



Changes in water temperature and oxygen: the effect of triploidy on performance and metabolism in large farmed Atlantic salmon

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ABSTRACT: In salmon farming, the use of sterile triploids (3N) can mitigate the problem of escapees interbreeding with wild salmon. However, triploid salmon appear less tolerant to high water temperatures and low oxygen levels compared to diploids (2N). To investigate how the thermal performance and physiology of large (2.5 kg) triploid Atlantic salmon *Salmo salar* L. differs from those of diploids, both ploidies were subjected to water temperatures between 3 and 18°C. The fish were exposed to reduced oxygen saturations ($O_{2\text{sat}}$ 70%), termed hypoxia, at 6 and 18°C. Triploids fed more than diploids between 3 and 9°C and at similar levels at 12°C. At 15°C, the feed intake significantly dropped in both ploidies, although more in triploids. During hypoxia, feed intake was higher in triploids at 6°C and equal to diploids at 18°C. The overall feed conversion ratio was similar between ploidies. Muscle energy phosphates were generally lower in triploids than diploids, while muscle glucose, blood haemoglobin and haematocrit were lower in triploids than diploids at $\geq 12^\circ\text{C}$. Plasma lactate levels tended to be higher in triploids and increased with increasing temperature and at hypoxia in both ploidies. Plasma cortisol increased in both ploidies at high temperatures and was highest in triploids under hypoxic conditions at 18°C. Triploids had a higher cataract score at the start of the experiment and developed more cataracts throughout the experiment. The present findings show that large triploid Atlantic salmon perform better at colder water temperatures compared to diploids and differ in parts of their physiological expression at increasing and high temperature.

KEY WORDS: Ploidy · Sterile · Growth · Feed intake · Thermal optimum · Energy metabolism · *Salmo salar*

INTRODUCTION

The use of sterile triploid Atlantic salmon *Salmo salar* L. mitigates genetic interactions between escaped farmed and wild fish and minimises unwanted sexual maturation before harvest (Taranger et al. 2010). In Norway, the number of escaped farmed

Atlantic salmon has ranged from 38 000 to 921 000 per year between 2005 and 2015 (Directorate of Fisheries Norway 2016). This has caused concerns regarding genetic interactions with wild stocks (Glover et al. 2012), and highlights the incentives for the use of sterile salmon in commercial aquaculture (Benfey 2016). Although the production of triploid salmon

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first started in the 1970s, implementation has been slow. The main concerns have been lower growth rates (Jungalwalla 1991, Friars et al. 2001, Taylor et al. 2013), reduced stress tolerance (Fraser et al. 2015) and increased mortality, particularly under suboptimal environmental conditions, when compared to diploids (Ojolic et al. 1995, Cotter et al. 2002, Hyndman et al. 2003b, Hansen et al. 2015, Sambraus et al. 2017a,b).

Most of the salmon grow-out phase takes place in seawater in floating net pens that have direct water exchange with the local environment. Therefore, the fish are exposed to large seasonal variations in water temperature that usually ranges between 2 and 19°C along the Norwegian coast (Oppedal et al. 2011). The lower and upper temperature limits for feeding in diploid Atlantic salmon are reported to be between 0 and 7, and 22 and 28°C, respectively (reviewed by Elliott & Elliott 2010), while the thermal optimum is between 13 and 19°C (Handeland et al. 2008, Hevrøy et al. 2013). The temperature ranges occur due to the inverse relationship between optimum temperature and body size in salmonids (McCauley & Huggins 1979). Previous studies have shown comparable growth rates in diploid and triploid Atlantic salmon during their first year at sea (Galbreath & Thorgaard 1995, Leclercq et al. 2011, Fjellidal et al. 2016), but this may be dependent on water temperature. For example, Sambraus et al. (2017a) recently demonstrated that triploid Atlantic salmon post-smolts (300 to 900 g) had higher feed intake than diploids at water temperatures between 3 and 9°C, but fed less than diploids at 15 and 18°C. To date, the thermal optimum of large triploid Atlantic salmon with regards to feed intake and growth is unknown.

A second environmental factor that may vary within open net pens is the concentration of oxygen ($\text{mg O}_2 \text{l}^{-1}$) in the water, which usually correlates with temperature. The environmental conditions in the net pens sets the limit for physiological function and production performance of the fish, primarily through their effects on fish metabolism (Fry 1971). Low levels of O_2 in the water will eventually lower aerobic capacity, nutrient digestion and growth. In commercial size net pens, the oxygen saturation ($\text{O}_{2\text{sat}}$, % of air saturation) has been found to vary between 30 and 130% (Crampton et al. 2003, Johansson et al. 2006, 2007). Under practical farming conditions, low O_2 concentrations, hypoxia, is most prevalent during periods with high water temperature (Oppedal et al. 2011, Burt et al. 2012), when the solubility of oxygen is low and fish metabolism is high. The combination of high water temperature and low $\text{O}_{2\text{sat}}$ has been

found to amplify the negative effect of triploidy on feed intake and growth in Atlantic salmon post-smolts, both under chronic (Hansen et al. 2015) and temporary reductions in $\text{O}_{2\text{sat}}$ (Sambraus et al. 2017a). Metabolic activity and hence oxygen demand is known to increase with temperature (Fry 1971) and decrease with increasing fish size and age (Forsberg 1994). Oxygen consumption has been documented in diploid post-smolts (Remen et al. 2012) and adult salmon (Lee et al. 2003) as well as in triploid post-smolts (Sambraus et al. 2017a). However, it is unknown how triploidy affects the response of large fish to reductions in $\text{O}_{2\text{sat}}$ at different temperatures. Physiological and clinical parameters are important when considering temperature tolerance in large diploid and triploid Atlantic salmon. For instance, haematocrit, plasma glucose, lactate and ions, along with white muscle creatine phosphate (CrP), adenosine triphosphate (ATP) and glucose have shown a response to increasing temperature in diploid and triploid Atlantic salmon post-smolts (Sambraus et al. 2017a) and brook trout *Salvelinus fontinalis* (Hyndman et al. 2003a,b) that may be enhanced in larger fish at the same temperatures.

Another concern with triploid salmon are higher incidences of ocular cataracts compared to diploids (Wall & Richards 1992, Taylor et al. 2015, Sambraus et al. 2017b). The pathology of this disorder in diploid salmon becomes more severe at high (Waagbø et al. 2010) and fluctuating (Bjerkås & Bjørnstad 1999) water temperature as well as in periods of fast growth (Bjerkås et al. 2001, Waagbø et al. 2010). In salmonids, as visual feeders, severe cataracts and poor vision may reduce welfare and feed intake, and limit affected fish in reaching their growth potential (Breck & Sveier 2001). Dietary histidine levels can mitigate cataract outbreaks in both ploidies, although the requirement is higher in triploids than diploids (Taylor et al. 2015, Sambraus et al. 2017b). The positive affect of histidine is associated with its metabolite N-acetyl-histidine (NAH) that functions as an osmolyte (Rhodes et al. 2010) and potentially an antioxidant (Remø et al. 2011) in the lens. In commercial Atlantic salmon aquaculture, severe cataracts can result in substantial financial losses (Menzies et al. 2002).

Regarding a previous experiment on the temperature effects of diploid and triploid post-smolts (Sambraus et al. 2017a), large Atlantic salmon are expected to perform similarly to the earlier life stage, although may have a lower thermal optimum for feed intake. In order to reduce the impact of escaped farmed salmon on wild stocks, the use of sterile

triploids is desirable. However, the industry may only convert to triploid Atlantic salmon if their performance is similar. The aim of the present study was to examine differences in performance parameters with an emphasis on commercial interest and the physiological status of large triploid Atlantic salmon compared to diploids. Both ploidies were exposed to a wide range of water temperatures, from 3 to 18°C, with additional periods of reduced oxygen saturation, 70 instead of 100% $O_{2\text{sat}}$ at 6 and 18°C. We investigated parameters related to production performance, white muscle energy stores, blood chemistry and ocular health.

MATERIALS AND METHODS

Fish stock and rearing conditions

The experiment was conducted at the Institute of Marine Research (IMR), Matre, Norway and approved by the Norwegian Animal Research Authority, in accordance with prevailing animal welfare regulations.

