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RESEARCH ARTICLE

Growth hormone transgenesis and polyploidy increase metabolic rate, alter the cardiorespiratory response and influence HSP expression in response to acute hypoxia in Atlantic salmon (*Salmo salar*) yolk-sac alevins

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ABSTRACT

Growth hormone (GH)-transgenic Atlantic salmon display accelerated growth rates compared with non-transgenics. GH-transgenic fish also display cardiorespiratory and metabolic modifications that accompany the increased growth rate. An elevated routine metabolic rate has been described for pre- and post-smolt GH-transgenic salmon that also display improvements in oxygen delivery to support the increased aerobic demand. The early ontogenic effects of GH transgenesis on the respiratory and cellular physiology of fish, especially during adverse environmental conditions, and the effect of polyploidy are unclear. Here, we investigated the effects of GH transgenesis and polyploidy on metabolic, heart and ventilation rates and heat shock protein (HSP) levels after exposure to acute hypoxia in post-hatch Atlantic salmon yolk-sac alevins. Metabolic rate decreased with decreasing partial pressures of oxygen in all genotypes. In normoxia, triploid transgenics displayed the highest mass-specific metabolic rates in comparison to diploid transgenics and non-transgenic triploids, which, in contrast, had higher rates than diploid non-transgenics. In hypoxia, we observed a lower mass-specific metabolic rate in diploid non-transgenics compared with all other genotypes. However, no evidence for improved O_2 uptake through heart or ventilation rate was found. Heart rate decreased in diploid non-transgenics while ventilation rate decreased in both diploid non-transgenics and triploid transgenics in severe hypoxia. Regardless of genotype or treatment, inducible HSP70 was not expressed in alevins. Following hypoxia, the constitutive isoform of HSP70, HSC70, decreased in transgenics and HSP90 expression decreased in all genotypes. These data suggest that physiological changes through GH transgenesis and polyploidy are manifested during early ontogeny in Atlantic salmon.

KEY WORDS: *Salmo salar*, Hypoxia, Growth hormone transgenesis, Polyploidy, Metabolic rate, Oxygen transport, Heat shock proteins

INTRODUCTION

Growth hormone (GH)-transgenic fish have gained increasing attention in the aquaculture industry as a result of accelerated growth rates (~twofold to 10-fold) compared with non-transgenic conspecifics (Devlin et al., 1994; Martinez et al., 1999; Shao Jun et al., 1992; Stokstad, 2002). Physiologically, faster growth entails a higher demand for O_2 through an increase in metabolism. In salmon, from the parr stage onwards, an increase in resting/routine metabolic rate has been verified in GH-transgenic fish (Cook et al., 2000; Lee et al., 2003; Stevens et al., 1998). In association with an increased O_2 demand, modifications to the cardiorespiratory physiology in GH-transgenic Atlantic salmon (*Salmo salar* L.) have been documented that include an increase in heart size, cardiac output, hemoglobin concentration, muscle aerobic enzyme activity (Deitch et al., 2006), gill surface area (Stevens and Sutterlin, 1999) and modifications to cardiorespiratory function (Blier et al., 2002; Cogswell et al., 2002).

Because of the concerns of non-native transgenes impacting wild fish stocks in the event of an escape from commercial aquaculture, it has been suggested that transgenic fish species should be reproductively sterile. In some fish species, including salmonids, polyploidy sporadically occurs as a natural evolutionary trait (Allendorf and Thorgaard, 1984; Ferris, 1984; Schultz, 1980) and is often artificially induced to produce commercial triploid, reproductively sterile progeny. The biological effects associated with triploidy in fish have, in part, been investigated and are mainly attributed to heterozygosity, cell size or gonadal development (Benfey, 1999; Maxime, 2008). As a consequence of the additional complement of chromosomes in triploid organisms, nuclear size is increased in the cells of most organs and tissues, which in turn, causes an increase in cell volume, with overall body size being maintained through hypoplasia in triploids (Benfey, 1999). In theory, sterile, triploid organisms have the potential for greater growth because of the abundance of genetic material as well as the diversion of energy from gonadal growth to somatic growth during sexual maturation. In practice, the evidence supporting increased growth rates in triploid organisms is inconclusive (Tiwary et al., 2004), because both slower (Galbreath et al., 1994) as well as equivalent growth rates have been observed in triploid populations relative to that of diploid conspecifics (McGeachy et al., 1995; Sacobie et al., 2012). In the past, poor performance of triploid fish in comparison to diploids, especially under conditions of high O_2 demand, has been suggested to be related to changes to their respiratory physiology and therefore hypoxia tolerance (Benfey, 1999). However, poor growth performance and increased prevalence of deformities in triploids has been refuted in more recent studies (O'Flynn et al., 1997; Oppedal et al., 2003; Sadler et al., 2001; Taylor et al., 2011), suggesting that triploid salmon can perform as well as diploid

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List of symbols and abbreviations

2n	diploid
3n	triploid
f_H	heart rate
f_V	ventilation rate
GH	growth hormone
HSP	heat shock protein
M	moles of O_2
$\dot{M}_{O_2}$	metabolic rate
$\dot{M}_{O_2}$/g	mass-specific metabolic rate
P_{O_2}	partial pressure of oxygen
Tx	transgenic
Nt	non transgenic
βw_{O_2}	solubility of oxygen in water

siblings. Differences in hematological parameters associated with triploidy, as well as a reduction in gill surface area and metabolism, have been reported for salmonid fish (Benfey, 1999), but the physiological effect of triploidy remains poorly understood. Triploid Atlantic salmon have been documented to display a reduction in O_2 carrying capacity (Graham et al., 1985) whereas triploid rainbow trout show higher rates of O_2 consumption (Oliva-Teles and Kaushik, 1987) and early collapse of the cardiovascular system's ability to deliver O_2 to the body during warming (Verhille et al., 2013). Triploid brook trout have been shown to have lower rates of O_2 consumption in comparison to diploid counterparts (Stillwell and Benfey, 1996).

Respiration in Atlantic salmon yolk-sac alevins at hatching is primarily cutaneous under normoxic conditions (Rombough, 1988; Wells and Pinder, 1996a; Wells and Pinder, 1996b; Pelster, 1999), and at this stage the larvae are exclusively dependent on the energy supply contained within the yolk sac. Hence, both O_2 supply and demand may be under different regulatory control than later stages of development or adulthood, and may be differentially affected by ploidal level or GH transgenesis.

Collectively, these findings suggest that in salmonids, GH transgenesis and triploidy have significant impacts on the O_2 cascade (i.e. the series of diffusive and conductive steps in the pathway of O_2 from the environment to the mitochondria). Further, such impacts might be exacerbated under adverse environmental conditions. In order to cope with the potential damaging and even lethal effects of environmental stress, fish respond with behavioural (e.g. evasion), physiological (e.g. release of stress hormones) and cellular [e.g. induction of heat shock proteins (HSPs)] strategies (Barton, 2002; Leblanc et al., 2012). The cellular stress response, characterized by an increase in the synthesis of HSPs, is generally exhibited by all organisms (Schlesinger, 1990; Feder and Hofmann, 1999). More recently, the temporal expression of HSPs throughout ontogeny has gained increased attention and it has been suggested that HSPs play a key role in early embryonic development in fish (Deane and Woo, 2011). Expression of genes encoding HSPs show spatial and temporal specificity during early development of zebrafish, and in the case of HSP70-4 and HSP90α, they appear to be required for normal tissue development (Krone et al., 2003).

Given that GH-transgenic Atlantic salmon show phenotypic differences in metabolism and cardiorespiratory function and that hypoxia/anoxia elicits a physiological stress response and alters HSP expression (Kregel, 2002), it is likely that genotypic differences resulting in changes to respiration will affect the heat shock response under conditions of low O_2 availability. The cellular stress response to low environmental O_2 levels is variable in fish, and high levels of tissue, cell and temporal specificity in terms of the levels of HSP expression have been reported (Airaksinen and Nikinmaa, 1995; Currie and Tufts, 1997; Currie and Boutilier, 2001; Currie et al., 2010). Furthermore, a decrease in HSP70 mRNA production in hepatic tissue of sparids (family Sparidae) has been reported after exogenous administration of growth hormone (Deane et al., 1999). Despite this body of research, we know very little regarding the effects of ploidy and/or transgenesis on the cellular stress response in fish.

