014^{ab}	0.089 ± 0.016^{b}	0.103 ± 0.029^{ab}	ns
Zinc (Zn)	2.4 ± 3.2	1.8 ± 0.6	3.3 ± 2.1	1.9 ± 1.0	2.4 ± 0.4	ns

Data are presented as means ± SD (5 dams × 5 DPO) and superscripts denote significant differences between treatments ($p < 0.05$; Linear Mixed Effects model). ns = not significant.

Table 8
Fatty acid composition of newly stripped eggs from female broodstock during post-ovulatory ageing.

	Days post-ovulation					Dam effect (p value)
	0 DPO	5 DPO	10 DPO	15 DPO	20 DPO	
Fatty acid (mg 100 g^{-1})						
Total saturated	1059.9 ± 71.6	1056.2 ± 170.7	1104.2 ± 171.8	1113.2 ± 68.1	1122.8 ± 179.7	ns
Total monoenes	2607.6 ± 323.7	2521.2 ± 346.0	2724.9 ± 400.5	2620.7 ± 150.4	2758.0 ± 123.2	ns
18:2n-6	629.2 ± 84.5	615.1 ± 98.5	664.6 ± 102.4	629.1 ± 30.8	668.8 ± 27.4	ns
20:4n-6	57.5 ± 6.9	57.9 ± 8.9	62.6 ± 9.7	62.8 ± 7.1	64.6 ± 8.1	ns
Total n-6 PUFA	868.7 ± 128.3	838.6 ± 117.2	911.1 ± 139.0	866.1 ± 49.3	907.4 ± 44.9	ns
18:3n-3	220.8 ± 20.3	224.8 ± 44.2	245.5 ± 47.3	229.5 ± 18.7	246.5 ± 20.4	ns
20:5n-3	454.7 ± 46.5	442.6 ± 58.0	466.7 ± 62.7	458.8 ± 36.7	477.0 ± 27.7	ns
22:6n-3	933.0 ± 68.3	987.0 ± 159.6	1030.3 ± 173.2	1017.0 ± 78.6	1050.2 ± 74.7	ns
Total n-3 PUFA	2112.6 ± 141.2	2162.6 ± 342.4	2285.3 ± 372.5	2237.5 ± 147.2	2325.9 ± 161.3	ns
Total PUFA	2996.4 ± 246.2	3016.0 ± 455.4	3205.1 ± 511.9	3115.9 ± 189.4	3253.2 ± 199.3	ns
Total	6663.9 ± 621.0	6593.4 ± 963.0	7034.2 ± 1079.8	6849.8 ± 386.0	7134.0 ± 442.1	ns

Data are presented as means ± SD (5 dams × 5 DPO) and superscripts denote significant differences between treatments ($p < 0.05$; Linear Mixed Effects model). ns = not significant.

Table 9
Lipid class content of newly stripped eggs from female brood fish during post-ovulatory ageing.

	Days post-ovulation					Dam effect (p value)
	0 DPO	5 DPO	10 DPO	15 DPO	20 DPO	
Lipid class (%)						
Diacylglycerol	1.9 ± 1.2^{a}	1.2 ± 0.1^{ab}	0.8 ± 0.1^{b}	0.7 ± 0.2^{b}	0.9 ± 0.2^{b}	ns
Cholesterol	9.9 ± 0.2^{c}	11.1 ± 0.2^{b}	10.0 ± 0.2^{bc}	10.5 ± 0.4^{bc}	13.0 ± 1.4^{a}	ns
Triacylglycerol	55.5 ± 2.0^{a}	55.6 ± 2.8^{a}	54.9 ± 1.4^{a}	49.2 ± 3.6^{b}	45.2 ± 3.2^{b}	ns
Sterol/Wax esters	3.7 ± 2.0^{ab}	3.0 ± 0.4^{b}	4.2 ± 1.1^{ab}	6.8 ± 1.7^{a}	4.2 ± 4.2^{ab}	ns
Total neutral lipids	70.9 ± 2.3^{a}	70.9 ± 2.5^{a}	70.0 ± 1.1^{ab}	67.2 ± 2.6^{b}	63.4 ± 0.5^{c}	ns
Pigmented material	1.5 ± 0.7	0.8 ± 0.5	0.7 ± 0.6	1.3 ± 0.1	1.2 ± 0.3	ns
Sphingomyelin	0.6 ± 0.1^{b}	0.6 ± 0.1^{b}	0.6 ± 0.1^{b}	1.4 ± 0.1^{a}	0.6 ± 0.2^{b}	ns
Phosphatidylcholine	22.4 ± 0.9^{b}	21.8 ± 1.5^{b}	22.3 ± 1.0^{b}	21.7 ± 2.1^{b}	28.0 ± 0.7^{a}	ns
Phosphatidic acid/Phosphatidyglycerol	0.7 ± 0.4^{c}	1.0 ± 0.3^{bc}	1.3 ± 0.1^{ab}	1.6 ± 0.3^{a}	1.0 ± 0.2^{bc}	ns
Phosphatidylethanolamine	3.9 ± 1.9^{b}	4.9 ± 0.6^{ab}	5.1 ± 0.3^{ab}	6.8 ± 0.7^{a}	5.8 ± 0.1^{a}	ns
Total polar lipids	29.1 ± 2.3^{c}	29.1 ± 2.5^{c}	30.0 ± 1.1^{bc}	32.8 ± 2.6^{b}	36.6 ± 0.5^{a}	ns
Neutral: Polar	2.5 ± 0.3^{a}	2.5 ± 0.3^{a}	2.3 ± 0.1^{ab}	2.1 ± 0.2^{bc}	1.7 ± 0.0^{c}	ns

Data are presented as means ± SD (5 dams × 5 DPO) and superscripts denote significant differences between treatments ($p < 0.05$; Linear Mixed Effects model). ns = not significant.