Fish eggs were provided by Aquagen (Trondheim, Norway) on 3 November 2009 ($n = 209\,700$). To induce triploidy, half of the eggs were subjected to hydrostatic pressure of 655 bar for 6 min and 15 s (TRC-APV, Aqua Pressure Vessel, TRC Hydraulics) at 37 min and 30 s after fertilisation at 8°C. The eggs of each ploidy were incubated separately in incubation trays (Sternier 2003, Sternier Fish Tech) in an isolated (UV treatment) flow-through system under darkness. Hatching started on 5 February 2010, and was completed by 18 February. On 26 April 2010, the yolk sack larvae of each ploidy were put into three 1.5 m fibreglass tanks under continuous light. The fish were continuously fed with a commercial start feed (NUTRA ST 0.5, Skretting) that increased in size up to 1.5 mm (Skretting). On 22 July 2010, the fish from each tank were mixed within ploidy and transferred to single, green, circular 3 m fibreglass tanks. To allow the fish to smolt as yearling smolts, the photoperiod was switched from 24 h light:0 h dark to simulated natural on 1 October. On 27 October 2010, all fish were vaccinated (Minova 6 Vet., Norvax(r), Intervet International) and transferred to larger single, green, 5 m fibreglass tanks. On 28 March 2011, a subsample of 2500 fish of each ploidy was transferred into separate tanks of the same type and reared until the change to seawater on 10 May 2011. In seawater, the fish were reared under continuous light until transfer to the experimental tanks.

The mean temperature in the period from fertilisation to start-feeding was 4.9°C. From start-feeding until the summer solstice (21 June 2010), the fish were reared in heated freshwater (mean 12.7°C) and changed to ambient freshwater until seawater transfer (mean 7.9°C, max. 15.3°C, min. 3.0°C). The ambient seawater temperature (10 May 2011 to 22 August 2012) ranged between 8.0 and 8.6°C. The oxygen saturation in the outlet was kept above 80% $O_{2\text{sat}}$ from hatching until transfer to the experimental tanks.

Triploidy was verified by measuring erythrocytes prior to the experiment (Benfey et al. 1984). Ten randomly chosen red blood cells of each blood smear sampled from 80 diploid and 80 triploid fish were measured (Image-Pro Plus, version 4.0, Media Cybernetics). The individual red blood cell diameter in triploids (mean 20.8 μm , min. 19.8 μm) had a larger mean and the range did not overlap with that of diploids (mean 16.8 μm , max. 18.1 μm), suggesting triploidisation occurred with 100% efficiency.

Experimental facilities

On 23 August 2012, mixed sex Atlantic salmon ($n = 120$ fish per ploidy) were measured for length and weight, and individually pit tagged (Trovan transponders ID 101, BTS Scandinavia) before being separated by ploidy and randomly distributed into 6 circular indoor tanks (40 fish per tank; 3 tanks per ploidy; 3 m diameter; 6300 l; 80 l min^{-1}). During the acclimation period and the experiment, all tanks were exposed to 24 h continuous light (Philips, TL-D 36W/ 33-640). Customised computer software (SD Matre, Normatic) controlled and regulated flow (Promag W flow meters, Endress and Hauser), temperature (TST 487-1A2B temperature probes), salinity (Liquisys MCLM223/ 253 probes), oxygen (Oxyguard 420 probe, Oxyguard International) and automatic feeding (Arvo-Tec T drum 2000). Until the start of the experiment on 13 September 2012 (Day 1) the fish were given an acclimation period in 34‰ seawater ($9.3 \pm 1^\circ\text{C}$). Oxygen levels were mainly regulated by the water flow, although supported by supersaturated water (400% O_2). Oxygen probes in the outflow of each tank were air calibrated weekly, taking temperature changes and atmospheric pressure variations into account. All water parameters were continuously measured and recorded as a mean of 5 min intervals per tank. The oxygen saturation was kept at 100% in the outflow during normoxia.

Feeding and experimental design

The fish were fed a commercial diet (Nutra Olympic 7 mm, Skretting) twice a day for 30 min (08:30 h; 14:00 h), aiming for 10 to 20% overfeeding per meal to ensure satiation. A sieve positioned in the outflow of each tank from the start of feeding to 10 min post feeding collected uneaten pellets. The sieve was removed from the outflow and left to air dry for 10 min to let excessive water drip off, and the content was subsequently weighed. The daily feed intake per tank was calculated by considering the amount of food supplied and the dry matter content of air-dried feed as well as the amount of collected uneaten pellets and the dry matter content of those. Due to the varying stability of feed pellets at different water temperatures, a recovery rate (%) of water-exposed pellets was included for each temperature the fish were exposed to (Helland et al. 1996).

Fifteen days after the start of the experiment, the temperature was decreased from 9 down to 6°C for 1 wk and then decreased again down to 3°C for 1 wk (Fig. 1). Thereafter, the temperature was increased by 3°C wk⁻¹ until it reached 18°C. Temperature changes were induced by the automated system at night. One additional week each at 6°C (Days 37 to 42) and at 18°C (Days 72 to 77) were included during which the fish were subjected to hypoxia. The water O_{2 sat} was decreased from 100% by 10% d⁻¹ until 70% O_{2 sat} was reached and this was maintained for 3.5 d until sampling. Hypoxia was induced by reduc-

ing the water inflow and closing the addition of supersaturated water. After both samplings at the hypoxic periods, the water O_{2 sat} was again increased to 100% in the outflow. After the final sampling (18_h°C, h: hypoxic conditions), the temperature gradually decreased from 18 to 9°C by 1°C d⁻¹ in order to examine how the fish recovered.

At the start of the experiment, 3 diploid and 1 triploid fish were removed due to lesions. During the experiment, 2 triploid fish did not recover from an anaesthetic bath after the sampling at 18°C. These fish were excluded from mortality calculations.

Sampling procedures

At the start of the experiment on 13 September 2012, all fish were weighed, measured and examined for cataracts. Samplings for white muscle, blood and plasma were conducted postprandially at 3°C (Day 29), 6°C (Day 36), 6_h°C (hypoxia; Day 42), 9°C (Day 50), 12°C (Day 57), 15°C (Day 64), 18°C (Day 71) and 18_h°C (hypoxia; Day 77). In order to minimise fish stress and maintain their physiological parameters, the water level was reduced and all fish were lightly anaesthetised (25 mg l⁻¹ Finquel®, Scanaqua) prior to netting individuals. After netting, each fish was killed by a blow to the head. A cooling pack stored at -80°C was placed with light pressure on the left fillet side. With a heparinised syringe (Heparin LEO, LEO Pharma), a blood sample was drawn from the caudal vein and divided into subsamples.

One part was instantly frozen in liquid nitrogen as whole blood, while the other was centrifuged for 2 min at 13 000 rpm (11 228 g) to allow blood and plasma to separate, which were subsequently separately frozen in liquid nitrogen. After the blood sample was drawn, the cooling unit was removed, and a sample of white muscle was taken from underneath the dorsal fin and immediately frozen in liquid nitrogen. Time from netting to flesh freezing was approximately 1 min. All samples were finally stored at -80°C until further analysis. The procedure was conducted with 3 fish per tank for each sampling. On Day 50, the sampled fish (n = 9 per ploidy) were examined for cataracts, and on Day 71, an additional 9 fish per ploidy (n = 18 per ploidy) were examined for cataracts to

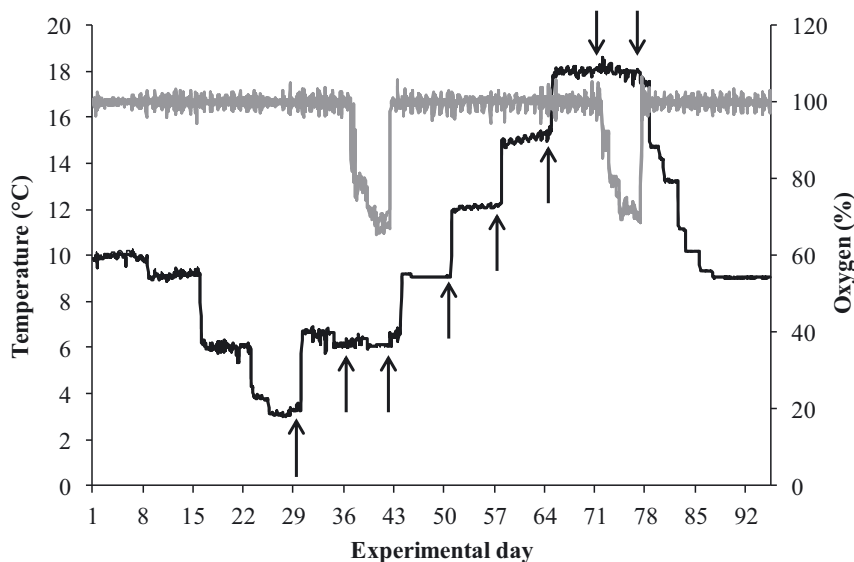


Fig. 1. Water temperature (black line) and water oxygen saturation (grey line; n = 24 measurements per day) in experimental tanks with large diploid and triploid Atlantic salmon *Salmo salar* in seawater. Arrows indicate the sampling time points

allow for individual comparison to the start of the experiment. At these 2 sampling points, lenses were dissected and additional white muscle samples taken for lens and muscle-free basic amino acid analysis.