In the present study, we tested for differences in metabolic rate, cardiorespiratory function and the expression of HSPs (HSP70, HSC70 and HSP90) between diploid and triploid, and transgenic and non-transgenic newly hatched Atlantic salmon yolk-sac alevins. We further investigated how these parameters are altered under hypoxic conditions. It was hypothesized that GH transgenesis will lead to accelerated growth rates that are reflected in an increase in oxygen uptake at this early developmental stage. This increase may be supported by improved O_2 delivery through cardiorespiratory modifications, especially when exposed to acute hypoxia. Despite the apparent susceptibility of triploid Atlantic salmon (Benfey, 1999; and citations within) to hypoxia, there is no clear evidence that this is the case in the present study. Therefore, it was hypothesized that the O_2 demand of triploids is unlikely to differ from that of diploids. A disturbance to cellular homeostasis by hypoxic exposure will elicit a cellular stress response that may be augmented by increased anabolic processes or an increased metabolism associated with GH transgenesis.

RESULTS

Treatments and measurements

Measurements of metabolic rate ($\dot{M}_{O_2}$) were performed at 8°C on diploid (2n) and triploid (3n) transgenic (2nTx and 3nTx) as well as 2n and 3n non-transgenic (2nNt and 3nNt) Atlantic salmon yolk-sac alevins [Series I, as described in Polymeropoulos et al. (Polymeropoulos et al., 2013)]. Subsequent measurements were conducted of $\dot{M}_{O_2}$, heart rate (f_H), as well as ventilation rate (f_V; see Series II). Mass-specific $\dot{M}_{O_2}$ ($\dot{M}_{O_2}$/g) was calculated on the basis of yolk-free, wet body mass.

Additionally, we tested the cellular stress response of alevins (see Series III) of the different genotypes (2nNt, 2nTx, 3nNt and 3nTx) to a hypoxic stress similar to that in Series I. Measurements of $\dot{M}_{O_2}$, as described in Series I, were repeated in 2nNt, 2nTx, 3nNt and 3nTx (n=5 for each) alevins at 8°C. Here, the alevins were successively exposed to decreasing levels of O_2 (hypoxic stress) as they consumed the O_2 in closed-system respirometry chambers. Experiments were performed until a P_{O_2} of 5 kPa was reached. After this procedure, experimental animals were transferred to normoxic water for 24 h of recovery. In parallel, 10 alevins per genotype were maintained under identical experimental conditions in normoxia (control group). For this purpose, the alevins were placed in multiwell plates [similar to that described in Polymeropoulos et al. (Polymeropoulos et al., 2013)], submerged in well-aerated water with only a thin mesh separating the alevins from escaping into the surrounding water. The control group was maintained within the same multiwell plate for a duration that matched that of the experimental animals (measurement + 24 h); this included a quick removal from the wells to mimic the handling stress to which the experimental animals were exposed. After the required interval, specimens were taken from the chambers, kept in ice water, measured for length and mass, and then decapitated and kept at −80°C until HSP immunodetection could be performed.

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Table 1. Comparisons of morphometric parameters of newly hatched Atlantic salmon (*Salmo salar*) yolk-sac alevins of different genotypes: diploid (2nNt), triploid (3nNt), diploid transgenic (2nTx) and triploid transgenic (3nTx) reared at 8°C

	2nNt (*n*=28)	2nTx (*n*=30)	3nNt (*n*=30)	3nTx (*n*=29)
Total body mass (g)	0.132±0.002	0.124±0.002	0.123±0.002	0.112±0.002[a]
Yolk-free body mass (g)	0.064±0.001[a]	0.060±0.001	0.058±0.001[b]	0.057±0.001[b]
Yolk mass (g)	0.068±0.002	0.064±0.002	0.065±0.002	0.055±0.002[a]
Caudal fin rays (*n*)	17.2±1.7	16.9±1.7	17.0±1.6	16.6±1.6
Total length (mm)	20.8±1.7	20.3±0.9	20.5±0.7	20.5±0.8

Data are means ± s.e.m. Different superscript letters indicate significant differences between groups at $P<0.05$, while their absence indicates no statistical difference.

Body mass and morphometry

Total body and yolk mass were lower in 3nTx yolk-sac alevins in comparison to all other genotypes (Table 1). Yolk-free body mass was highest in 2nNt alevins and differed from that of 3nNt and 3nTx alevins. The number of caudal fin rays as a measure of developmental stage and body length did not differ between genotypes. Yolk-sac alevins that were used in Series II or III did not differ statistically in any of the parameters measured from animals used in Series I.

Series I: mass-specific metabolic rate

Generally, $\dot{M}_{O_2}$/g decreased with increasing hypoxia, the decrease displaying a triphasic pattern. Within a P_{O_2} range of 15 to 11 kPa, values for $\dot{M}_{O_2}$/g within the groups remained constant (two-way repeated-measures ANOVA, P=0.1). Therefore, the data were separated into three sections (Fig. 1, dashed lines) and values for $\dot{M}_{O_2}$/g were pooled within a range of 3 kPa (P_{O_2}), resembling normoxic (21–19 kPa), intermediate hypoxic (14–12 kPa) and severely hypoxic (7–5 kPa) conditions. $\dot{M}_{O_2}$/g in 3nTx alevins was higher (~8%) than in 2nTx and 3nNt alevins while the latter two groups, in turn, had elevated metabolic rates (~8%) in comparison to 2nNt (one-way ANOVA; Fig. 1, inset). Within intermediate and severe hypoxia treatments, 2nNt alevins showed the lowest values for $\dot{M}_{O_2}$/g, while 2nTx, 3nNt and 3nTx alevins were not statistically different from each other (Fig. 1, inset).

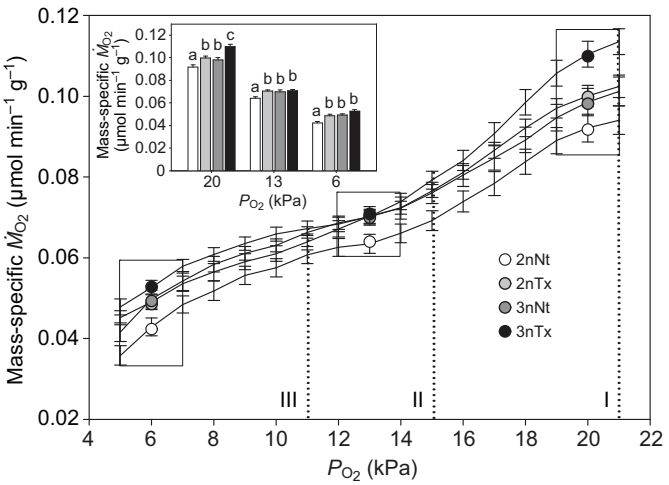

Fig. 1. Changes in metabolic rate ($\dot{M}_{O_2}$) in response to progressive, acute hypoxia (21→5 kPa). Responses of diploid (2nNt), triploid (3nNt), diploid transgenic (2nTx) and triploid transgenic (3nTx) Atlantic salmon (*Salmo salar*) yolk-sac alevins measured at 8°C. Dashed lines separate sections where $\dot{M}_{O_2}$ decreases (I, III) or remains constant (II) as determined by two-way repeated-measures ANOVA. Inset shows $\dot{M}_{O_2}$ data for normoxia, intermediate and severe hypoxia (data enclosed in boxes) and statistical differences determined by two-way ANOVA (P<0.05) are indicated using different letters. Data are means ± s.e.m. for the P_{O_2} ranges specified.

Series II: simultaneous measurement of mass-specific metabolic rate, heart rate and ventilation rate

The cardiorespiratory function in the two metabolically most distinct genotypes from Series I (2nNt and 3nTx) was subsequently studied. Measurements of $\dot{M}_{O_2}$/g, heart rate (f_H) and ventilation rate (f_V) were made on individual 2nNt and 3nTx (n=9 per group) alevins in normoxia, and the levels of intermediate and severe hypoxia as determined in Series I. As previously described in Series I (Fig. 1), here, $\dot{M}_{O_2}$/g in both groups decreased with declining levels of P_{O_2} compared with normoxic values (Fig. 2A,D). Using this experimental design, the overall $\dot{M}_{O_2}$/g in normoxia measured in Series II was ~1.75 times higher in comparison to Series I. As such, 3nTx alevins displayed elevated $\dot{M}_{O_2}$/g of ~26% in normoxia, compared with 16% in Series I, and as in Series I, remained elevated at all levels of P_{O_2} in comparison to 2nNt alevins.

3nTx and 2nNt alevins had a similar f_H in normoxia (Fig. 2B,E). Despite a small reduction in f_H in 2nNt alevins in intermediate hypoxia, it was not statistically different compared with normoxic values. However, in severe hypoxia, f_H in 2nNt alevins was reduced (~10%), while in 3nTx, f_H remained similar to measurements in normoxia. In contrast to these findings, both 3nTx and 2nNt yolk-sac alevins reduced their f_V under severe hypoxia (Fig. 2C,F). In normoxic and intermediate hypoxic conditions, no difference in f_V was detected between genotypes.