20 DPO compared to all other DPO and levels in 5 DPO were significantly >0 DPO. The percentages of triacylglycerol (TAG) in 0, 5 and 10 DPO were comparable and all significantly >15 and 20 DPO. There was a significant increase in percentage of sterol/wax esters in 15 DPO compared to 5 DPO with no other differences observed. Total neutral lipid percentage decreased in response to POA from 0 and 5 DPO to 15 and 20 DPO. Sphingomyelin was significantly higher in 15 DPO than all other DPO groups. Phosphatidylcholine was significantly greater in 20 DPO compared to all other DPO groups. The percentage of phosphatidic acid / phosphatidyglycerol was greater in 10 and 15 DPO compared to 0 DPO. The levels observed in 15 DPO eggs were also significantly >5 and 20 DPO. The percentages of phosphatidylethanolamine in 15 and 20 DPO were significantly >0 DPO. Collectively, the increase of sphingomyelin and phospholipid levels resulted in an increase of total polar lipids in response to POA. This transformation in both neutral and polar lipids resulted in a significant decrease in neutral: polar lipid ratio. The ratio in 0 and 5 DPO eggs was significantly >15 and 20 DPO. Further, a higher ratio was observed in 10 DPO compared to 20 DPO. No dam effect was observed in any of the individual lipid classes.

3.7.4. Carotenoids and vitamin E

Astaxanthin levels were significantly greater in 0 and 15 DPO eggs compared to 5 DPO (Table 10). Levels in 0 DPO eggs were also significantly >10 and 20 DPO. Canthaxanthin levels were significantly greater in 0 DPO compared to 5 and 10 DPO, however, comparable in 15 and 20 DPO. Adonirubin levels were significantly greater in 0 DPO compared to all other DPO groups. Lutein levels were significantly greater in 0 DPO compared to 5 and 20 DPO only. Collectively, total carotenoid content was significantly greater in 0 and 15 DPO compared to 5 DPO. Finally, total content in 0 DPO was significantly greater than at 10 and 20 DPO. There was no dam effect observed.

Table 10
Carotenoid levels in newly stripped eggs from female broodstock during post-ovulatory ageing.

	Days post-ovulation					Dam effect (p value)
	0 DPO	5 DPO	10 DPO	15 DPO	20 DPO	
Astaxanthin	6.7 ± 1.0^{a}	6.1 ± 0.8^{c}	6.3 ± 0.7^{bc}	6.5 ± 0.8^{ab}	6.3 ± 0.9^{bc}	ns
Canthaxanthin	0.7 ± 0.1^{a}	0.6 ± 0.1^{b}	0.6 ± 0.1^{b}	0.6 ± 0.1^{ab}	0.6 ± 0.1^{ab}	ns
Adonirubin	3.6 ± 0.5^{a}	3.2 ± 0.4^{b}	3.4 ± 0.4^{b}	3.4 ± 0.5^{b}	3.3 ± 0.5^{b}	ns
Lutein	0.8 ± 0.3^{a}	0.7 ± 0.2^{b}	0.7 ± 0.2^{ab}	0.7 ± 0.2^{ab}	0.7 ± 0.2^{b}	ns
Total carotenoids	11.7 ± 1.7^{a}	10.6 ± 1.4^{c}	11.0 ± 1.3^{bc}	11.2 ± 1.4^{ab}	11.0 ± 1.6^{bc}	ns

Data are presented as means ± SD (5 dams x 5 DPO) and superscripts denote significant differences between treatments ($p < 0.05$; Linear Mixed Effects model). ns = not significant.

Vitamin E (α-tocopherol) concentration was significantly reduced in 20 DPO eggs compared to 0, 5 and 10 DPO (Fig. 3). No dam effect was observed.

Table 11
Differentially abundant proteins in Atlantic salmon ovarian fluid when comparing different time points to 0 DPO.

Accession (UniProt)	Protein name	DPO compared)				Function
		0–5	0–10	0–15	0–20	
A0A1S3P927	Vitellogenin	1.5*	1.6*	1.6*	1.5	Reproduction
A0A1S3RF10	Fibrinogen gamma chain-like	1.3	1.8*	1.9*	2.0*	Heme
A0A1S3MVQ2	Hemopexin	1	1.1	1.2	1.4*	Heme
A0A1S3KK24	Histidine-rich glycoprotein-like	1	1.1	1.2*	1.2	Heme
A0A1S3SDM0	Uncharacterized protein LOC106608805	1	1.3	1.3	1.5*	Cell organisation
M4V2E4	Serine protease-like protein	1.2	1.4*	1.2	1.5*	Immune
A0A1S3N6L4	Apolipoprotein C-I-like	1.2	1.4	1.3	1.5*	Metabolism
P21848	Serum albumin 1	1.1	1.2	1.2	1.4*	Metabolism
A0A1S3MLW1	Zona pellucida sperm-binding protein 4-like	−1.1	−1.3	−1.6*	−2.1*	Reproduction
A0A1S3S8H0	Plasminogen (EC 3.4.21.7)	1	−1.2	−1.1	−1.4*	Heme
A0A1S3KYK4	Versican core protein	−1.2	−1.8*	−2.3*	−2.6*	Cell organisation
A0A1S3S665	bromodomain-containing protein 4-like isoform X5	−1.2	−1.9*	−1.1	−1.8*	Cell organisation
A0A1S3MWT4	Inhibin alpha chain	−1.3	−1.6*	−1.8*	−2.6*	Cell organisation
A0A1S3NZB8	dnaJ homolog subfamily C member 10-like	−1.3	−2.2*	−2.8*	−4.8*	Metabolism
A0A1S3PCZ2	Hyaluronidase (EC 3.2.1.35) (Hyaluronoglucosaminidase)	−1.2	−1.5	−1.5	−2.2*	Metabolism
C0H9I1	Fructose-bisphosphate aldolase (EC 4.1.2.13)	1	−1.2	−1.2	−1.6*	Metabolism
B5X3K2	Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12)	−1.1	−1.3*	−1.3*	−1.7*	Metabolism
A0A1S3SWR7	Zinc finger protein 239-like	−1.3	−1.9*	−2.3*	−2.3*	Transcription regulation
A0A1S3M8M8	Uncharacterized protein LOC106571128	−1.3	−1.2	−1.2*	−2.1*	Other

Changes in protein abundance are expressed as fold changes relative to 0 DPO (5 dams × 5 DPO), an asterisk (*) indicates a significant difference ($p < 0.05$; *t*-test).