To avoid additional handling, the whole fish stock was only measured and weighed at tank stocking, the start of the experiment and at the last sampling point at 18_n°C (Day 77). After the start of the experiment, handling and stress was reduced to a minimum in order to let the fish behave and feed undisturbed throughout the temperature changes. The experiment was terminated on 17 December 2012 (Day 96).

Measurements

Growth and feed conversion ratio

Body mass (to 10 g) and fork length (to 1 cm) were measured for each sampled fish. The specific growth rate (SGR, growth % d⁻¹) was calculated as $SGR = (e^q - 1) \times 100$ (Houde & Scheckter 1981), where $q = [\ln(W_2) - \ln(W_1)] (t_2 - t_1)^{-1}$ (Bagenal & Tesch 1978). W_2 and W_1 represent the live body weight at time points t_2 (sampling) and t_1 (start of the experiment), respectively. The condition factor (CF) was calculated as $CF = \text{weight [g]} / \text{length}^3 [\text{cm}] \times 100$. The feed conversion ratio (FCR) was calculated as $FCR = \text{feed intake [g]} / \text{weight gain [g]}$.

Muscle preparation and analysis (CrP, ATP, glycogen)

White muscle samples were directly transferred from -80°C into a freeze-drying unit at -55°C and dried for 3 d. The dried samples were superficially cleaned of blood and thoroughly ground with mortar and pestle into a fine powder and again stored at -80°C until analysis. CrP, ATP and glycogen were analysed as described by Solstorm et al. (2015). Briefly, for CrP and ATP analysis, perchloric acid was mixed with a sample of ground white muscle and immersed in an ultrasonic ice bath. The samples were mixed, neutralised and centrifuged, and the supernatant was filtered and transferred into HPLC glass vials for analysis that was performed instantly using a modified version of the method described by Sellevold et al. (1986) and Volonté et al. (2004). The samples were separated on a C₁₈ HPLC column at 30°C and the mobile phase was set at a constant flow rate (1.3 ml min⁻¹). The samples were recorded on a diode array at 215 nm. Stock solutions were prepared

in perchloric acid and diluted with the mobile phase to standard concentrations (CrP 0.400 µmol ml⁻¹, ATP 0.190 µmol ml⁻¹). The standard curves were created by increasing the injection volumes from 10 to 50 µl. After dilution with the mobile phase, the solution was stable for 30 min.

For glycogen extraction, HCl was added to a freeze-dried and ground white muscle sample and hydrolysed. The samples were centrifuged, and the supernatant was separated and frozen at -80°C. Another sample of freeze-dried, ground white muscle was mixed with HCl and thoroughly mixed to extract free glucose. After centrifugation, the supernatant was separated and frozen at -80°C. After thawing, all samples were neutralised and diluted in buffer to ensure a neutral pH balance. Analysis was performed in duplicates with the Cayman's Glucose Colorimetric Assay Kit (Cayman Chemical Company). The individual results were adjusted for exact ground sample weight and dilution volumes. Glycogen was calculated by subtracting free glucose concentrations. Due to a technical failure, the samples from the 6_n and 9°C samplings were lost.

Haematology

Haematocrit was measured by centrifuging whole blood heparinised capillary tubes for 2 min (Stat-Spin® Multipurpose Centrifuge). Whole blood haemoglobin concentrations were analysed with the QuantiChrom™ Haemoglobin Assay Kit (BioAssay Systems). Plasma cortisol was analysed with Cortisol Parameter Assay Kits (Cortisol ELISA, RE 52061, IBL). Plasma ion levels (Na⁺, K⁺, Cl⁻) were detected by ion selective electrodes, plasma triacylglycerols (TAG), glucose and lactate were analysed with standard kits using a COBAS c111 auto analyser (Roche Diagnostics). A pH meter (Radiometer PHM92 with PHC 3395-8 plasma electrode) was used to determine plasma pH.

Cataracts, and lens and muscle-free amino acids

Cataracts were detected with a Kowa SL-15 slit-lamp biomicroscope (Kowa Company) and scored according to the grading scale of Wall & Richards (1992). Depending on the degree of opacification, each lens was given a score from 0 to 4 and each fish a score from 0 to 8. Lens NAH was analysed as described by Breck (2004), adapted from O'Dowd et al. (1990), by reverse phase HPLC (Waters Corpora-

tion). Lens and white muscle-free basic amino acids were analysed according to Breck et al. (2005) by amino ninhydrin detection (Amersham Pharmacia Biotech) with Biochrom 20 plus (Biochrome).

Oxygen consumption

The measurements for oxygen consumption were performed as described by Sambraus et al. (2017a). In brief, oxygen consumption was estimated using the experimental tanks as open respirometers. The days for measuring the oxygen consumption were the first and the second last day of each temperature period. On those days, the inflow of oxygenated water was stopped for 30 min (11:15 to 11:45 h), leading to a gradual decline in oxygen content. Oxygen consumption rate (MO_2) was estimated from the equation:

$$MO_{2t} = \left(\text{Vol} \cdot \text{Sol} \cdot \frac{\text{Sat}_t - \text{Sat}_{t-1}}{\delta t \cdot 100} + \text{Flow} \cdot \text{Sol} \cdot \frac{100 - \text{Sat}_t}{100} + O_{2\text{flux}} \right) \cdot \text{biomass}^{-1} \quad (1)$$

where Vol is the tank volume (l, excluding fish volume), Sol is the solubility of oxygen at the prevailing temperature and conductivity (mg l^{-1}), and Sat_t is the oxygen saturation (%) at time t (the average saturation for 1 min intervals was used, $\delta t = 1$ min). Flow is the rate at which new aerated water replaces the tank water (l min^{-1}). Biomass is the estimated biomass of fish (kg) in the tank on the day of oxygen consumption measurements. The biomass for each tank is calculated by dividing the accumulated feed intake until the day of measurement by the individual FCR for each tank and then adding the measured tank biomass from the start of the experiment minus dead and sampled fish. Only the average consumption rate of the last 15 min of the 30 min period was used for the calculation. The oxygen flux ($O_{2\text{flux}}$) over the water surface during the progressive decline in oxygen was modeled as:

$$O_{2\text{flux}} = k \cdot (100 - \text{Sat}_t) \cdot \text{Sol} \cdot \frac{\text{Vol}}{100} \quad (2)$$

where k is the diffusion constant. The value of k was estimated by first oxygen-stripping the water in fishless tanks using N_2 gas and then measuring the $O_{2\text{sat}}$ increase as water exchanged with normoxic water at exchange rates matching those of the fish tanks (Remen et al. 2013). By inserting the O_2 flux equation into the MO_2 equation and letting $MO_2 = 0$, the increase in oxygen saturation after oxygen-stripping was modelled with different values of k .

The value of $k = 0.00135$ maximised the correlation between the observed and modelled increase in oxygen saturation after oxygen-stripping ($R^2 = 0.9997$).

Statistical analyses

All statistical analyses were conducted using Statistica™ 12.0 (Dell). Fish weight, length, condition factor and cataract score at stocking and at the start of the experiment were tested by 2-way nested ANOVAs with tank replicate (random effect) nested in ploidy. The effect of ploidy on growth performance, feed intake, white muscle energy parameters, blood and blood plasma, amino acids and lens-related parameters were tested using nested ANOVA designs with tank replicate as random effect, nested in ploidy and temperature period. Significant nested ANOVAs were followed by factorial ANOVA designs with ploidy and temperature period as categorical variables to detect possible interactions and followed by Student-Newman-Keuls (SNK) post hoc tests to detect differences within and between diploids and triploids at the different temperatures. A significance level of 5% ($p < 0.05$) was used for all tests. All data were tested for normality using a Kolmogorov-Smirnov test prior to statistical analysis. Homoscedasticity of all data was inspected visually. Two variables, plasma triglycerides and plasma lactate, were log-transformed in order to ensure normality and homoscedasticity.

