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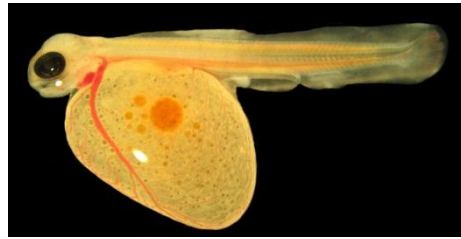
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The effects of oxygen and temperature on the physiology of hatching stage Atlantic salmon



Elias T. Polymeropoulos

(Diploma in Biology)

Submitted in fulfilment of the requirements for the degree of Doctor of
Philosophy (PhD)

University of Tasmania

July 2013

"doing what little one can to increase the general stock of knowledge is as respectable an object of life, as one can in any likelihood pursue"

(Charles Darwin)

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Author 2 and author 3 assisted with refinement and presentation

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“The research associated with this thesis abides by the international and Australian codes on human and animal experimentation, the guidelines by the Australian Government's Office of the Gene Technology Regulator and the rulings of the Safety, Ethics and Institutional Biosafety Committees of the University.”

Cover image: Atlantic salmon (*S. salar*) yolk sac alevin.

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Abbreviations

2n	=	diploid
3n	=	triploid
bpm	=	beats per minute
βO_2	=	solubility of oxygen
f_{H}	=	heart rate
f_{V}	=	ventilation rate
GH	=	growth hormone
GO_2	=	oxygen conductance
Hb	=	hemoglobin
Hsp	=	heat shock protein
KO_2	=	Krogh's diffusion coefficient
kPa	=	kilopascal
MO_2	=	metabolic rate
PO_2	=	partial pressure of oxygen
T_{a}	=	ambient temperature
Tx	=	transgenic
Nt	=	non transgenic

Abstract

Environmental change and its impact on the form, function and adaptive responses of fish, especially in relation to maternal effects or individual genotype, is of major interest to biologists. Oxygen levels and temperature have profound effects on the physiology of fish, and early developmental stages are particularly susceptible to environmental challenges. In this thesis the metabolic, cardiorespiratory and cellular stress responses to acute or chronic changes of these variables in Atlantic salmon (*Salmo salar*) embryos and yolk-sac alevins were investigated.

Eggs and alevins matched their metabolic demand to acute changes in oxygen levels without altering cardiorespiratory function unless oxygen reached critically low levels (5 kPa). In contrast, chronic hypoxia (10.5 kPa for 15 days), but not hyperoxia (28 kPa for 15 days) resulted in functional and structural modifications that enabled metabolic rate to return to the normoxic (21 kPa) pre-exposure level while maintained in hypoxia (10.5 kPa); this indicating a metabolic compensation. On return to acute normoxia, irrespective of the measurement temperature (4, 8, 12°C), metabolic rate was elevated above the pre-hypoxic value observed in normoxia, presumably a result of the structural and/or functional modifications that occurred in hypoxia.

In addition, differences in metabolism and responses to hypoxia were influenced by maternal factors such as egg size, which in turn are determined by maternal body size. Egg size-dependent differences in metabolic rate of embryos were

present and larger embryos from repeat spawning females exhibited increased tolerance to hypoxia, as represented by lower critical oxygen levels for hatching than observed in embryos from smaller eggs from maiden spawners (13 kPa vs. 17 kPa). This result suggests an advantage in embryos from larger eggs whereby the embryo can obtain sufficient oxygen due to the larger surface area of the egg in respect to the embryo's metabolic rate.

Genetic modification for growth (growth hormone transgenesis) or triploidy (three sets of paired chromosomes) increased metabolic rate above a diploid conspecific (both by 8%). This effect was additive in triploid transgenic alevins that also displayed an altered cardiorespiratory response to acute, severe hypoxia (5 kPa). In addition, acute hypoxia did not elicit a cellular stress response, but was associated with differential (reduced) expression of cellular stress proteins (heat shock proteins) that in one case (Hsp90) was dependent on the growth hormone transgenic genotype.

Taken together, the above observations demonstrate that extrinsic as well as intrinsic factors have substantial effects on the physiology of developing fish, and that the overall response to extrinsic factors contains a temporal component. Given the associated effects are reflected in changes in metabolism, it is likely that the effects will significantly impact growth, survival and performance of the developing fish.