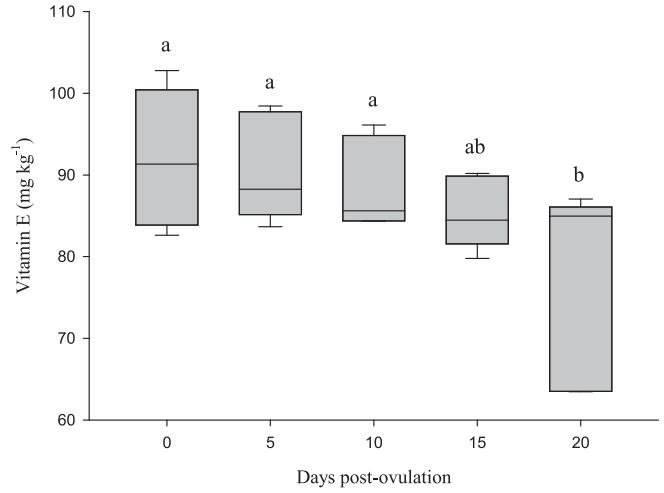

Fig. 3. Vitamin E (α-tocopherol) concentration in newly stripped eggs from female broodstock during post-ovulatory ageing. Data are presented as means ± SD (5 dams × 5 DPO) and superscripts denote significant differences between treatments ($p < 0.05$; Linear Mixed Effects model).

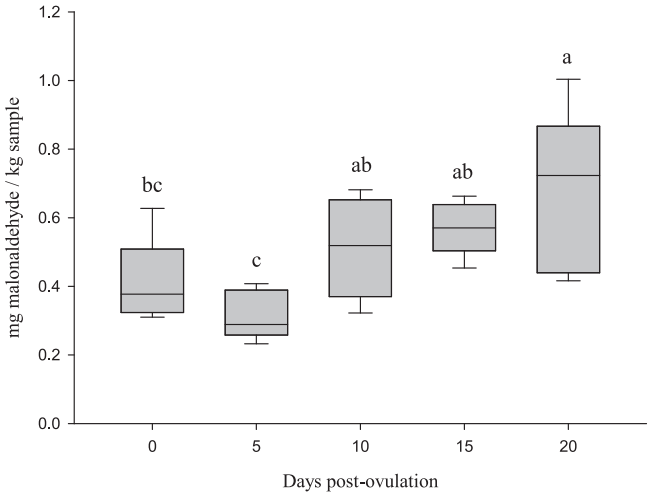

Fig. 4. TBARS concentration in newly stripped eggs from female broodstock during post-ovulatory ageing. Data are presented as means ± SD (5 dams × 5DPO) and superscripts denote significant differences between treatments ($p < 0.05$; Linear Mixed Effects model).

3.7.5. TBARS

The level of lipid-peroxidation aldehydes, expressed by TBARS content, was significantly higher in 20 DPO eggs than 0 and 5 DPO (Fig. 4). TBARS levels in 10 and 15 DPO were also significantly >5 DPO. No dam effect was observed.

3.8. Gene expression

The expression of all genes involved in energy metabolism (*atp5f1c*, *atp5f1* and *coxII*), one gene involved in cell cycle regulation (*ccnb3)* and apoptosis (*bclx*) was significantly reduced in 20 DPO in comparison to 0 DPO ($p < 0.05$) (Figs. 5, 6 and 7). Expression of all genes gradually decreased from 0 DPO to 20 DPO. No dam effect was observed in these genes. The expression of *mkk6a* reached minimum levels in 15 DPO, and a peak was observed in 20 DPO (Fig. 6b). Finally, the expression of *tbb5*, *calm2*, *cata*, *hsp70*, *sod1* and *sod2* did not show significant differences with DPO and no dam affect was observed (Figs. 6 and 8).

3.9. Correlations

Ovarian fluid osmolality correlated positively with egg diameter in both diploids ($r^2 = 0.954$, $p < 0.05$) and triploids ($r^2 = 0.961$, $p < 0.01$). The change in egg lipid class composition was not correlated with any subsequent trait in diploid fish, however the decreasing neutral: polar lipid ratio correlated positively in triploids with egg survival ($r^2 = 0.929$, $p < 0.05$). The decrease in neutral: polar lipid ratio also correlated negatively with TBARS ($r^2 = 0.918$, $p < 0.05$).

4. Discussion

The present study showed that post-ovulatory ageing (POA) has an impact on the survival and performance of diploid and triploid Atlantic salmon, which can be correlated to several oocyte quality parameters resulting from oocyte ageing. The results suggest that triploids are more sensitive to oocyte ageing as egg groups exposed to increased POA resulted in a reduced fertilisation success and survival during early egg incubation, and an increased variance in individual growth. An