RESULTS

Effect of temperature and $O_{2\text{sat}}$ on feed intake, oxygen consumption and growth

At stocking, there was no difference between both ploidies in body weight (2N: 2253 ± 34 g; 3N: 2248 ± 56 g) or length (2N: 57.3 ± 0.3 cm; 3N: 58.1 ± 0.5 cm), but triploids had a significantly lower condition factor than diploids (2N: 1.18 ± 0.00 ; 3N: 1.14 ± 0.00 ; $p < 0.001$; 2-way nested ANOVA). At the end of the acclimation period (9°C), triploids were significantly heavier (2N: 2307 ± 29 g; 3N: 2494 ± 63 g; $p = 0.015$), longer (2N: 57.7 ± 0.2 cm; 3N: 59.0 ± 0.5 cm; $p = 0.002$) and had a higher condition factor (2N: 1.18 ± 0.00 ; 3N: 1.21 ± 0.00 ; $p = 0.022$, 2-way nested ANOVA) than diploids.

There was an effect of temperature and an interaction between temperature and ploidy on mean daily

Table 1. p-values of growth performance and condition (specific growth rate, length growth, condition factor, mean daily feed intake, oxygen consumption), white muscle (creatine phosphate [CrP], ATP, glycogen), blood (haemoglobin, haematocrit) and plasma parameters (cortisol, chloride, sodium, triacylglycerol, glucose, lactate and pH) in Atlantic salmon *Salmo salar* for Figs. 2 to 7. Each parameter was tested in a nested ANOVA design (temperature [T], ploidy [P]) with tank as random factor, nested in ploidy and sampling point (temperature). Significant nested ANOVAs were followed by factorial ANOVAs (T × P) to detect possible interactions between the treatments. DM: dry mass. Values in **bold** indicate a significant effect (p < 0.05)

Parameter	Nested ANOVA		Factorial ANOVA
	T	P	T × P
Mean daily feed intake (% biomass)	<0.001	0.689	<0.001
Oxygen consumption (mg O ₂ kg ⁻¹ min ⁻¹)	<0.001	0.268	0.820
Specific growth rate (% growth d ⁻¹)	<0.001	<0.001	0.021
Length growth (mm d ⁻¹)	<0.001	<0.001	0.409
Condition factor	<0.001	0.033	<0.001
CrP (μmol g DM ⁻¹)	<0.001	<0.001	0.985
ATP (μmol g DM ⁻¹)	<0.001	<0.001	0.569
Glycogen (μmol g DM ⁻¹)	<0.001	0.011	0.011
Haemoglobin (mg dl ⁻¹)	<0.001	<0.001	0.152
Haematocrit (%)	<0.001	<0.001	0.197
Cortisol (ng ml ⁻¹)	<0.001	0.582	<0.001
Chloride (mmol l ⁻¹)	<0.001	0.329	0.538
Sodium (mmol l ⁻¹)	<0.001	0.991	0.370
Potassium (mmol l ⁻¹)	0.851	0.748	0.549
Triacylglycerol (mmol l ⁻¹)	<0.001	0.150	0.304
Glucose (mmol l ⁻¹)	<0.001	0.038	0.085
Lactate (mg dl ⁻¹)	<0.001	0.065	0.893
pH	<0.001	0.560	0.360

feed intake (Table 1). Excluding the hypoxic periods, feeding increased from 3 to 12°C and decreased with further increasing temperature in both ploidies (Fig. 2A). Triploids had higher feed intake than diploids at the initial 9°C period and the second period at 6°C, while diploids fed more at 15 and 18°C, when feeding dropped more in triploids than diploids.

Reducing O_{2 sat} from 100 to 70% at 6°C had no negative effect on the feed intake in either ploidy (Fig. 2A; see Fig. S1A in the Supplement at www.int-res.com/articles/suppl/q010p157_supp.pdf). However, the same reduction in O_{2 sat} at 18°C caused a significant decrease in feed intake in both ploidies that was more pronounced in diploids than in triploids (Fig. 2A; see Fig. S1B in the Supplement). In the recovery period after 18_h°C, there was no effect of ploidy on feed intake (see Fig. S1C in the Supplement). The feed conversion ratio from the start of the experiment to the final sampling was 1.00 ± 0.03 and 1.01 ± 0.02 in diploids and triploids, respectively.

The oxygen consumption rate (Fig. 2B) was affected by temperature (Table 1) and increased from 3 to 9°C and again to 15°C in both ploidies.

There was a significant effect of temperature and ploidy on the SGR (Fig. 3A), length growth (mm d⁻¹; Fig. 3B) and the condition factor (Fig. 3C), and of the interaction between temperature and ploidy on the SGR and the condition factor (Table 1). Triploids had higher SGRs than diploids from the start of the experiment until the second sampling at 6°C (Fig. 3A), and faster length growth than diploids at 18°C. For all remaining fish that were measured at 18_h°C, there was no effect of ploidy on the SGR in diploid and triploid males (nested ANOVA, p = 0.814) and females (p = 0.721). The condition factor was significantly affected by temperature and ploidy, and there was an interaction between both parameters (Table 1). Triploids had a significant higher condition factor than diploids at 3 and 9°C.

Mortality was low with only 2 diploid fish during the acclimation period and 1 diploid (15°C) and 1 triploid (18°C) fish during the experiment.

White muscle energy analyses

White muscle energy phosphates (CrP and ATP) and glycogen were significantly affected by temperature period and ploidy (Fig. 4A–C, Table 1). Muscle CrP concentrations decreased from 3 to 6°C in both ploidies, and also from 6 to 18_h°C in triploids. ATP levels in diploids were not affected by temperature, whereas triploid fish had higher levels at 3 and 12°C compared to 18°C, and levels at 15°C were lower compared to diploids. There was an effect of the interaction between temperature and ploidy on muscle glycogen and levels at 12°C were higher in diploids than triploids (Table 1, Fig. 4C).

Haematology

There was a significant effect of temperature and ploidy on blood haemoglobin (Fig. 5A) and haematocrit (Fig. 5B, Table 1). With increasing temperature, haemoglobin increased in diploids (3 to 15°C and 9 to 18°C), but not triploids. Haematocrit increased in both ploidies, although this was more amplified in diploids than triploids. Diploids had higher haemoglo-

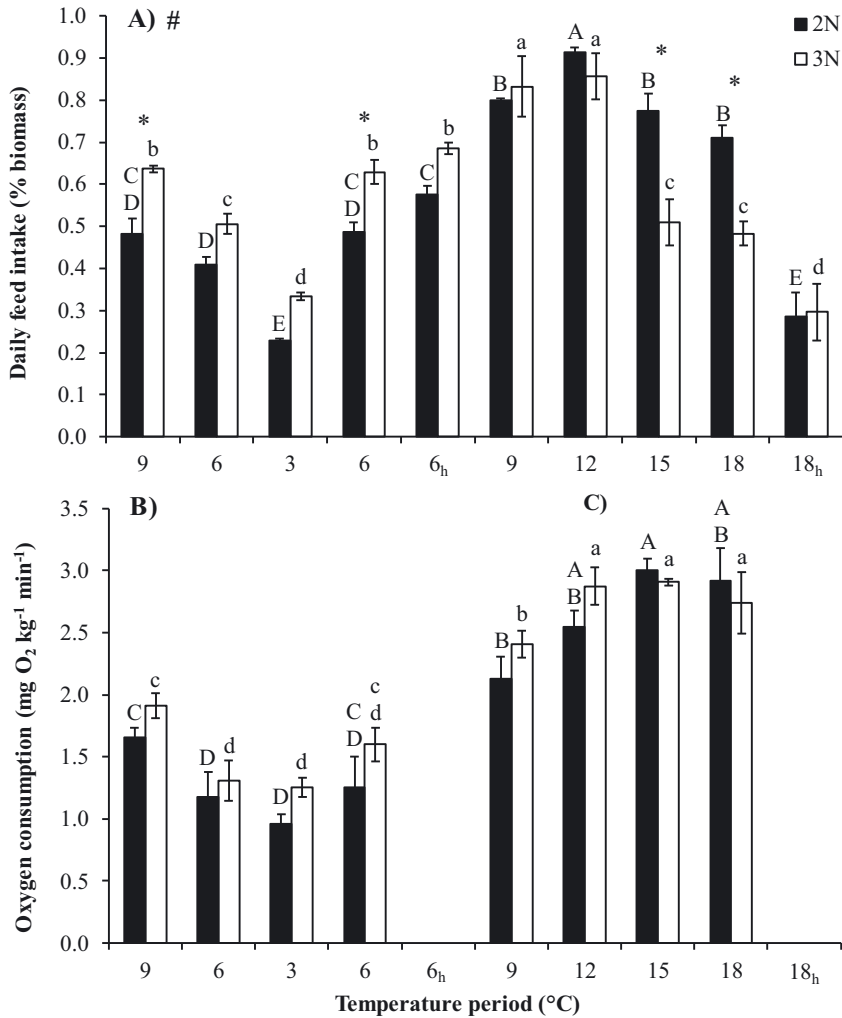


Fig. 2. Mean \pm SE (A) daily feed intake and (B) oxygen consumption in triplicate groups of large diploid and triploid Atlantic salmon *Salmo salar* in seawater. The oxygen consumption was not measured during hypoxic periods (h). Different uppercase (lowercase) letters indicate significant differences between temperature periods in diploids (triploids). *Significant difference between ploidies at the specific sampling point (Student-Newman-Keuls, $p < 0.05$); #: significant interaction between temperature period and ploidies (factorial ANOVA, $p < 0.05$)