1. General introduction: Effects of environmental and inherent factors on metabolic, cardiorespiratory function and heat shock protein expression in fish

1.1. Thermal tolerance and oxygen limitation

In the aquatic environment, an animal's performance is determined by its ability to cope with environmental fluctuations such as changes in temperature or O₂ levels. The scope of an animal's aerobic performance (aerobic scope) defines the limits within which an animal can sustain aerobic metabolism, and the circulatory and ventilatory systems have the capacity to match O₂ demand (Pörtner 2002, Pörtner and Knust 2007). The aerobic scope is defined as the difference between standard and maximum metabolic rate and peaks at a specific thermal optimum (Fig. 1.1a). Various environmental factors such as temperature, O₂, CO₂ concentration or pollutants have the potential to limit the aerobic scope of a species and consequently reduce its fitness. Some species will tolerate a reduction in the aerobic scope to a great degree, dependent on their capacity for anaerobic metabolism and other defence mechanisms (e.g. antioxidants, heat-shock proteins). Beyond the limits of these compensatory mechanisms, denaturation of proteins and ultimately death will occur. It has been found that the aerobic thermal window (the thermal limits within which the aerobic scope can be maximised) is the narrowest during early development and reproductive life stages (Pörtner and Farrell 2008), which makes these stages particularly sensitive to environmental challenges (Fig. 1.1b). This is a result of the high energetic cost associated with growth during development and the diversion of energy to raise the offspring during reproduction. The narrow thermal window in embryos and larvae might also be associated with the transition from simple cutaneous to

ventilatory and circulatory O₂ supply in some species. Understanding the effects of a changing environment on the metabolism and cardiorespiratory function of a species has a pivotal role when assessing the environmental impact on a particular species.

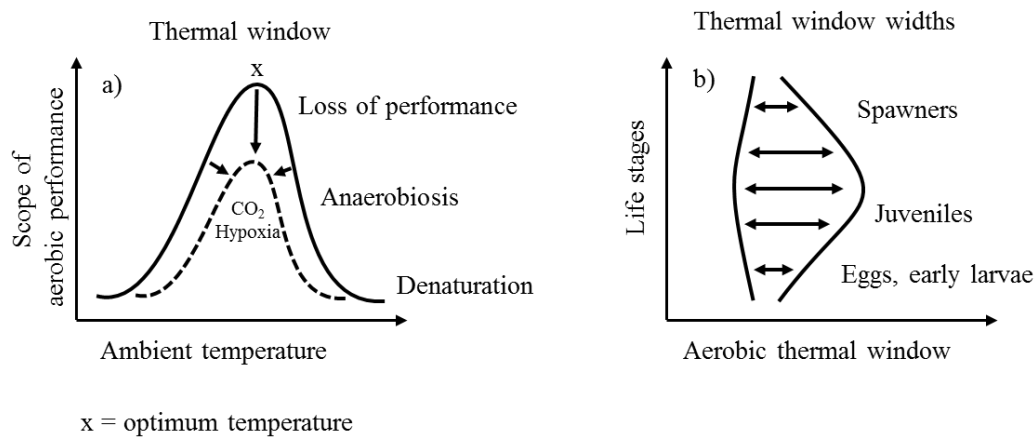


Figure 1.1. Temperature effects on aquatic animals (adapted from Pörtner and Farrell 2008). The aerobic scope is the difference between minimum and maximum metabolic rate. a) Within a certain thermal range, aerobic performance is maximised (X). The further outside this thermal optimum, the organism becomes progressively hypoxemic¹ (functional hypoxia²), aerobic performance is reduced, anaerobic metabolism is induced and ultimately denaturation occurs. Overall, the aerobic scope and aerobic thermal window is reduced by environmental changes (e.g. hypoxia or hypercapnia³). b) The aerobic thermal window shows ontogenic plasticity with early stages of development and reproductive life stages having the narrowest thermal windows and highest susceptibility to environmental perturbations.

1.2. Gas exchange in water breathing vertebrates

In aquatic vertebrates O₂ is obtained from the water and is transferred to the cells, where it is used in mitochondria to generate cellular energy (Adenosine triphosphate, ATP) via oxidative phosphorylation. In fish, water is actively pumped across the gills, while O₂ passively diffuses down its partial pressure

¹ Hypoxemia refers to the deficiency of oxygen in the blood

² Hypoxia is a relative term and refers to levels of O₂ below normal, generally considered as sea-level (PO₂ = 21 kPa).

³ Hypercapnia refers to levels of CO₂ above normal

gradient via countercurrent O_2 exchange into the gill capillaries (Fig. 1.2). There, O_2 binds to hemoglobin (Hb) of erythrocytes and is transported to the metabolising tissues by cardiovascular convection where it diffuses into the cells. In the mitochondria, the O_2 is used to oxidise organic fuels to produce cellular energy (ATP) and CO_2 as a waste product. In steady state, the flow rate of O_2 through the system and therefore the rate of O_2 consumption (⁴an indirect measure of metabolic rate, \dot{M}_{O_2}) is equal at all steps and can be quantified at the convective or diffusive stages using the Fick principle (1870, Fig. 1.2).