Series III: HSP levels

The $\dot{M}_{O_2}$/g pattern in Series III was similar to that observed in Series I and was therefore incorporated within Series I results. Western blot analysis of whole-animal samples revealed that HSP70 (inducible isoform) was undetectable in the control or hypoxia-treated alevins. In contrast, HSC70 (constitutive isoform) and HSP90 were detectable but no differences in relative expression of HSC70 between genotypes were observed in the control samples kept in normoxic conditions (Fig. 3A). However, HSC70 levels in acute hypoxia-treated GH-transgenic alevins were significantly lower than in non-transgenics (P=0.031). Likewise, relative HSP90 expression in control groups was similar but significantly lower than that in all hypoxia-treated alevins (P<0.001; Fig. 3B).

DISCUSSION

In the present study, we show that exposure to acute hypoxia generally leads to a decrease in metabolic rate in Atlantic salmon yolk-sac alevins when measured at 8°C. In addition, we present evidence that GH transgenesis and triploidy result in elevated mass-specific metabolic rates in normoxia as well as acute hypoxia, and the combination of GH transgenesis and triploidy appear to have additive effects. Furthermore, we confirmed higher metabolic rates of triploid transgenic alevins compared with diploid non-transgenic alevins in a separate methodological approach while simultaneously measuring heart rate and ventilation rate. Neither of these latter

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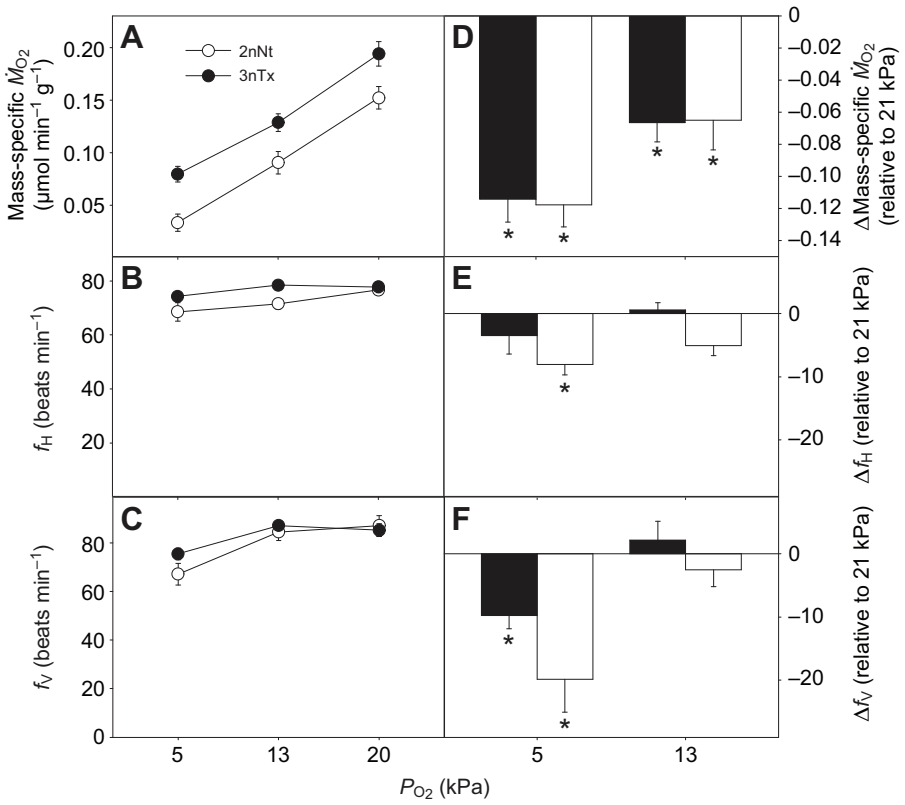

Fig. 2. Cardiorespiratory response to acute hypoxia. Changes in $\dot{M}_{O_2}$ (A), heart rate f_H (B) and ventilation rate f_V (C). Responses of diploid (2nNt) and triploid transgenic (3nTx) Atlantic salmon (*Salmo salar*) yolk-sac alevins in response to a stepwise (P_{O_2} of 21, 13 and 5 kPa) challenge of hypoxia are shown. Right panels show relative changes in $\dot{M}_{O_2}$ (D), f_H (E) and f_V (C) at 13 and 5 kPa with respect to values measured in normoxia (21 kPa). Asterisks indicate a significant difference (P<0.05) in respective parameters compared with normoxic (control) conditions. All values are expressed as means ± s.e.m.

measures correlated with metabolic rate at this ontogenic stage. Hence, the increase in metabolic rate through GH transgenesis and triploidy is not reflected in improved O_2 uptake through heart or ventilation rate and therefore must relate to other physiological mechanisms to ensure O_2 delivery. These might include morphological changes such as gill structure or cutaneous thickness, changes in haematological parameters such as hemoglobin concentration, or changes in cardiac stroke volume. The difference in metabolic rate among groups was also not reflected in altered expression of HSPs (HSP70, HSC70 and HSP90) under control conditions, and hypoxia did not appear to elicit a cellular stress response. However, hypoxia resulted in differential decreased HSP levels among groups with transgenics showing reduced levels of HSC70 while HSP90 expression was significantly decreased under hypoxic conditions across all genotypes.

Series I and II

The $\dot{M}_{O_2}$ curves in alevins that were measured in Series I using the closed-system approach generally showed a triphasic pattern. Here, an initial decline in $\dot{M}_{O_2}$ with decreasing P_{O_2} (21–14 kPa) is followed by a period where $\dot{M}_{O_2}$ is decreasing at a slower rate (14–9 kPa) in intermediate hypoxia before decreasing at a faster rate with more severe hypoxia (9–5 kPa). This pattern was unexpected, as it does not fully comply with either classic oxygen conformity or regulation curves for $\dot{M}_{O_2}$ (see Richards, 2009).

It is possible that the initial, steep decrease in $\dot{M}_{O_2}$ is related to an increased activity of the unconstrained alevin at the beginning of the measurement, where the alevins have not fully settled or reached resting $\dot{M}_{O_2}$ after handling. However, once the animals were put in the respirometry chambers, they settled on the mesh within a few minutes and did not move (E.T.P., personal observation). In addition, when $\dot{M}_{O_2}$ was measured in the open-flow respirometry system and heart rate and ventilation rates were measured simultaneously, it was verified that resting heart and ventilation rates (the lowest constant rate observed throughout the experiment) were established, usually within ~30 min. The period of the initial steep decline in the closed system is significantly longer (~90–120 min) and therefore unlikely to be related to activity.

The switch to a period of relative oxyregulation (slower decline in $\dot{M}_{O_2}$) is difficult to explain. In section II, the alevins show a

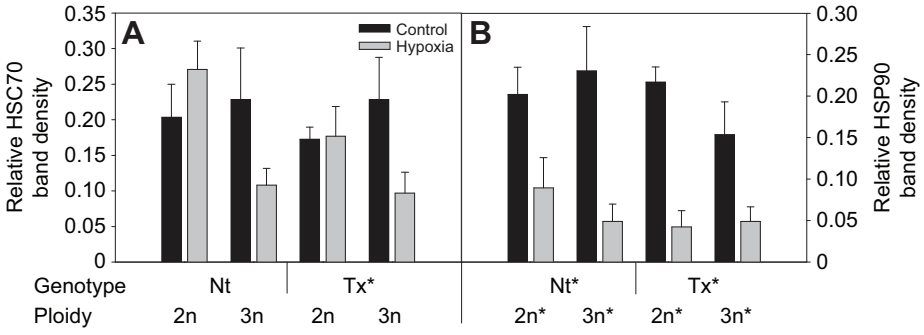

Fig. 3. Heat shock protein (HSP) levels. Relative quantification of (A) HSC70 and (B) HSP90 in diploid (2nNt), triploid (3nNt), diploid transgenic (2nTx) and triploid transgenic (3nTx) Atlantic salmon (*Salmo salar*) yolk-sac alevin whole-body homogenates in normoxia (control, n=10 per group) and after acute, progressive hypoxia exposure (hypoxia, n=5 per group). The bar graphs depict protein band density obtained from densitometric scans of immunoblots. Asterisks indicate a significant difference (P<0.05) from respective control conditions within each genotype. All values are expressed as means ± s.e.m.