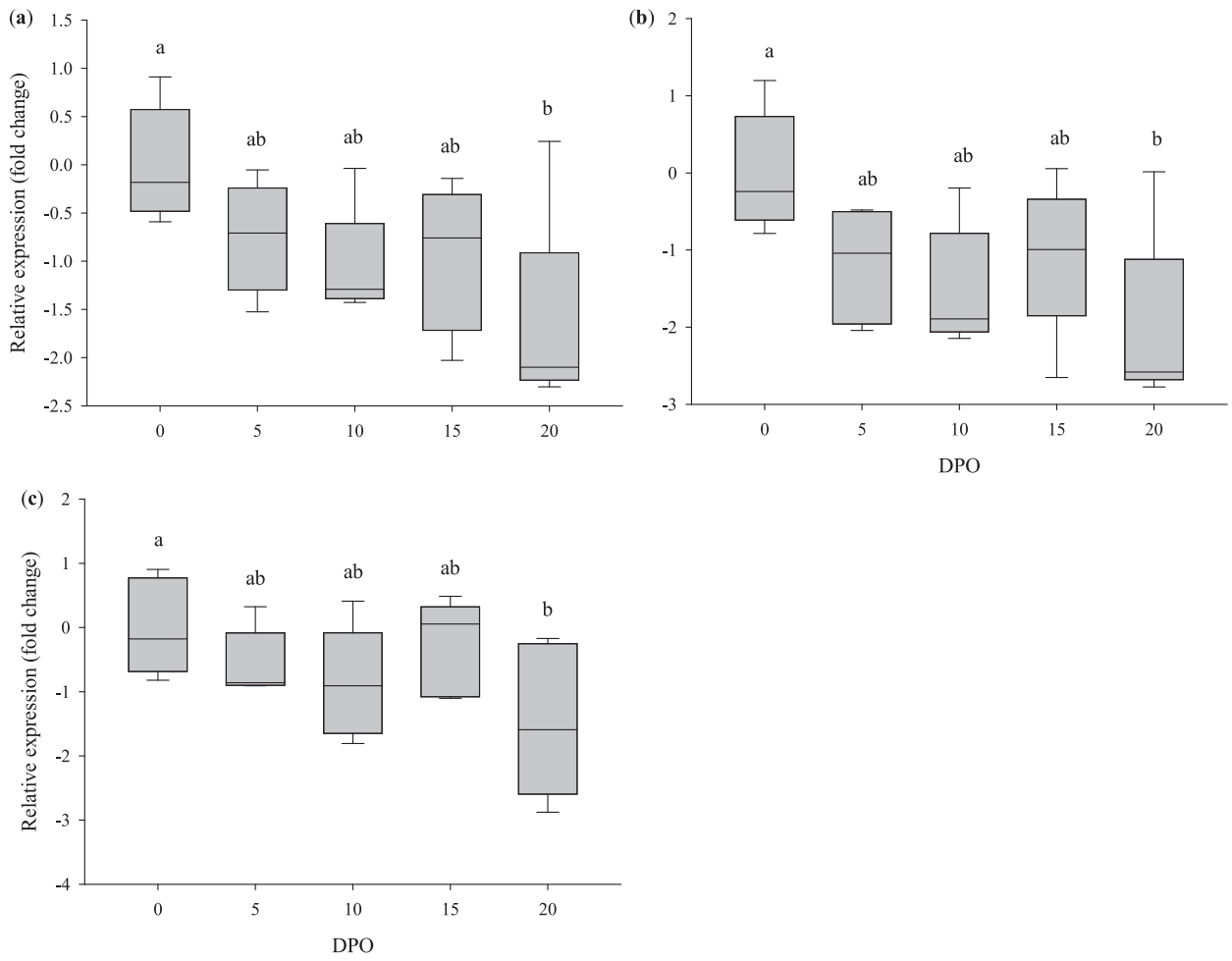

Fig. 5. mRNA expression of genes associated with energy metabolism; (a) *atp5f1*, (b) *atp5f1c*, and (c) *coxII* in newly stripped eggs from female broodstock during post-ovulatory ageing expressed as fold change relative to 0 DPO. Data were normalised using the geometric mean of *b2m*, *ef1a* and *rpl1* reference genes. Data are expressed as means ± SD (6 eggs × 5 dams × 5DPO) and different superscripts denote significant differences between DPO ($p < 0.05$; Linear Mixed Effects model).

increased trend in deformity prevalence was also observed with increasing POA in both ploidy. Several ovarian fluid and newly stripped egg quality criteria were assessed as potential biomarkers to determine the future success of a batch in the event of over ripening of eggs. Ovarian fluid osmolality, neutral: polar lipid ratio, vitamin E concentration and TBARS were identified as good candidate biomarkers to investigate further. Finally, 19 proteins identified from the ovarian fluid, several involved in cell organisation, metabolism and reproduction showed significant changes in abundance in relation to POA.

The reduced fertilisation rate of salmonid eggs observed in the present study in response to POA confirmed previous findings (Bromage et al., 1992; Mommens et al., 2015). Fish oocytes are activated when in contact with freshwater resulting in egg swelling due to water absorption which restrict then access to the micropyle for spermatozoa (Billard and Cosson, 1992). Osmotic swelling was observed in this study in relation to DPO with an apparent increase in egg diameter. This was correlated with a significant increase in ovarian fluid osmolality during DPO suggesting that water was entering the egg, which is a likely contributing factor to the reduced fertilisation rate. Increased swelling has been shown to decrease chorion thickness in *Oncoryhnchus mykiss* as a result of increased tension within the egg (Samarin et al., 2015). No change to the chorion thickness was observed in other studies performed in *Oncorhynchus mykiss* (Lahnsteiner, 2000) and Caspian brown trout (*Salmo trutta caspius*) (Bahre Kazemi et al., 2010), however an increase in perivitelline space was reported. It is important to note that fertilisation rates assessed at 120°days were lower than expected especially in eggs from dams partially stripped at 5 DPO compared to the same dams stripped at 10 DPO. Similarly, survival in eyed eggs assessed at 300°days post fertilisation was reduced in broodstock stripped for the first time at 0 DPO (and 5 DPO) mainly due to large variability between dams. These results can be explained by the increased inter variability between dams which suggest other factors at play (e.g. silo effect) during the early incubation of these egg batches. Importantly, while fertilisation rate was assessed according to a standardised protocol for salmon hatcheries, it remains a crude assessment of fertilisation success as some eggs may have been initially fertilised and died. This highlights the challenge of assessing egg quality in fish at such early developmental stages (Migaud et al., 2013). In the present study, broodstock were partially stripped to test for the effect of DPO and milt from a single male was preserved to prevent any paternal effect. To test if the partial stripping procedure had any detrimental impact on fertilisation rate or egg viability from 20 DPO dams, separate eggs from dams that had also ovulated 20 days prior but with no partial stripping and fertilised with newly cannulated milt from a second male were also used. There was no difference in fertilisation rate or egg viability between both 20 DPO groups. However, milt quality decreased during the egg stripping period which may subsequently have led to reduced fertilisation rates. This suggests that the short-term preservation strategy involving Ringer solution and cold storage at 4 °C was not robust enough to keep optimal milt motility for up to 20 days and another approach should be considered if repeated i.e. cryopreservation. Despite this, results confirmed that POA and the associated ovarian fluid and oocyte quality are contributing factors explaining the