bin concentrations than triploids at 18°C and higher haematocrit at 15 and 18_h°C. Both ploidies had a significant increase in haemoglobin and haematocrit when O_2 sat was decreased from 100 to 70% at 18°C (Fig. 5A,B).

Plasma clinical chemistry

There was a significant effect of temperature on plasma cortisol (Fig. 6A), chloride (Fig. 6B) and sodium (Fig. 6C, Table 1). Plasma cortisol increased in both ploidies when the temperature increased

from 15 to 18°C and more so in diploids, resulting in higher levels at 18°C. When O_2 sat was decreased from 100 to 70% at 18°C, ploidies differed as plasma cortisol in triploids increased. Plasma chloride was lowest in both groups at 9°C and levels in triploids were higher during hypoxia at 18°C compared to normoxia. Plasma sodium increased from 3 to 6°C in both ploidies and from 15 to 18°C in diploids. During hypoxia, levels in both groups were lower compared to normoxia at 6°C. Plasma potassium was not affected by temperature period or ploidy and the values ranged between 2.67 (15°C) and 3.00 mM (18°C) (2.82 ± 0.10 mM; mean \pm SD) in diploids and 2.74 (12°C) and 3.04 mM (18_h°C) (2.85 ± 0.12 mM) in triploids (data not shown).

There was a significant effect of temperature on plasma TAG (Fig. 7A), glucose (Fig. 7B), lactate (Fig. 7C) and pH (Fig. 7D), and a significant effect of ploidy on plasma glucose (Table 1). In triploids, TAG was higher at 6 and 9°C compared to 15 and 18°C. Plasma glucose in triploids increased from normoxia to hypoxia at 18°C and levels were higher compared to diploids. Plasma lactate increased from 15 to 18°C in diploids and from 15 to 18_h°C in triploids. The plasma pH in both ploidies was higher at 9°C than 6 and 12°C, and lower at 18°C than 15°C in triploids. Compared to normoxia, hypoxia resulted in a lower blood pH at 6°C in triploids and 18°C in diploids.

Cataract development, and lens and muscle-free amino acid composition

At the start of the experiment, triploids had a significantly higher mean cataract score compared to diploids (nested ANOVA, $p < 0.001$). Diploids ($n = 118$) had a mean score of 0.5 ± 0.0 , and the mean score of affected individuals was 1.2 ± 0.1 , representing 40.3% of the diploid population. The respective scores in triploids ($n = 120$) were 2.1 ± 0.1 and 2.5 ± 0.1 (85.8%). The cataract assessment at the end of the 9°C period on Day 50 ($n = 9$ per ploidy), using a subsample of the fish that were evaluated at the start

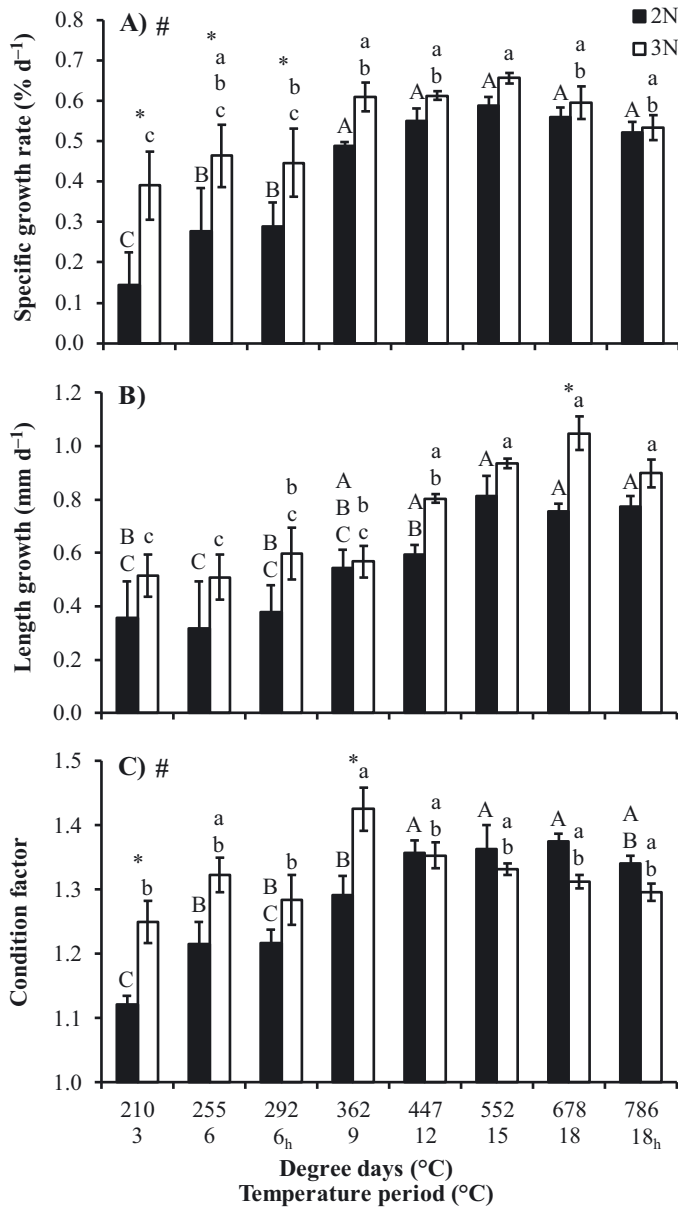


Fig. 3. Mean ± SE (A) specific growth rate, (B) length growth and (C) condition factor of large diploid and triploid Atlantic salmon *Salmo salar* in seawater. See Fig. 2 for explanation of symbols

of the experiment, showed that diploid individuals had a lower cataract score compared to the start, while triploids had an increase in the sum score from 2.1 ± 0.2 to 3.0 ± 0.6 (Table 2). A new subsample on Day 71 (n = 18 per ploidy) revealed an individual increase in cataract score in triploids from 2.5 ± 0.3 at the start of the experiment to 3.1 ± 0.2 , in contrast to a reduced mean cataract score in diploids (0.5 ± 0.1 to 0.4 ± 0.1). The cataract occurrence on Day 50 was positively correlated with the individual SGR in tri-