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greater ability to maintain $\dot{M}_{O_2}$, which could be indicative of a switch to a more 'O_2-efficient' metabolic pathway, which is triggered at a specific P_{O_2}. This pathway might involve the reallocation of ATP to different ATP-consuming pathways (i.e. protein synthesis/degradation, ion transport) that are more critically involved in the hypoxic metabolic response. During the exposure to a gradually decreasing P_{O_2}, the alevins might have also adjusted to hypoxia through other pathways of ATP production that support the maintenance of $\dot{M}_{O_2}$. Even though the alevins appear to settle relatively quickly in the respirometers, a measure of activity (e.g. frequency of fin movement) during an experiment is likely to add further insight into the $\dot{M}_{O_2}$ pattern observed. In addition, the analysis of changes in substrate utilisation or anaerobic enzyme activity (e.g. lactate dehydrogenase, pyruvate kinase, creatine phosphokinase) might add further insight into changes in metabolic pathways. Another simple explanation for the triphasic pattern could be that if section I corresponds to an elevated $\dot{M}_{O_2}$ because of an unsettled state of the animal, section II might then correspond to the true settled state. The decrease in $\dot{M}_{O_2}$ in section III might then be analogous to the critical O_2 tension, where cellular changes are made to further reduce metabolic rate as part of 'metabolic depression'.

In adult salmonids, the physiological response to acute hypoxia is constituted by metabolic depression, cholinergic bradycardia, as well as an increase in ventilation (Burggren and Pinder, 1991; Holeton and Randall, 1967; Randall, 1982) to counteract the reduced availability of O_2 and high aerobic demand typical for salmonids. In salmonid and other lower order vertebrate larvae, however, this cardiorespiratory response appears to be absent (Holeton, 1971; McDonald and McMahon, 1977). At this early developmental stage, diffusion of O_2 across the skin is the primary avenue for O_2 uptake and the reliance on gills as the main respiratory organ occurs at the end of the larval stage (Wells and Pinder, 1996a; Wells and Pinder, 1996b). This precondition might provide an explanation for the difference in respiratory response to hypoxia in comparison to adult fish, but also supports the notion of an uncoupling of heart rate and ventilation rate from metabolic rate, as has been observed in other vertebrates such as zebrafish larvae (Barrionuevo and Burggren, 1999) and the chicken embryo (Mortola et al., 2009).

The uncoupling of heart and ventilation rate from metabolic rate questions the relevance of convective O_2 transport in the O_2 cascade and the importance of gill ventilation at this early ontogenic stage. It has been suggested that the embryonic heart beat might primarily serve alternative functions such as nutrient transport and angiogenesis (Burggren, 2004), whereas the gills serve in ionoregulation (Fu et al., 2010). Alternatively, the absence of a cardiorespiratory response to hypoxia could be explained by a lack of chemosensory feedback at this life stage. In a recent study on the ontogeny of rainbow trout cardiac control, this hypothesis was refuted as adrenergic and cholinergic control of the heart is present shortly after hatching, and the latter can be delayed by chronic hypoxic exposure (Miller et al., 2011).

In the present study, we observed bradycardia and a decrease in ventilation in response to severe hypoxia in diploid Atlantic salmon alevins. In contrast, 3nTx alevins did not display this bradycardic response, but a decrease in f_V at a low P_{O_2} was observed in both genotypes. This result implies that although there was no general coupling of f_H or f_V to $\dot{M}_{O_2}$, the chemosensory precondition for a cardiorespiratory response to hypoxia is met, but an increase in metabolism did not lead to increased O_2 uptake through heart or ventilation rate.

The differences in $\dot{M}_{O_2}$/g between Series I and Series II are most likely due to the different experimental designs where the relatively greater constraint of the animal in Series II might have led to an elevation of $\dot{M}_{O_2}$/g. Even though the overall reduction in $\dot{M}_{O_2}$ with hypoxia is similar between genotypes in Series II (unlike in Series I), we observed a more severe hypoxic cardiorespiratory response in 2nNt through a decrease in heart rate and a trend towards lower ventilation rate. The lack of a bradycardic response in 3nTx could be indicative of a higher tolerance to severe hypoxia despite elevated $\dot{M}_{O_2}$, and a smaller relative reduction in $\dot{M}_{O_2}$ in severe hypoxia compared with normoxic values (60% in 3nTx versus 80% in 2nNt).

If O_2 uptake was purely limited by physical constraints and diffusive distances were similar between genotypes of similar body mass, the same response in f_H and f_V could be expected. As this is not the case, the animal must be using physiological strategies to maximize O_2 uptake. These may include improved O_2 extraction capabilities via an increase in cardiac output (increase in cardiac stroke volume), hyperventilation through an increase in ventilation volume or an increased hemoglobin concentration. Equally, changes to the diffusive properties of the skin (e.g. diffusion distance) could affect O_2 uptake and therefore the cardiorespiratory response. These possibilities require further experimentation.

It is generally accepted that GH transgenesis, leading to accelerated growth rates, is associated with an increase in metabolism in juvenile and adult salmon (Deitch et al., 2006). In the present study, it was demonstrated that in normoxia, GH transgenesis and triploidy leads to an increase in metabolism in alevins, which appears to be additive when GH transgenesis and ploidy are combined. This raises a question about the interactive effects of GH transgenesis and triploidy on metabolic physiology, which so far, has not been investigated. Whereas the increased metabolism as a consequence of GH transgenesis is physiologically conclusive in the light of a greater energy requirement for developmental and anabolic processes, alterations to respiratory mechanisms due to triploidy remain elusive. It has been hypothesized that the larger cell size of most tissues and the distinct morphology characteristic of triploids (Benfey, 1999) would have an impact on O_2 transport to the cell and therefore on overall metabolism (Maxime, 2008). According to Fick's law of diffusion (Dejours, 1981), the increase in cell volume of triploid organisms should increase the distance for O_2 diffusion to reach the centre of the cell. A greater O_2 gradient must then be established to meet the O_2 demand, which, in turn, increases O_2 uptake. Despite numerous accounts of alterations to hematological parameters in the literature, in particular the size and shape of triploid erythrocytes, the effects of triploidy on metabolic rate in fish remain inconclusive and do not appear to follow a consistent pattern (Benfey, 1999; Maxime, 2008). If triploidy affects the hematology of fish, it stands to reason that further investigation of O_2 binding properties of embryonic/larval hemoglobin in triploid fish should help answer this question.

Series III

On a cellular level, we observed decreases in predominantly constitutive HSPs after exposure to acute, progressive hypoxia, and for HSC70 this decrease was genotype dependent (transgenic, polyploid). Expression of the stress-inducible HSP70 was not observed, thus, hypoxia does not appear to induce HSPs at least at this early developmental stage. The effects of hypoxia on HSP induction in fish are variable. Our finding is consistent with that of Zarate and Bradley (Zarate and Bradley, 2003), who showed that HSP30, HSP70 and HSP90 mRNA were not induced with hypoxia in Atlantic salmon. Similarly, anoxia did not induce HSP70 in the red blood cells of rainbow trout (Currie and Tufts, 1997). In contrast, HSPs were induced with hypoxia in rainbow trout gill epithelial

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cells (Airaksinen and Nikinmaa, 1995). Likewise, the air breathing mudminnow, *Umbra limi*, responded to hypoxia and 6 h of air exposure with significant increases in HSP70 and HSP90 in liver tissue (Currie et al., 2010). In the anoxia-tolerant western painted turtle, long-term but not short-term anoxia led to an induction of HSPs (constitutive HSP73, inducible HSP72 and HSP90) in several tissues (Ramaglia and Buck, 2004). Hypoxic submergence also resulted in elevated levels of HSP70 in the hearts of the hypoxia-tolerant common frog, *Rana temporaria* (Currie and Boutilier, 2001). These latter studies suggest that the induction of HSPs may be at least part of the strategy of low O_2 tolerance, protecting proteins from damage associated with low O_2 and/or the sudden return of O_2 to tissues. These studies also illustrate the complexity of the HSP response to hypoxia/anoxia and highlight the necessity of improving our understanding of its temporal, spatial and species-specific regulation.

Atlantic salmon are considered hypoxia intolerant, and thus it may not be surprising that HSP induction was not observed. The aerobic thermal limit, and thus the susceptibility to hypoxia, is thought to be greater during early life stages when the O_2 supply shifts from cutaneous to ventilatory and circulatory systems. It is possible that the lack of a heat shock response (e.g. HSP70) with hypoxia observed here contributes to the O_2 sensitivity at the alevin stage. It is also possible that the decrease in constitutive HSPs with hypoxia might be indicative of a reduced ability for protein synthesis as a result of the lack of sufficient energy obtained via aerobic pathways during hypoxic hypometabolism. That said, the red blood cells of adult rainbow trout are able to mount an inducible heat shock response under severe hypoxia (Currie and Tufts, 1997) and during pharmacological inhibition of both oxidative phosphorylation and glycolysis (Currie et al., 1999), suggesting that the cells of adult salmonids, at least, are not energetically limited in terms of their heat shock response. Protein synthesis in earlier developmental stages, however, may be more susceptible to these energetic constraints.