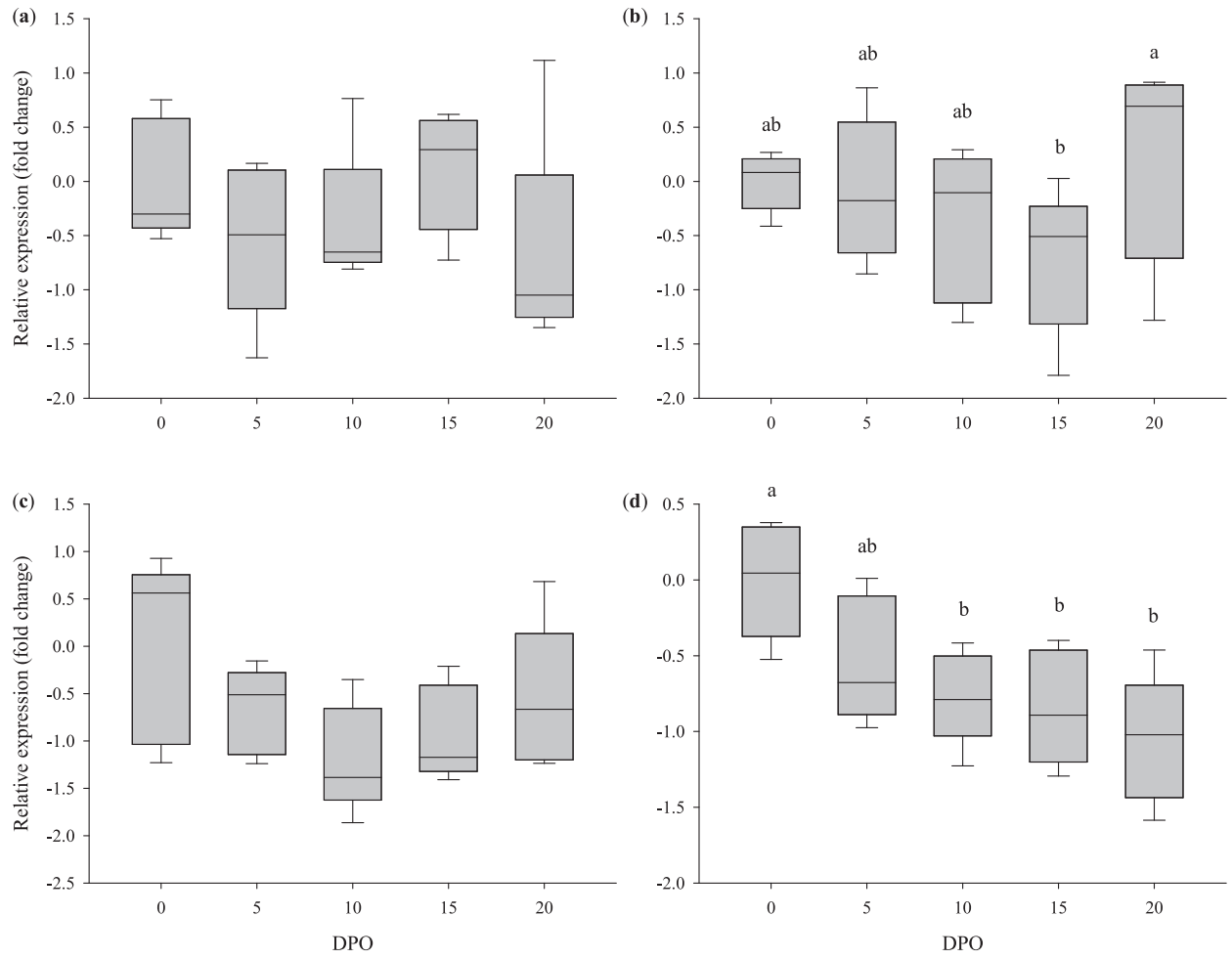

Fig. 6. mRNA expression of genes associated with the cell cycle; (a) *calm2*, (b) *mkk6a*, (c) *tbb5*, and (d) *ccnb3* in newly stripped eggs from female broodstock during post-ovulatory ageing expressed as fold change relative to 0 DPO. Data were normalised using the geometric mean of *b2m*, *ef1a* and *rpl1* reference genes. Data are expressed as means ± SD (6 eggs × 5 dams × 5 DPO) and different superscripts denote significant differences between DPO ($p < 0.05$; Linear Mixed Effects model).

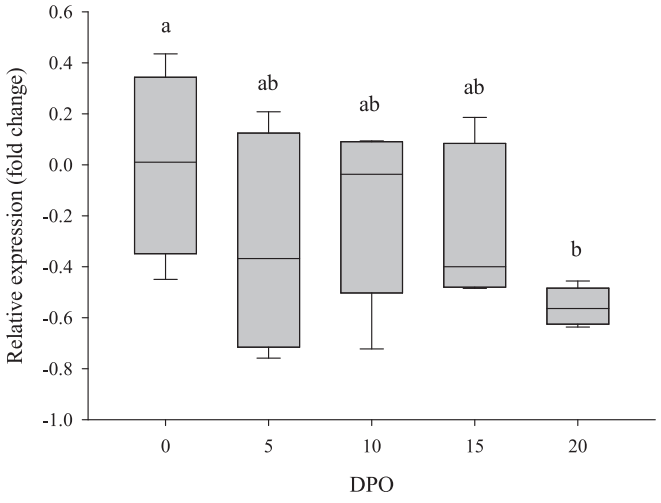

Fig. 7. mRNA expression of *bclx*, an antiapoptopic gene, in newly stripped eggs from female broodstock during post-ovulatory ageing expressed as fold change relative to 0 DPO. Data were normalised using the geometric mean of *b2m*, *ef1a* and *rpl1* reference genes. Data are expressed as means ± SD (6 eggs × 5 dams × 5 DPO) and different superscripts denote significant differences between DPO ($p < 0.05$; Linear Mixed Effects model).

reduction in fertilisation rate.

As suggested by Bromage et al. (1992) and Taylor et al. (2011) for both ploidy, salmonid eggs batches with a low fertilisation rate generally experience lower survival also during embryogenesis, which was supported by the current study results as egg survival decreased significantly in both ploidy with increased DPO. Therefore, careful production planning with reducing the duration to fertilisation post-ovulation can result in higher numbers of viable eggs for culture. This however contrasts with findings in coho salmon (*Oncorhynchus kisutch*) showing older eggs (4 to 11 DPO) stored in vivo (but not in vitro) were more viable than freshly ovulated eggs and yielded higher frequencies of triploids (Devlin et al., 2010). The authors suggested that egg viability is enhanced by retention of eggs in the peritoneal cavity. Such contrasting results may be explained by differences between species given coho salmon has one of the longest POA window (20 days, Fitzpatrick et al., 1987) compared to Atlantic salmon (6–8 days, Bromage et al., 1994) and rainbow trout (<7 days, Lahnsteiner, 2000) (Migaud et al., 2013). Furthermore, 6.7% of diploid eggs that were exposed to 15 DPO underwent spontaneous triploidy while none were detected in 0, 5 and 10 DPO groups suggesting an effect of POA on spontaneous triploidy. This has been previously reported in the literature (Aegerter and Jalabert, 2004). A large-scale assessment of domesticated Atlantic salmon produced in Norwegian aquaculture showed 2% spontaneous triploidy and confirmed that triploidy can occur naturally (Glover et al., 2015). However, the frequency of naturally-occurring triploid Atlantic salmon in wild Norwegian and Russian populations was shown to be much