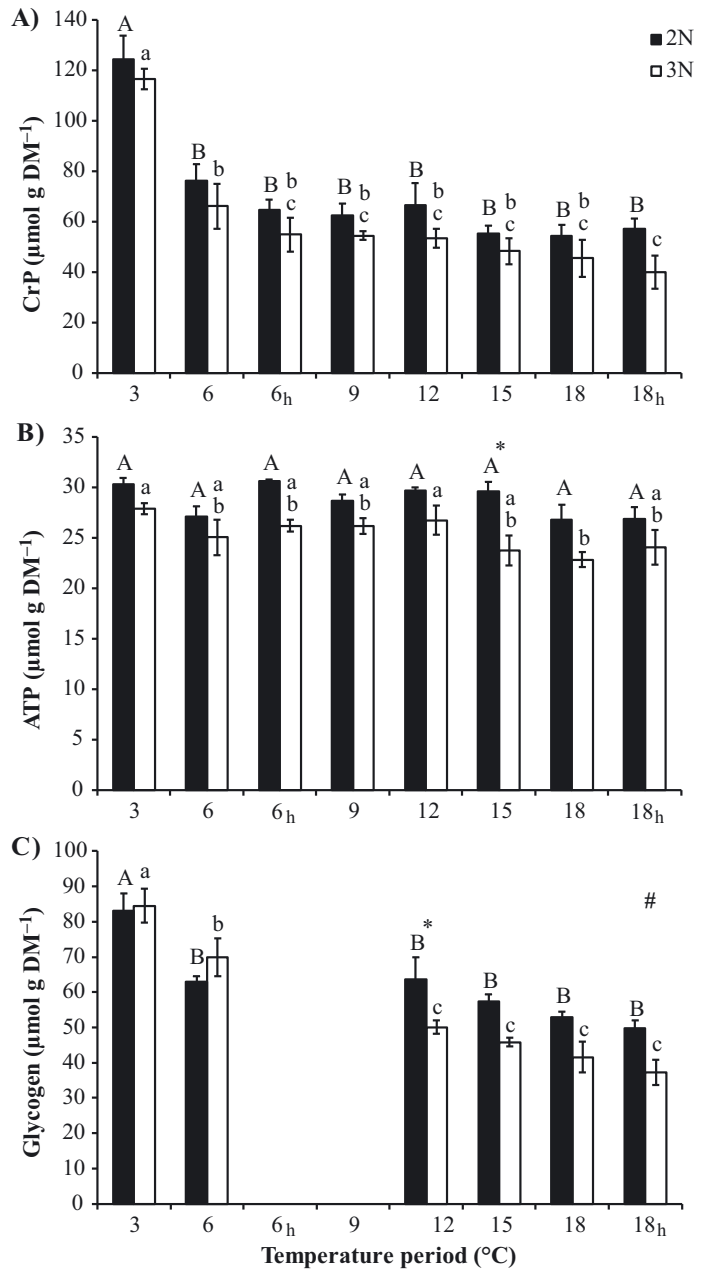


Fig. 4. Mean ± SE white muscle (A) creatine phosphate (CrP), (B) adenosine triphosphate (ATP) and (C) glycogen levels in large diploid and triploid Atlantic salmon *Salmo salar* in seawater. n.d.: no data due to technical failure. DM: dry mass. See Fig. 2 for explanation of symbols

ploids ($y = 11.727x - 4.2965$; $R^2 = 0.5583$). There was no difference in lens histidine and NAH between ploidies at both samplings (Table 2).

In triploids, histidine was significantly elevated among white muscle-free basic amino acids at both sampling points (Table 2). Carnosine was significantly higher in triploids than diploids at 9°C on Day 50, but not at 18°C on Day 71. There was no sig-

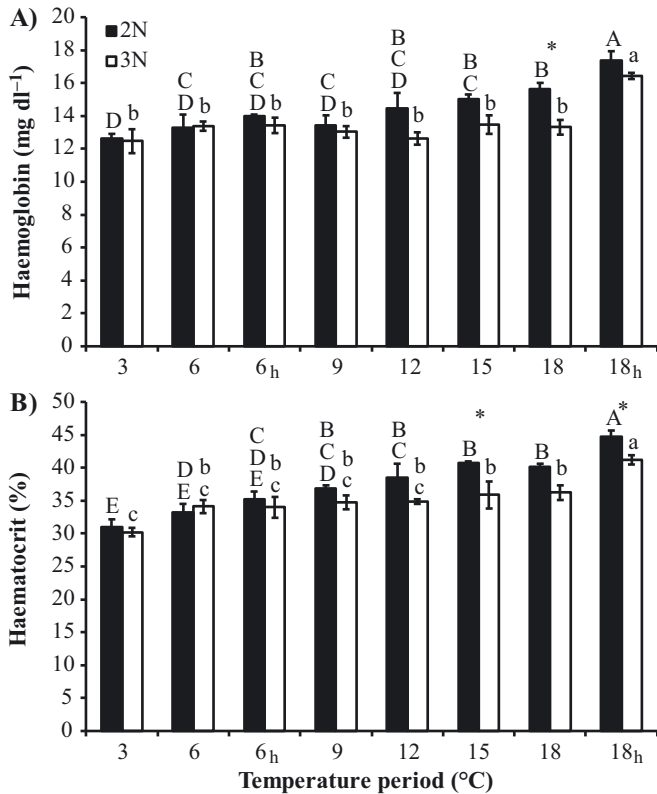


Fig. 5. Mean \pm SE blood (A) haemoglobin and (B) haematocrit levels in large diploid and triploid Atlantic salmon *Salmo salar* in seawater. See Fig. 2 for explanation of symbols

nificant difference between ploidy levels in the remaining amino acids at either sampling point (Table 2).

DISCUSSION

Our objective was to determine how large triploid Atlantic salmon perform under a broad range of temperatures and periods of reduced oxygen compared to diploids. For many of our growth and physiological indicators, we found that triploids performed as well as or better than diploids between 3 and 12°C, including under hypoxia. At and above 15°C, triploids performed worse compared to diploids with regards to feed intake and showed initial signs of physiological stress and limitations, although without mortalities, during the most challenging period. Therefore, farming triploids would appear a viable alternative to diploids in areas where temperatures do not exceed 15°C for prolonged periods.

We found triploids had greater feed intake than diploids at lower temperatures, but lower feed intake at higher temperatures. The same ploidy effect with

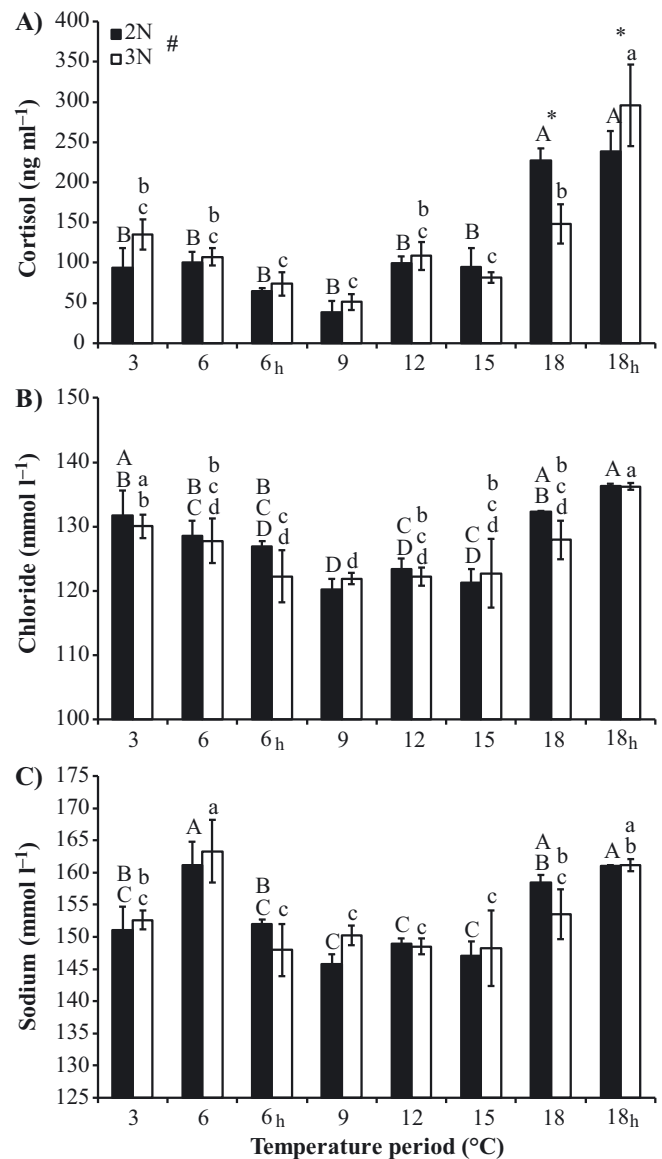


Fig. 6. Mean \pm SE plasma (A) cortisol, (B) chloride and (C) sodium levels in large diploid and triploid Atlantic salmon *Salmo salar* in seawater. See Fig. 2 for explanation of symbols

temperatures has been observed before, but appears more pronounced in the current study on large fish compared to previous work on post-smolts (Hansen et al. 2015, Sambraus et al. 2017a). This is most likely due to the narrower thermal window of larger compared to juvenile fish (Pörtner & Farrell 2008). We found no ploidy effect on the FCR, similar to previous studies with salmonids (Habicht et al. 1994, Yamamoto & Iida 1994, Hansen et al. 2015), although Fraser et al. (2013) reported a lower biological FCR in triploids when reared in seawater. Feed conversion in triploids may differ at different temperatures. This