Interestingly, the decrease in HSC70 observed during hypoxia appears to be restricted to GH-transgenic alevins. As 3nNt larvae display a similar increase in metabolic rate as 2nTx larvae, this finding cannot be explained by differences in metabolism. Our knowledge of the cellular and molecular consequences resulting from GH transgenesis is limited. It is possible that protein synthesis is preferentially directed towards muscle growth in transgenic animals over constitutive HSPs, particularly under harsh environmental conditions (e.g. hypoxia). Interestingly, the differences in metabolic rate between groups was not reflected in differences in HSP levels. Differences in HSP/HSC levels with hypoxia may have been anticipated because of the higher demand for O_2 in, for example, 3nTx over all other groups. However, it appears that generally the HSP levels in normoxia or hypoxia are not influenced by the initial metabolic rate. Future studies should examine the effects of long-term exposure to hypoxia on the cellular stress response throughout development in transgenic animals to improve our understanding of the involvement of HSPs in normal growth and development as well as in hypoxia tolerance.

Conclusions and perspectives

This is the first study examining the metabolic, cardiorespiratory and cellular stress response to hypoxia of a GH-transgenic Atlantic salmon under consideration of the effects of polyploidy. We conclude that GH transgenesis or artificially induced triploidy increase metabolism and together alter cardiorespiratory function under O_2-limiting conditions in Atlantic salmon during early development. Hypoxia did not induce a cellular stress response, which may have a negative effect on the ability of the animal to deal with harsh environments. We see an important role in further investigating respiratory and cellular responses to environmental stress in triploid and GH-transgenic vertebrates during ontogeny and comparing them with juvenile and adult life stages. It is likely that physiological differences during early life stages are carried on to adulthood and might affect robustness or susceptibility to environmental stress in an aquaculture environment.

MATERIALS AND METHODS

Experimental animals

Atlantic salmon eggs used in this study were all fertilized on the same day and hatched at ~400 degree days post-fertilisation (50 days at 8°C post-fertilisation). Yolk-sac alevins were reared under standard hatchery conditions (8°C, in darkness) at AquaBounty Canada (Fortune, PEI, Canada) in heath-stack incubators fed with flow-through, UV-treated ground water. Four groups of alevins were used: diploid (2nNt), triploid (3nNt), diploid transgenic (2nTx) and triploid transgenic (3nTx). Transgenic Atlantic salmon carry a single copy of the α-form of a salmon growth hormone transgene (opAFP-GHc2) that is stably integrated at the α-locus in the EO-1α lineage and exhibit a rapid growth phenotype (AquaBounty Technologies, 2010). Triploidy in Atlantic salmon was induced by a hydrostatic pressure shock for 5 min at a constant 65.5 MPa initiated at 300°C minutes after fertilisation, which prevents the extrusion of the second polar body during meiotic development and renders the animals sterile with an effectiveness of better than 99%.

Yolk-sac alevin mass and morphometry

Yolk-sac alevin (alevin) dimensions were measured using a horizontal dissecting microscope (Leica EZ4D, Wetzlar, Germany) with an integrated ocular micrometer. Alevins were killed immediately following each experiment. The total wet mass of each alevin was measured to the nearest 0.001 g prior to yolk-sac removal. The wet, yolk-free, body and wet yolk mass of each alevin was measured in grams following the separation of the yolk sac from the alevin body using micro-operating scissors. Total body length (mm) and number of caudal fin rays [*n*, as a proxy for developmental stage (Gorodilov, 1996)] were measured under a dissecting microscope.

Series I: measurement of metabolic rate in alevins

The rate of oxygen consumption (≅metabolic rate=$\dot{M}_{O_2}$) in alevins was measured using fluorescence-based closed-system respirometry. Each alevin was placed in an individual well in a polystyrene multiwell plate (24 wells×3.3 ml, Oxodish®, PreSens, Regensburg, Germany) that was filled with air-equilibrated water from the rearing trays (partial pressure of O_2, P_{O_2}=21 kPa). Each well contained an optode to monitor O_2 levels and was fitted with a stainless steel mesh positioned 3 mm from the bottom to ensure the alevin did not contact the optode [as described in Polymeropoulos et al. (Polymeropoulos et al., 2013)]. Four wells remained empty of animals for calibration purposes: two were filled with normoxic water from the rearing trays and two with a zero solution (1% sodium sulphite solution, which acted as an O_2 scavenger). Each well was sealed with a glass coverslip using vacuum grease, care being taken to exclude all air bubbles. The multiwell plate was placed on a SensorDishReader (SDR, PreSens) on an orbital mixer inside a temperature-controlled cabinet. The orbital mixer was set to the lowest speed (70 rpm) required to move a small (2 mm Ø) stainless steel ball around the perimeter at the bottom of each well ensuring the water in each well was gently mixed; the alevins remained motionless on the mesh. The P_{O_2} in each well was measured at 2 min intervals starting from 21 kPa conditions until the P_{O_2} reached 5 kPa. Movement response to physical disturbance confirmed that all alevins were alive at the end of each experiment. Before every measurement, optodes were calibrated by alternatively filling the wells with 1% sodium sulphite solution containing 10^{-4} mol l^{-1} cobalt chloride (P_{O_2}=0 kPa) and air-equilibrated water (P_{O_2}=21 kPa).

The $\dot{M}_{O_2}$ (μmol O_2 min^{-1}) for each egg or alevin was calculated from the decline in P_{O_2} over time in each well, after taking into account the diffusive flux of O_2 that occurs through the polystyrene Oxodish® well plate (see Polymeropoulos et al., 2013).

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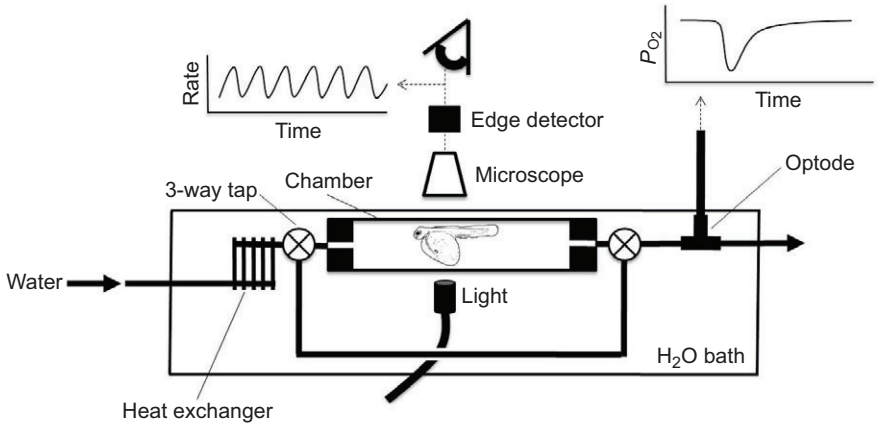

Fig. 4. Respirometry setup. Depicted is a diagram of the respirometry setup in combination with the optophysiological system to simultaneously measure $\dot{M}_{O_2}$, f_H and f_V in individual Atlantic salmon (*Salmo salar*) alevins at different levels of P_{O_2}. In principle, the respirometry chamber containing the alevin was sealed for a period of ~30 min while f_H and f_V were recorded, each recorded at a separate time. $\dot{M}_{O_2}$ was calculated from the time integral of the gas concentration curve, derived from the P_{O_2} recording, after flushing the chamber.

Mass-specific $\dot{M}_{O_2}$ ($\dot{M}_{O_2}$/g) was adjusted for yolk-free wet body mass (g) to account for differences in body mass among individuals. The volume of the chamber was corrected for the volume displaced by each individual (according to its total wet mass assuming a density of 1 g ml^{-1}), the stainless steel ball bearing and mesh.

Series II: simultaneous measurement of metabolic rate, heart rate and ventilation rate in individual alevins

Simultaneous measurements of $\dot{M}_{O_2}$, heart rate (f_H) and ventilation rate (f_V) were performed on individual alevins using a combined optophysiological and respirometry system (Fig. 4). Individual alevins were placed in a sealed flow-through respirometry chamber (1.5 ml) under the exclusion of air; the chamber being partially submerged in a temperature-controlled waterbath (Polyscience, Niles, IL, USA; accuracy ±0.025°C) at the respective temperature. Water temperature in the chamber was measured with a thermocouple (accuracy ±0.1°C, traceable to a National Standard). Normoxic water (P_{O_2}=21 kPa) was pumped through the chamber at a constant flow rate of 1.5 ml min^{-1} using a peristaltic pump (Living Systems Instrumentation, Burlington, VT, USA), the water first passing through a heat exchanger in the water bath to maintain the required temperature.