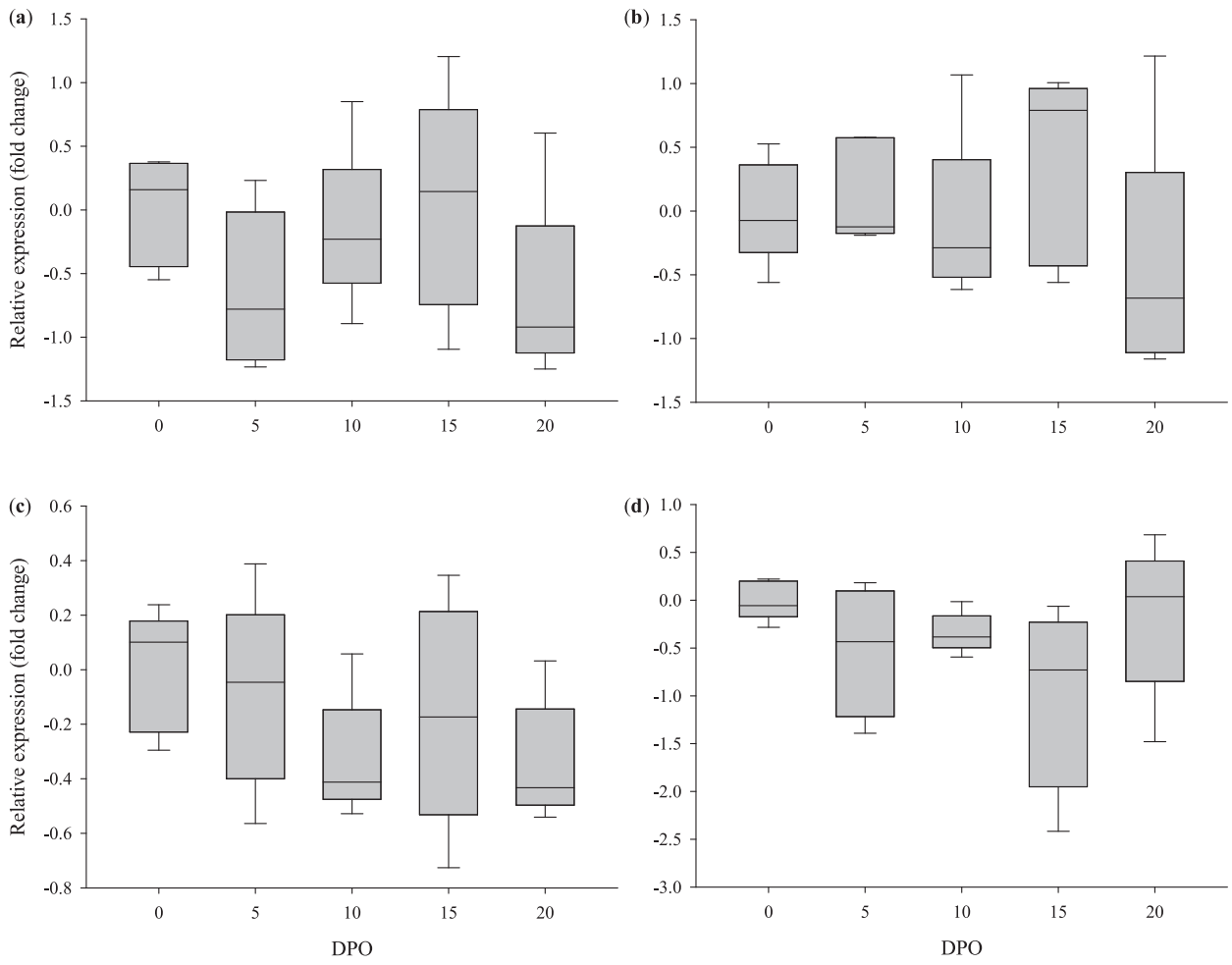

Fig. 8. mRNA expression of genes associated with defence against oxidative stress; (a) *sod1*, (b) *sod2*, (c) *cata*, and (d) *hsp70* in newly stripped eggs from female broodstock during post-ovulatory ageing expressed as fold change relative to 0 DPO. Data were normalised using the geometric mean of *b2m*, *ef1a* and *rpl1* reference genes. Data are expressed as means ± SD (6 eggs × 5 dams × 5 DPO) and different superscripts denote significant differences between DPO ($p < 0.05$; Linear Mixed Effects model).

lower (0.017%, Jørgensen et al., 2018). In rainbow trout eggs incubated at 12 °C, over 50% spontaneous triploids were found after 14 DPO (Aegerter and Jalabert, 2004). Exposing these eggs to triploid induction will most likely result in mortalities, as suggested in the present study.

Overall survival from hatch to the end of the trial was comparable between ploidy irrespective of POA status, although a DPO effect was observed between hatch to first feeding and a ploidy effect was found from first feeding to end of the trial. POA did not appear to have an impact on fry weight at first feeding although diploids appeared to have higher body weight than triploids as previously reported (Galbreath et al., 1994; McGeachy et al., 1995; Taylor et al., 2011). Subsequent growth was affected as triploids exhibited a slower growth rate than diploids in 0 and 5 DPO groups but were comparable in 10 and 15 DPO groups. POA had no effect on the growth rate of diploids, but triploids had a greater growth rate in 10 and 15 DPO groups compared to 5 DPO. The growth rates were reflected in the average final weights and lengths of the fish, however, the population variance appeared to be affected especially with a larger variance in triploid length. A decrease in condition factor was also observed in both ploidy with increasing ageing. Results confirmed the hypothesis that there is a POA effect resulting in a larger spread of fish size. However, at the parr stage, fish from the five experimental dams were pooled according to ageing status and thus this variation observed may also be a result of family influence.