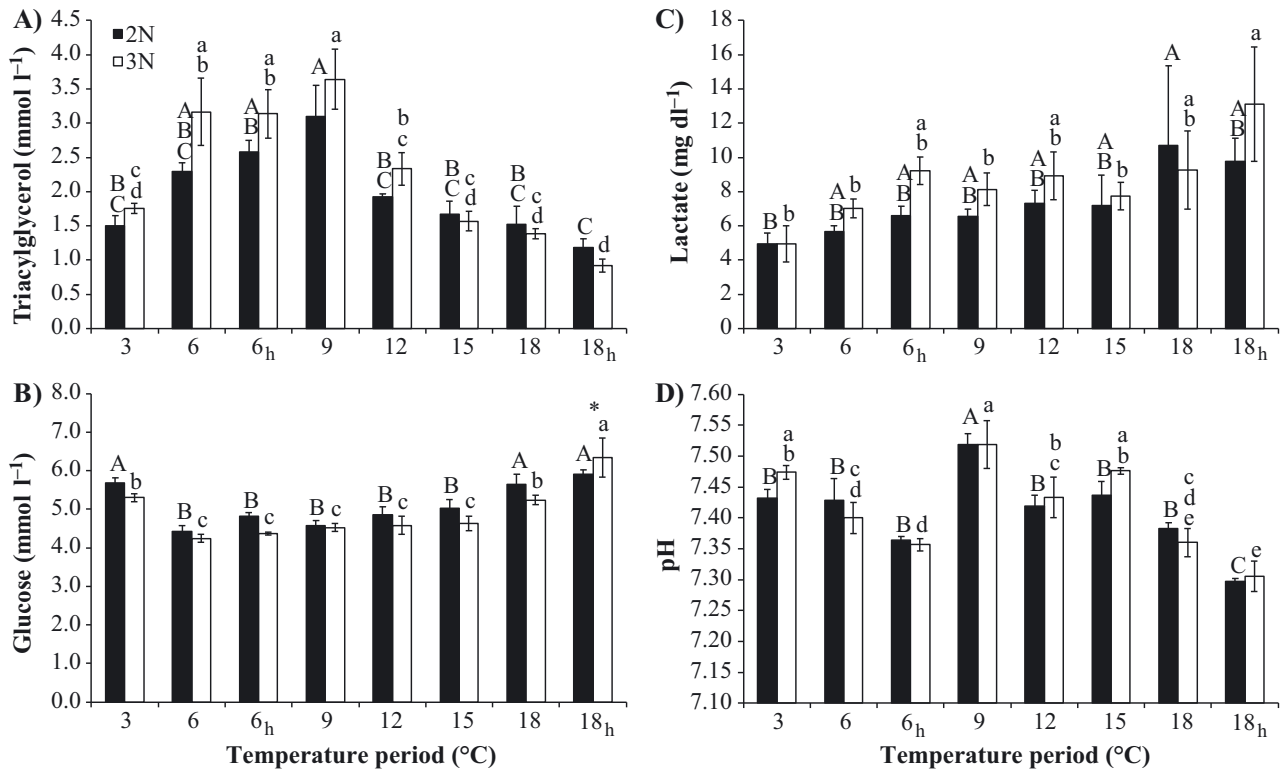


Fig. 7. Mean ± SE plasma (A) triacylglycerol, (B) glucose, (C) lactate and (D) pH levels in large diploid and triploid Atlantic salmon *Salmo salar* in seawater. See Fig. 2 for explanation of symbols

Table 2. Cataract score (0 to 8) and mean ± SE lens and muscle-free basic amino acids (μmol g⁻¹) on Day 50 (9°C) and 71 (18°C) in triplicate groups of diploid and triploid Atlantic salmon *Salmo salar* in seawater. The cataract score for Day 0 is the mean score for the same fish. NAH: N-acetyl-histidine. p-values in **bold** indicate significantly different means between ploidies on the given day (p < 0.05)

Sample	Parameter	Day 50			Day 71		
		Diploid	Triploid	p-value	Diploid	Triploid	p-value
Lens	Cataract score (Day 0)	0.3 ± 0.2	2.1 ± 0.2	<0.001	0.5 ± 0.1	2.5 ± 0.3	<0.001
	Cataract score	0.0 ± 0.0	3.0 ± 0.6	<0.001	0.4 ± 0.1	3.1 ± 0.2	<0.001
	Histidine	0.70 ± 0.10	0.83 ± 0.08	0.820	0.74 ± 0.12	0.80 ± 0.07	0.670
	NAH	7.48 ± 0.62	6.75 ± 0.53	0.436	7.69 ± 0.71	6.59 ± 0.70	0.644
Muscle	Histidine	0.55 ± 0.12	0.94 ± 0.07	0.006	0.75 ± 0.03	1.08 ± 0.06	0.024
	Anserine	15.0 ± 0.4	15.1 ± 0.2	0.973	14.9 ± 0.3	14.6 ± 0.2	0.520
	Carnosine	0.51 ± 0.07	0.77 ± 0.09	0.008	0.19 ± 0.02	0.13 ± 0.01	0.501
	Ornithine	0.04 ± 0.00	0.04 ± 0.01	0.527	0.03 ± 0.01	0.04 ± 0.01	0.695
	Arginine	0.19 ± 0.01	0.24 ± 0.02	0.134	0.13 ± 0.00	0.16 ± 0.02	0.246
	Lysine	0.59 ± 0.03	0.88 ± 0.16	0.120	0.73 ± 0.11	0.96 ± 0.05	0.261

could not be measured under the present setup, but it has been documented in diploid Atlantic salmon (Handeland et al. 2008), indicating a need for further research on feeding efficiency.

The specific growth rate reflected the feed intake and the difference between ploidies decreased with

an increasing number of degree days at higher water temperatures. This was concomitant with a shift from a higher to lower mean condition factor in triploids. Triploidy in salmonids is often associated with a lower condition factor during the seawater phase compared to diploids (Leclercq et al. 2011, Taylor et

al. 2015, Fjellidal et al. 2016, Smedley et al. 2016). The reasons for this may be multifactorial and can include weight loss due to smoltification (Sambraus et al. 2017b) or, as observed in the present study, increasing length growth when weight growth is stagnating or decreasing. Nevertheless, the present data on feed intake, growth rate and condition factor show that triploids can outgrow diploid Atlantic salmon at cold and moderate water temperatures.

In the present study, large triploids had the highest feed intake at 9 and 12°C compared to 12°C in diploids. The optimum temperature for growth in diploid Atlantic salmon has been found to be 14°C in post-smolts (170 to 300 g, Handeland et al. 2008) and $\leq 13^\circ\text{C}$ in fish of 1.6 kg (Hevrøy et al. 2013), compared to 12°C in triploids (Sambraus et al. 2017a). The present findings not only confirm a lower temperature optimum in triploids, but are also in accordance with a negative relationship between optimal temperature for feed intake and fish size.

Current theory suggests that the upper temperature limit in fish is determined by metabolic processes. Higher temperature increases metabolism and oxygen consumption, and hence reduces the scope for activity. Once routine metabolism is higher than the capacity for oxygen uptake, the capacity for feeding and hence the scope for growth decreases. Further, when energy costs exceed energy intake, the fish may become hypoxic if feeding continues; therefore, feeding activity is reduced (Claireaux et al. 2000, Hevrøy et al. 2012, Remen et al. 2012). The more pronounced drop in feed intake from 12 to 15°C in triploids compared to diploids in the present study also points to a lower temperature optimum in triploids. Although the feed intake in triploids was only two-thirds of that of diploids at 15 and 18°C, both ploidies had similar oxygen consumption rates at those temperatures. As triploids also had lower levels of muscle glycogen than diploids at $\geq 12^\circ\text{C}$, this indicates that, under a regime of increasing temperature, triploids begin to utilise endogenous energy sources at an earlier point, i.e. at a colder temperature, than diploids. Atkins & Benfey (2008) demonstrated that the routine metabolic rate in triploid Atlantic salmon is higher at low temperatures and lower at high temperatures when compared to diploids. Although we found no differences in the present study, we did see a similar trend that may however also be influenced by feed intake and specific dynamic action, respectively.