To minimize handling stress, alevins were acclimated within the chamber under constant flow of normoxic water for 1 h before measurement. The chamber was then sealed for ~30 min prior to flushing with water at the original incoming P_{O_2} until the P_{O_2} within the chamber had returned to the original P_{O_2} (~8 min). The process of sealing and flushing was repeated for each experiment, where the desired incoming P_{O_2} (kPa) was achieved by bubbling water with the required mixture of O_2 and N_2 (high-precision low-flow gas blender, V10040A, Bird, San Diego, CA, USA) and was confirmed with an optochemical O_2 sensor (Oxygen Meter, HQ10, Hach, Loveland, CO, USA). It was assumed that the constant movement of the pectoral fins and opercular pumping during periods where the chamber was sealed allowed sufficient mixing of the water and prevented the formation of hypoxic boundary layers surrounding the alevin.

For the measurement of $\dot{M}_{O_2}$, an optochemical O_2 sensor in a flow-through cell (FTC-PSt3, PreSens) in combination with an O_2 analyser (Fibox, PreSens) was used. This was connected in series downstream to the respirometry chamber and maintained at the same ambient temperature (Fig. 1). In the closed but intermittently flushed respirometry system, the rates of O_2 consumption (μmol min^{-1}) were calculated, as previously described (Clark et al., 2005). Following a period where the chamber was sealed, it was then flushed with fresh water and the $\dot{M}_{O_2}$ was determined from the time integral of the resulting P_{O_2} curve ($\int P_{O_2}$; kPa min^{-1}) multiplied by the oxygen capacitance (βw_{O_2}; μmol l^{-1} kPa^{-1}), the flow of water ($\dot{V}$; ml min^{-1}) used to flush the chamber, and the reciprocal of the time (t; min) during which the compartment was sealed:

$$\dot{M}_{O_2} = \beta w_{O_2} \cdot \int P_{O_2} \cdot \frac{\dot{V}}{t}. \quad (1)$$

Mass-specific $\dot{M}_{O_2}$ was calculated by adjusting for wet yolk-free body mass (g).

The validation of the intermittantly sealed flow-through respirometry system is described in detail in the Appendix.

During periods when the chamber was sealed, f_H and f_V were measured using optophysiological methods. For this purpose, a stereomicroscope (Stemi SV6, Zeiss, Germany) was equipped with a CCD camera (SSC-DC50P, Sony, Japan), which was situated above the transparent respirometry chamber to visually monitor the fish (Fig. 4). Video information was processed by a video dimension analyser (V94, Living Systems Instrumentation) and displayed on a computer via a video capture USB card (Roxio, 315-E). In principle, the instrument operates on the relative optical density changes of border structures (i.e. border of beating heart or moving operculum) and generates a linear voltage signal that can be read by a digital interface (PowerLab 8/sp, ADInstruments, Dunedin, New Zealand). Separate measurements of f_H and f_V were performed for each level of P_{O_2} within the first 5 min of the chamber being sealed. Within this time frame, the P_{O_2} within the sealed chamber did not drop lower than 1.25 kPa from the initial value. This was verified by measuring the P_{O_2} within the chamber using a microoptode. Therefore, the measurements for f_H and f_V are representative of the desired P_{O_2}. A minimum of 30 consecutive heart beats and operculum movements were recorded using LabChart®7 Pro (ADInstruments). Movement response to physical disturbance confirmed that all alevins were alive at the end of each experiment.

Series III: HSP immunodetection

Whole alevins were ground in liquid nitrogen and soluble protein was extracted as in LeBlanc et al. (LeBlanc et al., 2012). Briefly, 25–35 mg of ground sample was homogenized in tissue homogenization buffer (50 mmol l^{-1} Tris, Sigma-Aldrich; 2% SDS, Bio-Rad; 0.1 mmol l^{-1} protease inhibitor cocktail, Sigma-Aldrich) using a PowerGen 125 polytron (Fisher Scientific). Samples were then pushed through a 27 G needle several times and spun at 14,000 ***g*** for 10 min in a Sorvall Legend RT microcentrifuge (Mandel). The supernatant, containing the soluble protein, was removed and stored at −80°C. The soluble protein concentration of each sample was measured using the DC protein assay kit (Bio-Rad) based on the Lowry method (Lowry et al., 1951).

To determine HSP levels, immunodetection via western blot was performed using the Novex Midi Gel System (Invitrogen). Briefly, 15 μg of soluble protein per sample were loaded onto 4–12% Bis-Tris gels, electrophoresed and transferred to PVDF membranes using the Iblot system (Invitrogen) according to the manufacturer's instructions. Commercial standards (HSC70 SPP-751, HSP90 SPP-770; Assay Designs) or a red blood cell sample from a heat-shocked (HSP70) rainbow trout were loaded onto each gel to allow direct comparison between gels. For immunodetection, rabbit salmonid-specific primary antibodies against HSP70 (AS05061), HSC70 (AS05062) and HSP90 (AS05063) were used at a final concentration of 1:50,000 (Agrisera). The HSP70 antibody detects only the inducible form of HSP70 and does not cross-react with the constitutively expressed isoform (HSC70), while the HSP90 antibody does not distinguish between the HSP 90α, HSP 90βa and HSP 90βb isoforms of the protein (Rendell et al., 2006). A goat anti-rabbit antibody (SAB-300; Enzo Life Sciences) was used at a concentration of 1:50,000 as secondary antibody.

Protein bands were visualized using the ECL Advance Western Blotting Detection Kit (GE Healthcare) and a Versadoc Imaging System (Bio-Rad).

Bands were then quantified using ImageLab software (Bio-Rad) and the band density of each sample was divided by the band density of the appropriate standard to obtain the relative band density. After each round of immunoblotting, antibodies were stripped from the membranes using acid stripping buffer (0.4 mol l^{-1} glycine, Sigma-Aldrich; 0.07 mol l^{-1} SDS, Bio-Rad; 0.018 mol l^{-1} Tween 20, Sigma-Aldrich; pH 2.2) for 2×30 min, followed by 3×5 min washes in PBS (1.37 mol l^{-1} NaCl, 27 mmol l^{-1} KCl, 43 mmol l^{-1} $Na_2HPO_4{\cdot}7H_2O$, 14 mmol l^{-1} KH_2PO_4; Sigma-Aldrich) and membranes were re-probed using a different antibody.

Statistical analysis

Morphometrical parameters were compared between genotypes using one-way ANOVA. Comparisons of $\dot{M}_{O_2}$ in Series I and $\dot{M}_{O_2}$, f_H and f_V in Series II between and within groups were performed using two-way repeated-measures ANOVA with P_{O_2} and genotype (2nNt, 2nTx, 3nNt and 3nTx) as factors and the level of significance set to $P<0.05$. For HSC70 and HSP90, three-way ANOVAs with treatment (hypoxia or control), ploidy (2n or 3n) and transgenesis (transgenic or non-transgenic) as factors were performed. All pairwise multiple comparisons procedures were performed using adjusted Bonferroni *post hoc* modified *t*-tests.

APPENDIX

Validation of the intermittantly sealed flow-through respirometry system

The single chamber respirometer with a flow of water through it approximates a first-order linear system (see Frappell et al., 1989). Hence, following a period where the chamber has been sealed and the P_{O_2} within the chamber has declined as a result of oxygen consumption, flushing the chamber with fresh water will result in an exponential rise of the P_{O_2} in the outflowing water as it approaches the incoming P_{O_2} (noting that oxygen consumption is continually occurring but with a high enough flow of water the difference between inflowing and outflowing P_{O_2} is effectively zero) (Fig. A1).

The accuracy of the respirometer was determined by an instantaneous injection of 0.5 ml of anoxic water into the system upstream from the chamber (volume=1.5 ml) through which water of a known P_{O_2} (21, 13 or 5 kPa) flowed at a constant rate (1.5 ml min^{-1}). From the time integral of the resulting P_{O_2} curve, multiplied by the O_2 capacitance (βw_{O_2}; mmol l^{-1} kPa^{-1}) and flow of water (volume $time^{-1}$), the number of moles of O_2 injected can be determined (M; Fig. A2). All tests were performed in triplicate and averaged.

The accuracy of the system was calculated by comparing theoretical (the number of moles replaced in chamber water of a given P_{O_2} following injection of a known volume of anoxic water) and calculated numbers of moles. The system could reliably determine 0.0036±0.0009 nmol in a 0.5 ml injection, which as the injected water flushed through the system perturbed the P_{O_2} to ~4 kPa, this being similar to the perturbation seen when flushing the chamber during experiments.

The error associated in determing M using this respirometry system was below 7% (21 kPa=~1.5%; 13 kPa=~4.2%; 5 kPa=~7.0%).