As reported in Mommens et al. (2015), an increased occurrence of opercula shortening was observed with oocyte ageing in which they concluded stocking density was the likely cause. In the present study, opercula shortening occurrence increased in diploids with POA while in triploids this remained stable irrespective of egg ageing. While the stocking density was comparable for 0, 5 and 10 DPO groups, there was a lower number stocked for 15 DPO due to increased egg mortality prior to stocking. Despite this, there was no difference between diploid and triploid tanks, which suggests that stocking density was not the cause of opercula shortening occurrence. Samarin et al. (2016) showed increased abnormalities of Northern pike (*Esox lucius*) larvae in response to POA, however it was only opercula shortening in diploid juveniles in the present study, which appeared to be affected. Mommens et al. (2015) also reported an increase in cranial malformations of juveniles in response to POA, however this could not be investigated in the present study. It appears that malformations in juveniles that may have resulted from POA were not associated with jaw or vertebral deformities. Further studies on the impact of POA on specific deformities should be performed.

POA and ploidy clearly impacted fish performance in the present study and the identification of informative biomarkers of quality in "green' eggs would be very useful. Previous investigations have shown a decrease ovarian fluid pH in response to increased POA in salmonids (Lahnsteiner et al., 1999; Lahnsteiner, 2000; Aegerter and Jalabert, 2004; Bahre Kazemi et al., 2010; Mommens et al., 2015). In contrast, pH increased in the present study from 0 to 5 DPO and remained constant thereafter. A decrease in pH is likely a result of the breakdown of the egg

membrane which allows the egg content to infiltrate the ovarian fluid (Dietrich et al., 2007). The comparable pH from 5 to 20 DPO in this study suggests no cell membrane breakdown occurred during these ageing stages. Mommens et al. (2015) found a decrease in pH only between 22 and 28 DPO, which was a longer duration than any of the treatments in the present study. Ovarian fluid osmolality increased linearly with DPO and correlated positively with egg diameter in both ploidies. An increase in osmolality was also observed in the study by Mommens et al. (2015), who suggested potential leakage of osmolytes from over-ripe eggs. However, contrasting results were reported in rainbow trout with no osmolality changes shown in some studies (Lahnsteiner, 2000; Aegerter and Jalabert, 2004), and an effect of POA suggested by Rime et al. (2004) potentially indicating egg protein fragments accumulate in the fluid in response to POA. Another suggestion could be water ingression into the eggs. As explained earlier, when eggs are immersed in water, or in this case held in the ovarian fluid for prolonged periods of time, water can enter the egg via the micropylar canal (Billard and Cosson, 1992), resulting in a higher concentration of solute in the ovarian fluid. An increase in viscosity of the ovarian fluid may further reduce the sperm motility and impact on fertilisation.

Proteomic analysis of the ovarian fluid was performed in the current study and a total of 52 proteins were identified and quantified including several proteins showing significant changes in abundance during POA. In general terms, proteins in this biofluid originate from secretory epithelia in the ovaries and filtered blood plasma and might contain also proteins leaked from the oocytes. From a biomarker perspective, proteins that are secreted, extracellular or are leaked from the oocytes might be particularly informative specially within the context of POA. Versican core protein, an extracellular proteoglycan that binds and stabilises hyaluronan, and provides structure to tissues (Russell and Robker, 2007) was significantly lower in ovarian fluid as POA progressed. This protein is essential for final oocyte maturation and ovulation and was also reported to protect cells from apoptosis induced by oxidative stress (Russell and Robker, 2007). Another protein that decreased with POA was inhibin alpha chain, which is a secreted protein that has been previously reported to be involved in mediating gonadotropin- and MIH-induced final oocyte maturation in zebrafish (Wu et al., 2000). On the other hand, other proteins directly associated in reproduction that were significantly impacted by POA included the zona pellucida sperm-binding protein 4-like (ZP4) and vitellogenin. ZP4 is a component of the zona pellucida, an extracellular protective matrix surrounding oocytes which mediates sperm binding and may act as a sperm receptor in vertebrates (Killingbeck and Swanson, 2018). Results obtained in the present study (up to 2.1 down-regulation of ZP4 in ovarian fluid during POA) contrast with previous results obtained in zebrafish in which several ZP proteins were upregulated in eggs of poor quality (Yilmaz et al., 2017). Further investigations linking ZP proteins from eggs and ovarian fluid to reproductive success are therefore required. Finally, vitellogenin increased in the ovarian fluid in all POA groups compared to 0 DPO as already reported in previous studies (Rime et al., 2004). Increased vitellogenin fragments, as suggested by Rime et al. (2004) are likely associated with leakage from some of the oocyte components into the ovarian fluid suggesting that the abundance of vitellogenin fragments could be a reliable indicator of POA. While results from the proteomic analysis need to be validated, the data obtained does indicate that there are significant changes in the protein makeup of the ovarian fluid during POA that could be used as biomarkers for egg quality and even more importantly that changes in protein composition are most likely associated with reproductive success as highlighted by other authors (Johnson et al., 2020; Rime et al., 2004).

In the present study, proximate as well as lipid, mineral and pigment composition of eggs obtained from the different DPO treatments was performed. Surprisingly, the moisture content of the eggs did not increase with POA. This may be explained by (i) the low volume of water that is able to enter an egg and/or (ii) when processing egg samples for moisture content, if not dried efficiently, it may be possible that ovarian fluid remains. Similarly, no effect was found in energy, total lipid or associated fatty acid composition. Differences were found in protein and energy content between DPO groups, however no trend was observed. Although total lipid was not impacted by POA, the composition of lipid classes changed. The neutral: polar lipid ratio decreased with egg ripening which was driven by either an increase in phosphate groups or a decrease in diacylglycerol (DAG) and triacylglycerol (TAG). Fraser (1989) discussed how fish larvae catabolise yolk reserves to maintain basal metabolism when experiencing environmental stressors. This energy reserve is predominantly comprised of TAG, which is an important lipid storage utilised for early development in Atlantic salmon alevins (Cowey et al., 1985). Therefore, the decrease in TAG observed with POA may be a factor explaining poor survival and growth performance. A decrease in TAG levels was also evident in *Salmo trutta caspius* eggs over 30 DPO (Bahre Kazemi et al., 2010). It is likely that increases in reactive oxygen substances (ROS), particularly hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and peroxynitrite ($ONOO^-$), are important factors of POA, inducing oxidative stress and lipid peroxidation (Lord and Aitken, 2013; Samarin et al., 2015). Lipid peroxidation has a detrimental impact on cell membranes and enzymatic activity (Lord and Aitken, 2013). Changes to these normal functions are likely causing reduced survival and abnormal development as the decrease of neutral: polar lipid ratio was correlated with the decrease in egg survival in triploids in the present study. Also, if membrane fluidity is compromised, this may reduce the efficiency of sperm-egg interaction. Thiobarbituric acid reactive substances (TBARS) are formed as a by-product of lipid peroxidation and an increase was observed in response to POA in the current study, which correlated with the oxidation observed in the decrease in neutral: polar lipid ratio. The connection between oxidative stress and oocyte ageing appears to be well-established, however the impact on antioxidant activity is less explored and warrants further studies.