Lowering the O_2 sat from 100 to 70% had no negative effects on feeding at 6°C, only at 18°C, when both ploidies significantly reduced feeding but eventually fed at similar rates during hypoxia and the recovery

period at 100% O_2 sat thereafter (see Fig. S1 in the Supplement). This contrasts with previous work where hypoxic conditions at similar high temperatures reduced feeding more in triploid than diploid post-smolts (Hansen et al. 2015, Sambraus et al. 2017a). Furthermore, there was no mortality recorded in the present study when environmental conditions were challenging compared to the high mortality in other studies that exposed triploid salmonids to suboptimal conditions such as high temperature and/or reduced oxygen (Ojolick et al. 1995, Hyndman et al. 2003b, Hansen et al. 2015, Sambraus et al. 2017a). This may indicate an increased tolerance to hypoxia at later life stages in triploids or solely the fact that the intensity in the present experiment (70% O_2 sat) was lower compared to the chronic (70% O_2 sat) or more intense exposure (60% O_2 sat) to hypoxia, or the higher temperatures in the studies mentioned above.

Triploid salmon had significantly lower concentrations of white muscle energy phosphates compared to diploids. This would result in a reduced ability to cope with exercise in triploids (Hyndman et al. 2003b). Triploid salmon may have limited capacity to up-regulate CrP directly and/or the enzyme creatine kinase to produce sufficient CrP when ATP is available. Further, there was no hypoxia effect in either ploidy on these parameters at 6 or 18°C, as previously found by Sambraus et al. (2017a). Triploid Atlantic salmon post-smolts also responded with decreasing muscle CrP concentrations at high temperatures (18°C), although the exposure to hypoxia (60% O_2 sat) had no further effect (Sambraus et al. 2017a). Muscle glycogen decreased in both ploidies with increasing temperature and was higher in diploids at water temperatures of 12°C and above. The observed decrease in muscle glycogen between 6 and 18°C in triploids is of the same order of magnitude as that observed in triploid post-smolts within the same temperature range (Sambraus et al. 2017a). Temperature also had an effect on white muscle glycogen concentrations in resting triploid brook trout that had approximately 12% less glycogen at 19 than 9°C (Hyndman et al. 2003a,b). It appears that at cold temperatures and low metabolism, triploids successfully maintain muscle glycogen. However, at higher temperatures, the metabolism may utilise more, especially at times when feed intake in triploids is lower than in diploids.

There was a temperature and ploidy effect on blood haemoglobin and haematocrit in which diploids had higher concentrations than triploids with increasing water temperature. This has implications for the oxygen carrying capacity and responds to a reduced metabolic scope in triploids at high temperatures as

previously suggested for triploid chinook salmon *Oncorhynchus tshawytscha* (Bernier et al. 2004). In the literature, blood haemoglobin concentrations in triploid salmonids have been reported to be both equal (Stillwell & Benfey 1994, 1996) and lower (Benfey & Sutterlin 1984, Small & Randall 1989, Yamamoto & Iida 1994, Kobayashi et al. 2009) compared to diploids, as has the blood-oxygen affinity (Graham et al. 1985, Verhille & Farrell 2012). However, lower blood haemoglobin concentrations in triploids, as measured particularly at high water temperatures in the present study, ultimately limits oxygen transport to the tissues. Because efficient ATP synthesis is highly dependent on an oxygen-rich aerobic environment, this may be a cause for lower ATP and subsequently CrP levels in triploids. These factors make triploids susceptible to warm temperatures and may explain increased mortality in previous studies with such conditions (e.g. Ojolic et al. 1995, Hyndman et al. 2003b, Hansen et al. 2015, Sambras et al. 2017a).

Under normoxic conditions, plasma lactate concentrations were similar in both ploidies, but lactate tended to be higher in triploids during hypoxia. Triploidy has been found to have little to no effect on plasma lactate in brook trout (Hyndman et al. 2003a) and Atlantic salmon (Sadler et al. 2000, Fraser et al. 2014, Sambras et al. 2017a). The large variation in plasma lactate levels in triploids at 18°C and hypoxia gives reason to assume that there may be a wide variation in tolerance within the population, families or individuals. It has been shown that family has a significant effect on different performance parameters (Friars et al. 2001) and also on the tolerance level of temperature and hypoxia (Anttila et al. 2013) in salmonids. As triploid families perform similarly well as diploids (Taylor et al. 2013), it may be possible to selectively breed triploid families that are more tolerant to hypoxia.

Growth rates, haematological and plasma parameters, and the lack of considerable mortalities, support the assumption that the fish in the present study were somewhat stressed, although within a zone of physiological tolerance. Nonetheless, the primary (plasma cortisol) and secondary (plasma glucose) stress responses were significantly higher in triploids compared to diploids at the combination of high temperatures and hypoxia. The stress responses in diploids and triploids have been reported to be similar after stressful events in brook trout (Biron & Benfey 1994, Benfey & Biron 2000) and Atlantic salmon (Sadler et al. 2001, Fraser et al. 2014), in experiments conducted at moderate water temperatures and following physical stress. It is possible that high mortality in

previous experiments, where triploid salmonids were exposed to challenging chronic or acute conditions, is attributable to a combination of high stress and altered energy metabolism and haematology when the water temperature is high.

The occurrence of cataracts at the start of the experiment and also the development of lens opacities thereafter was higher in triploids than diploids, which is in accordance with previous studies (Leclercq et al. 2011, Taylor et al. 2013, 2015, Sambras et al. 2017b). This did not only include the incidence of cataract-affected fish, which was higher in triploids, but also the severity when compared to diploids. The fact that both diploid subsamples had a lower cataract score during the experiment compared to the start of the experiment suggests a reversible type of cataract that may have been induced by an osmotic disturbance (e.g. due to anaesthetics) at the initial sampling. Causes of cataracts in Atlantic salmon can be multifactorial, but identified risk factors include smoltification (Waagbø et al. 1996, Bjerkås & Sveier 2004), increasing or elevated seawater temperatures (Bjerkås & Bjørnstad 1999, Bjerkås et al. 2001), accelerated growth (Bjerkås et al. 2001, Breck & Sveier 2001, Waagbø et al. 2010) and nutritional aspects (Bjerkås et al. 2006). Cataracts were more frequently observed in Atlantic salmon in the 1990s after the removal of blood meal from the diets (Wall 1998). Blood meal is rich in the amino acid histidine, and omitting blood meal from the diets reduced the concentration of dietary histidine and thereby tissue imidazoles, such as the cataract-mitigating NAH in the lens (Rhodes et al. 2010, Waagbø et al. 2010). In this study, there was no significant effect of ploidy on either lens histidine or lens NAH concentrations, although mean lens NAH concentrations were over 10% lower in triploids at both sampling points. Low levels of lens NAH have been shown to correlate with cataract formation in both diploid and triploid Atlantic salmon (Waagbø et al. 2010, Taylor et al. 2015), and it is possible that the small difference in lens NAH between ploidies led to a cataract increase in triploids but not diploids. Severe cataract formation has negative effects on feed intake and growth (Breck & Sveier 2001, Ersdal et al. 2001, Breck et al. 2003, Taylor et al. 2015), and therefore not only affects fish welfare, but also economical aspects (Menziés et al. 2002). Both ploidies in the present experiment were fed commercial diets for diploids, but it has been shown that triploid salmon have an altered nutritional requirement for certain nutrients compared to diploids (e.g. phosphorous: Fjellidal et al. 2016, histidine: Tay-

lor et al. 2015, Sambraus et al. 2017b) which may have made triploids more susceptible to cataracts than diploids. With regards to histidine, the demand for adjusted triploid diets particularly applies at times when nutrient requirement is increased e.g. in periods of fast growth or at warm water temperatures (Sambraus et al. 2017b). It is probable that the cataract development in triploids would have been reduced if a diet with elevated concentrations of histidine was fed.

CONCLUSION

The findings of the present study demonstrate that large triploid Atlantic salmon feed and grow equally well or better than diploids at cold and moderate water temperatures, but less so than diploids at 15 and 18°C. When exposed to 70% oxygen saturation at 6 and 18°C, feed intake in triploids was higher than or similar to diploids and no mortalities occurred. This is important for the aquaculture industry to find suitable locations for the grow-out of triploid Atlantic salmon. Triploids had generally less muscle energy stores and, with increasing water temperature, lower haemoglobin and haematocrit levels than diploids. This may impair fish performance and result in mortality under more severe environmental conditions than the ones tested in the present study. Most of the physiological parameters tested did not indicate a significant disadvantage of triploidy in responding to increasing temperature and reduced levels of dissolved oxygen. The low overall mortality and lack of mortality during hypoxia at high temperatures contrast with other studies, but suggest that large triploid salmon are, within thresholds, more tolerant to O_{2sat} fluctuations compared to earlier life stages and thus may substitute diploid Atlantic salmon on a commercial scale.

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