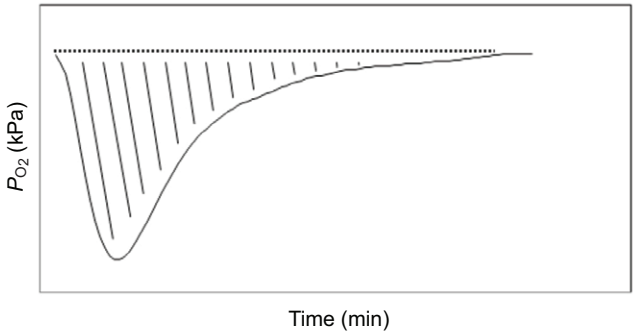

Fig. A1. Example of the P_{O_2} profile of outflowing water leaving the respirometry chamber against time that occurs when the chamber, previously sealed and in which O_2 is depleted by the O_2 consumption of the animal inside, is flushed with fresh incoming water (dotted line). The dashed area represents the time integral that is used to calculate $\dot{M}_{O_2}$.

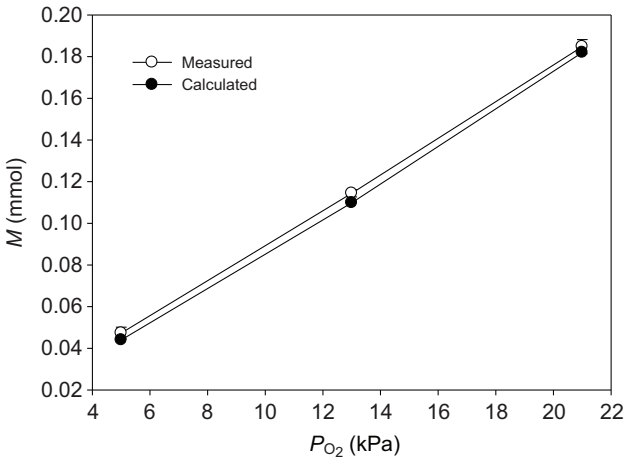

Fig. A2. Comparison between theoretical and calculated number of moles of O_2 (M) when injecting anoxic water into the respirometer. Data are means ± s.e.m.

Competing interests

The authors declare no competing financial interests.

Author contributions

E.T.P. conceived, designed and executed the experiment(s), interpreted the findings being published, and drafted and revised the article; D.P. conceived the experiments, and drafted and revised the article; S.L. designed and executed the experiment(s), interpreted the findings being published, and drafted and revised the article; N.G.E. conceived and designed the experiments, interpreted the findings being published, and drafted and revised the article; S.C. conceived and designed the experiments, interpreted the findings being published, and drafted and revised the article; P.B.F. conceived and designed the experiments, interpreted the findings being published, and drafted and revised the article.

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References

Airaksinen, S. and Nikinmaa, M. (1995). Effect of hemoglobin concentration on the oxygen-affinity of intact lamprey erythrocytes. *J. Exp. Biol.* **198**, 2393-2396.
Allendorf, F. W. and Thorgaard, G. H. (1984). *Evolutionary Genetics of Fishes* (ed. B. J. Turner), pp. 1-53. New York, NY: Springer.
Aquabounty Technologies (2010). *Report Prepared for the FDA Veterinary Medicine Advisory Committee*. Silver Spring, MD: FDA.
Barrionuevo, W. R. and Burggren, W. W. (1999). O_2 consumption and heart rate in developing zebrafish (*Danio rerio*): Influence of temperature and ambient O_2. *Am. J. Physiol.* **276**, R505-R513.
Barton, B. A. (2002). Stress in fishes: a diversity of responses with particular reference to changes in circulating corticosteroids. *Integr. Comp. Biol.* **42**, 517-525.
Benfey, T. J. (1999). The physiology and behavior of triploid fishes. *Rev. Fish. Sci.* **7**, 39-67.
Blier, P. U., Lemieux, H. and Devlin, R. H. (2002). Is the growth rate of fish set by digestive enzymes or metabolic capacity of the tissues? Insight from transgenic coho salmon. *Aquaculture* **209**, 379-384.
Burggren, W. W. (2004). What is the purpose of the embryonic heart beat? Or how facts can ultimately prevail over physiological dogma. *Physiol. Biochem. Zool.* **77**, 333-345.
Burggren, W. W. and Pinder, A. W. (1991). Ontogeny of cardiovascular and respiratory physiology in lower vertebrates. *Annu. Rev. Physiol.* **53**, 107-135.