Several antioxidants are present in fish eggs to prevent such oxidative stress. Although not the primary function, carotenoids can serve as an antioxidant as they scavenge and stabilize singlet oxygen and peroxyl radicals (Palace and Werner, 2006). In the present study, individual carotenoids and total carotenoid content were consistently higher at 0 DPO. Vitamin E is also an important antioxidant in teleost eggs (Palace and Werner, 2006). Like carotenoid content, vitamin E content decreased in response to POA in the present study. Fish are unable to synthesis their own carotenoids and vitamin E and therefore enough maternally deposited levels must be present in the yolk reserves to protect them from ROS experienced with increasing POA. The oxidation process and potentially antioxidant function may serve as useful biomarkers in determining the future health of an egg batch. Nevertheless, in our study we did not find a significant correlation between POA and the expression of detoxification genes involved in oxidative stress neutralisation, which agrees with recent investigations in goldfish (Samarin et al., 2019b). However, a set of genes involved in energy metabolism (*atp5f1c*, *atp5f1* and *coxII*) significantly decreased with POA in the current study. Similarly, in mouse oocytes, a decrease in the expression of ATP-related genes has also been observed (Hamatani et al., 2004). These results support Samarin et al. (2018) hypothesis, which links oocyte ageing with the onset of mitochondrial dysfunction. Apoptosis is another cellular process that seems to be upregulated in ageing oocytes. Cell death is the final stage of the oocyte ageing process and consequently the expression of molecules involved in apoptosis regulation is expected. Previous research in fish species have reported higher transcript levels of apoptotic genes in over-ripened eggs (Lord et al., 2013; Samarin et al., 2018, 2019a) whereas the expression of anti-apoptotic genes decreased with eggs ageing in mice (Gordo et al., 2002) and pigs (Ma et al., 2005). In our study, we also observed a significant decrease in the expression of an anti-apoptotic gene (*bclx*) with POA in salmon eggs. In addition, the expression of a cyclin (*ccnb3*) was also downregulated in salmon eggs from 10 DPO. Contradictory results have been reported regarding the expression of genes involved in cell cycling related to oocyte ageing. In rainbow trout, previous investigations have

observed an upregulation of cyclins with the ageing process (Aegerter et al., 2005) whereas other studies in mice have reported a decreased expression of cell cycle-related genes in aged oocytes (Xu et al., 1997). Therefore, the impact of POA on the regulation of cell cycling genes in salmon eggs requires further investigation.

5. Conclusions

Post-ovulatory ageing appeared to have little effects on most biochemical analyses performed on oocytes however differences in several parameters were shown (e.g. TBARS, vitamin E, some gene transcripts). Between 10 and 15 DPO, there appeared to be a significant oxidation of lipids with decreased neutral: polar lipid ratio and increased TBARS. These results agree with those of Mommens et al. (2015), who suggested that oocyte quality in Atlantic salmon remains stable for up to two weeks post-ovulation. Egg survival and fertilisation were negatively impacted by POA, however with no differences between ploidy. A difference in performance of juveniles was observed with triploids showing improved average weights and condition factor with increased POA. This may be a result from increased triploid mortality in 10 and 20 DPO during the egg stages subsequently leaving the most robust and better performing individuals. However, variation in size within the triploid groups was greater than in diploids and both ploidies had a decreasing condition factor in response to POA. The increased variation in triploid size poses significant stock management issues. It would be beneficial to fertilise eggs for triploid production within 5 days post-ovulation to reduce the consequences associated with a higher variation in fish size. Triploids may be more sensitive to POA, which could potentially be a confounding factor with the shock associated with triploid induction. A reliable biomarker which may predict higher mortalities and increased growth variation would be advantageous for triploid Atlantic salmon aquaculture. Lipid peroxidation and the secondary by-product TBARS levels in unfertilised eggs may be used as an indication of the future survival and performance of a triploid batch. In addition, several gene (egg) and protein (ovarian fluid) markers appear to be related to POA and indirectly egg quality although their reliability as egg quality predictors must be further investigated. New data from the present study can be applied to industry protocols in order to improve the quality of egg batches, with triploids in particular, by ensuring the time to stripping post-ovulation is kept to within 10 days, but preferably soon after ovulation for optimal oocyte quality.

CRediT authorship contribution statement

M. Clarkson: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft. **J.F. Taylor:** Conceptualization, Methodology, Formal analysis, Investigation, Writing – review & editing, Supervision. **J.C. Mota-Velasco:** Conceptualization, Resources. **A. Hamilton:** Conceptualization, Resources. **A. Albalat:** Formal analysis, Writing – review & editing. **B. Morro:** Data curation. **W. Mullen:** Formal analysis. **A. Latosinska:** Formal analysis. **L. Chalmers:** Formal analysis. **L.M. Vera:** Formal analysis. **H. Migaud:** Conceptualization, Resources, Methodology, Validation, Investigation, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Herve Migaud reports financial support was provided by Innovate UK. Herve Migaud reports financial support was provided by Biotechnology and Biological Sciences Research Council.

Data availability

The authors do not have permission to share data.

Acknowledgements

The authors acknowledge funding from the SALMOTRIP+ project (INNOVATE UK/BBSRC Grant Award 38306-273244). Additional funding for MC was provided by BBSRC grant (BB/M013049/1, SALMOTRIP+: Optimisation and implementation of sterile triploid salmon in Scotland).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2023.740305.

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