Clark, T. D., Ryan, T., Ingram, B. A., Woakes, A. J., Butler, P. J. and Frappell, P. B. (2005). Factorial aerobic scope is independent of temperature and primarily modulated by heart rate in exercising Murray cod (*Maccullochella peelii peelii*). *Physiol. Biochem. Zool.* **78**, 347-355.
Cogswell, A. T., Benfey, T. J. and Sutterlin, A. M. (2002). The hematology of diploid and triploid transgenic Atlantic salmon (*Salmo salar*). *Fish Physiol. Biochem.* **24**, 271-277.
Cook, J. T., Sutterlin, A. M. and McNiven, M. A. (2000). Effect of food deprivation on oxygen consumption and body composition of growth-enhanced transgenic Atlantic salmon (*Salmo salar*). *Aquaculture* **188**, 47-63.
Currie, S., Bagatto, B., DeMille, M., Learner, A., LeBlanc, D., Marks, C., Ong, K., Parker, J., Templeman, N., Tufts, B. L. et al. (2010). Metabolism, nitrogen excretion, and heat shock proteins in the central mudminnow (*Umbra limi*), a facultative air-breathing fish living in a variable environment. *Can. J. Zool.* **88**, 43-58.
Currie, S. and Boutilier, R. G. (2001). Strategies of hypoxia and anoxia tolerance in cardiomyocytes from the overwintering common frog, *Rana temporaria*. *Physiol. Biochem. Zool.* **74**, 420-428.
Currie, S. and Tufts, B. L. (1997). Synthesis of stress protein 70 (HSP70) in rainbow trout (*Oncorhynchus mykiss*) red blood cells. *J. Exp. Biol.* **200**, 607-614.
Currie, S., Tufts, B. L. and Moyes, C. D. (1999). Influence of bionergetic stress on heat shock protein gene expression in nucleated red blood cells of fish. *Am. J. Physiol.* **276**, 990-996.
Deane, E. E., Kelly, S. P., Lo, C. K. M. and Woo, N. Y. S. (1999). Effects of GH, prolactin and cortisol on hepatic heat shock protein 70 expression in a marine teleost *Sparus sarba*. *J. Endocrinol.* **161**, 413-421.
Deane, E. E. and Woo, N. Y. S. (2011). Advances and perspectives on the regulation and expression of piscine heat shock proteins. *Rev. Fish Biol. Fish.* **21**, 153-185.
Deitch, E. J., Fletcher, G. L., Petersen, L. H., Costa, I. A. S. F., Shears, M. A., Driedzic, W. R. and Gamperl, A. K. (2006). Cardiorespiratory modifications, and limitations, in post-smolt growth hormone transgenic Atlantic salmon *Salmo salar*. *J. Exp. Biol.* **209**, 1310-1325.
Dejours, P. (1981). *Principles of Comparative Respiratory Physiology*. Amsterdam: Elsevier; North-Holland Biomedical Press.
Devlin, R. H., Yesaki, T. Y., Biagi, C. A., Donaldson, E. M., Swanson, P. and Chan, W. K. (1994). Extraordinary salmon growth. *Nature* **371**, 209-210.
Feder, M. E. and Hofmann, G. E. (1999). Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annu. Rev. Physiol.* **61**, 243-282.
Ferris, S. D. (1984). Tetraploidy and the evolution of the catostomid fishes. In *Evolutionary Genetics of Fishes* (ed. B. J. Turner), pp. 55-93. New York, NY: Springer.
Frappell, P. B., Blevin, H. A. and Baudinette, R. V. (1989). Understanding respirometry chambers: what goes in must come out. *J. Theor. Biol.* **138**, 479-494.
Fu, C., Wilson, J. M., Rombough, P. J. and Brauner, C. J. (2010). Ions first: Na^+ uptake shifts from the skin to the gills before O_2 uptake in developing rainbow trout, *Oncorhynchus mykiss*. *Proc. Biol. Sci.* **277**, 1553-1560.
Galbreath, P. F., Jean, W. S., Anderson, W. and Thorgaard, G. H. (1994). Freshwater performance of all-female diploid and triploid Atlantic salmon. *Aquaculture* **128**, 41-49.
Graham, M. S., Fletcher, G. L. and Benfey, T. J. (1985). Effect of triploidy on blood oxygen content of Atlantic salmon. *Aquaculture* **50**, 133-139.
Gorodilov, Y. N. (1996). Description of the early ontogeny of the Atlantic salmon, *Salmo salar*, with a novel system of interval (state) identification. *Environ. Biol. Fishes* **47**, 109-127.
Holeton, G. F. and Randall, D. J. (1967). The effect of hypoxia upon the partial pressure of gases in the blood and water afferent and efferent of the gills of rainbow trout. *J. Exp. Biol.* **46**, 317-327.
Holeton, G. F. (1971). Respiratory and circulatory responses of rainbow trout larvae to carbon monoxide and to hypoxia. *J. Exp. Biol.* **55**, 683-694.
Kregel, K. C. (2002). Heat shock proteins: modifying factors in physiological stress responses and acquired thermotolerance. *J. Appl. Physiol.* **92**, 2177-2186.
Krone, P. H., Evans, T. G. and Blechinger, S. R. (2003). Heat shock gene expression and function during zebrafish embryogenesis. *Semin. Cell Dev. Biol.* **14**, 267-274.
LeBlanc, S., Höglund, E., Gilmour, K. M. and Currie, S. (2012). Hormonal modulation of the heat shock response: Insights from fish with divergent cortisol stress responses. *Am. J. Physiol.* **302**, 184-192.
Lee, C. G., Devlin, R. H. and Farrell, A. P. (2003). Swimming performance, oxygen consumption and excess post-exercise oxygen consumption in adult transgenic and ocean-ranched coho salmon. *J. Fish Biol.* **62**, 753-766.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**, 265-275.
Martinez, R., Arenal, A., Estrada, M. P., Herrera, F., Huerta, V., Vazquez, J., Sanchez, T. and De La Fuente, J. (1999). Mendelian transmission, transgene dosage and growth phenotype in transgenic tilapia (*Oreochromis homorum*) showing ectopic expression of homologous growth hormone. *Aquaculture* **173**, 271-283.
Maxime, V. (2008). The physiology of triploid fish: current knowledge and comparisons with diploid fish. *Fish Fish.* **9**, 67-78.
McDonald, D. G. and McMahon, B. R. (1977). Respiratory development in Arctic char *Salvelinus alpinus* under conditions of normoxia and chronic hypoxia. *Can. J. Zool.* **55**, 1461-1467.
McGeachy, S. A., Benfey, T. J. and Friars, G. W. (1995). Freshwater performance of triploid Atlantic salmon (*Salmo salar*) in New Brunswick aquaculture. *Aquaculture* **137**, 333-341.
Miller, S. C., Gillis, T. E. and Wright, P. A. (2011). The ontogeny of regulatory control of the rainbow trout (Oncorhynchus mykiss) heart and how this is influenced by chronic hypoxia exposure. *J. Exp. Biol.* **214**, 2065-2072.
Mortola, J. P., Wills, K., Trippenbach, T. and Al Awam, K. (2009). Interactive effects of temperature and hypoxia on heart rate and oxygen consumption of the 3-day old chicken embryo. *Comp. Biochem. Physiol.* **155**, 301-308.
O'Flynn, F. M., McGeachy, S. A., Friars, G. W., Benfey, T. J. and Bailey, J. K. (1997). Comparisons of cultured triploid and diploid Atlantic salmon (*Salmo salar* L.). *ICES J. Mar. Sci.* **54**, 1160-1165.
Oliva-Teles, A. and Kaushik, S. J. (1987). Nitrogen and energy metabolism during the early ontogeny of diploid and triploid rainbow trout (*Salmo gairdneri* R.). *Comp. Biochem. Physiol.* **87**, 157-160.
Oppedal, F., Taranger, G. L. and Hansen, T. (2003). Growth performance and sexual maturation in diploid and triploid Atlantic salmon (*Salmo salar* L.) in seawater tanks exposed to continuous light or simulated natural photoperiod. *Aquaculture* **215**, 145-162.
Pelster, B. (1999). Environmental influences on the development of the cardiac system in fish and amphibians. *Comp. Biochem. Physiol.* **124**, 407-412.
Polymeropoulos, E. T., Elliott, N. G., Wotherspoon, S. J. and Frappell, P. B. (2013). Respirometry: Correcting for diffusion and validating the use of plastic multiwell plates with integrated optodes. *Physiol. Biochem. Zool.* **86**, 588-592.
Ramaglia, V. and Buck, L. T. (2004). Time-dependent expression of heat shock proteins 70 and 90 in tissues of the anoxic western painted turtle. *J. Exp. Biol.* **207**, 3775-3784.
Randall, D. J. (1982). The control of respiration and circulation in fish during exercise and hypoxia. *J. Exp. Biol.* **100**, 275-288.
Rendell, J. L., Fowler, S., Cockshutt, A. and Currie, S. (2006). Development-dependent differences in intracellular localization of stress proteins (hsps) in rainbow trout, *Oncorhynchus mykiss*, following heat shock. *Comp. Biochem. Physiol.* **1**, 238-252.
Richards, J. G. (2009). Metabolic and molecular responses of fish to hypoxia. In *Fish Physiology*, Vol 27 (ed. J. G. Richards, A. Farrell and C. Brauner), pp. 443-486. Amsterdam: Elsevier; Academic Press.
Rombough, P. J. (1988). Growth, aerobic metabolism, and dissolved oxygen requirements of embryos and alevins of steelhead, *Salmo gairdneri*. *Can. J. Zool.* **66**, 651-660.
Sacobie, C. F. D., Glebe, B. D., Barbeau, M. A., Lall, S. P. and Benfey, T. J. (2012). Effect of strain and ploidy on growth performance of Atlantic salmon, *Salmo salar*, following seawater transfer. *Aquaculture* **334-337**, 58-64.
Sadler, J., Pankhurst, P. M. and King, H. R. (2001). High prevalence of skeletal deformity and reduced gill surface area in triploid Atlantic salmon (*Salmo salar* L.). *Aquaculture* **198**, 369-386.
Schlesinger, M. J. (1990). Heat-shock proteins. *J. Biol. Chem.* **265**, 12111-12114.
Schultz, R. J. (1980). Role of polyploidy in the evolution of fishes. In *Biological Relevance: Polyploidy* (ed. W. H. Lewis), pp. 313-340. New York, NY: Plenum Press.
Shao Jun, D., Gong, Z., Fletcher, G. L., Shears, M. A., King, M. J., Idler, D. R. and Hew, C. L. (1992). Growth enhancement in transgenic Atlantic salmon by the use of an 'all fish' chimeric growth hormone gene construct. *Biotechnology* **10**, 176-181.
Stevens, E. D. and Sutterlin, A. (1999). Gill morphometry in growth hormone transgenic Atlantic salmon. *Environ. Biol. Fishes* **54**, 405-411.
Stevens, E. D., Sutterlin, A. and Cook, T. (1998). Respiratory metabolism and swimming performance in growth hormone transgenic Atlantic salmon. *Can. J. Fish. Aquat. Sci.* **55**, 2028-2035.
Stillwell, E. J. and Benfey, T. J. (1996). Hemoglobin level, metabolic rate, opercular abduction rate and swimming efficiency in female triploid brook trout (*Salvelinus fontinalis*). *Fish Physiol. Biochem.* **15**, 377-383.
Stokstad, E. (2002). Transgenic species: engineered fish: friend or foe of the environment? *Science* **297**, 1797-1799.
Taylor, J. F., Preston, A. C., Guy, D. and Migaud, H. (2011). Ploidy effects on hatchery survival, deformities, and performance in Atlantic salmon (*Salmo salar*). *Aquaculture* **315**, 61-68.
Tiwary, B. K., Kirubagaran, R. and Ray, A. K. (2004). The biology of triploid fish. *Rev. Fish Biol. Fish.* **14**, 391-402.
Verhille, C., Anttila, K. and Farrell, A. P. (2013). A heart to heart on temperature: impaired temperature tolerance of triploid rainbow trout (*Oncorhynchus mykiss*) due to early onset of cardiac arrhythmia. *Comp. Biochem. Physiol.* **164**, 657.
Wells, P. R. and Pinder, A. W. (1996a). The respiratory development of Atlantic salmon: I. Morphometry of gills, yolk sac and body surface. *J. Exp. Biol.* **199**, 2725-2736.
Wells, P. R. and Pinder, A. W. (1996b). The respiratory development of Atlantic salmon: II. Partitioning of oxygen uptake among gills, yolk sac and body surfaces. *J. Exp. Biol.* **199**, 2737-2744.
Zarate, J. and Bradley, T. M. (2003). Heat shock proteins are not sensitive indicators of hatchery stress in salmon. *Aquaculture* **223**, 175-187.