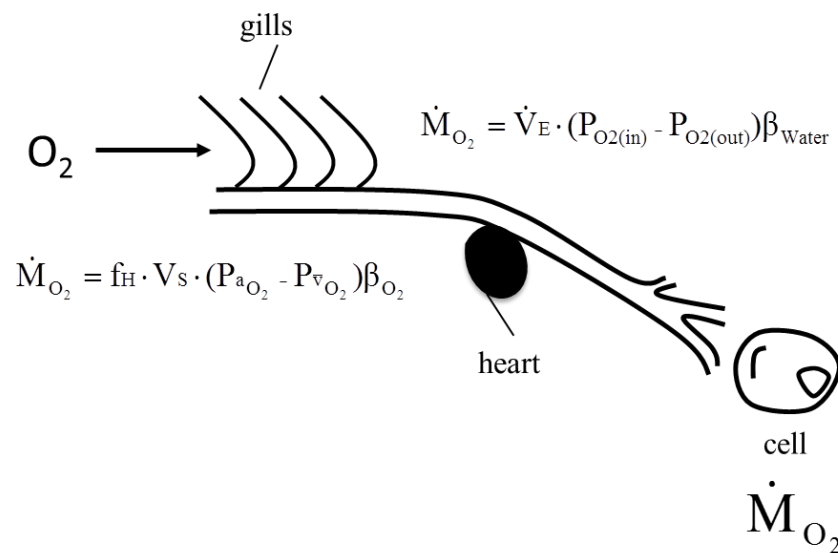


Figure 1.2. A simplified schematic of the oxygen cascade in fish. O_2 is moving into the gills via diffusive and is distributed to cells via convective pathways. The metabolic rate (\dot{M}_{O_2}) can be quantified at each step as a product of the conductance of O_2 multiplied by the partial pressure difference, using derivations of the Fick principle. The \dot{M}_{O_2} at each step is equal and ultimately established by the \dot{M}_{O_2} at the mitochondria in the cell. Abbreviations: \dot{M}_{O_2} , metabolic rate; \dot{V}_E , minute ventilation; $P_{O_2(in)}$, inspired partial pressure of O_2 (PO_2); $P_{O_2(out)}$, is the expired PO_2 ; β_{water} , capacitance of O_2 in water; f_H , heart rate; V_S , cardiac stroke volume, $P_{a_{O_2}}$, arterial PO_2 ; $P_{v_{O_2}}$, venous PO_2 ; β_{O_2} , capacitance of O_2 in blood.

⁴ Within this thesis the terms O_2 consumption and metabolic rate will be used interchangeably

The fundamental equation for O₂ convection described by Fick (1870) is:

$$\dot{M}O_2 = f_H \cdot V_S \cdot ([O_2]_A - [O_2]_V) \quad \text{eqn. 1}$$

where $\dot{M}O_2$ is the metabolic rate, f_H is heart rate and V_S is stroke volume (and their product is the cardiac output, \dot{Q}) and $[O_2]_A$ and $[O_2]_V$ are respectively the concentrations of O₂ in arterial and mixed venous blood. The parameters required to determine $\dot{M}O_2$ can be relatively easily measured. Therefore, it is possible to calculate $\dot{M}O_2$ when the O₂ saturation of hemoglobin and \dot{Q} of the animal is known (Fig. 1.3).

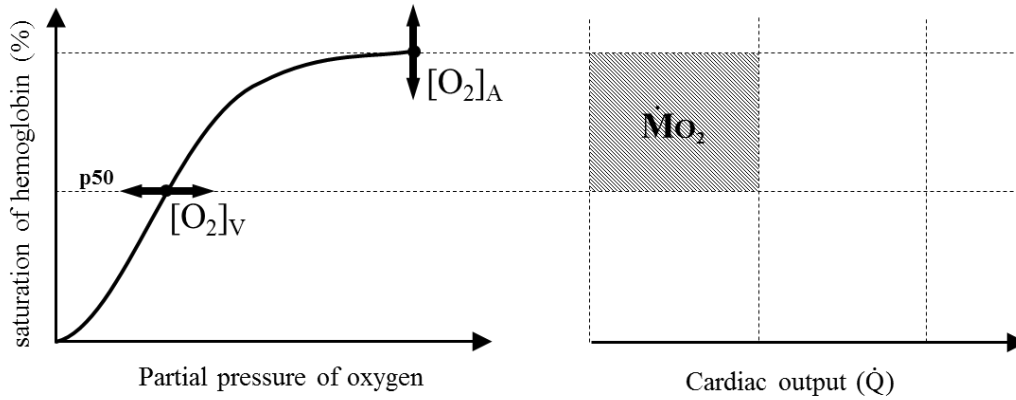


Figure 1.3. Simplified concept of O₂ convection as described by Fick (1870) where metabolic rate (gray shaded area, $\dot{M}O_2$) is a function of arterial and venous concentrations of O₂ ($[O_2]_A$ and $[O_2]_V$) and cardiac output (\dot{Q}). Changes to \dot{Q} , assuming $[O_2]_A$ and $[O_2]_V$ remain constant, must alter $\dot{M}O_2$ and vice versa.

1.3. Hypoxia and temperature

Low environmental O₂ (hypoxia) occurs naturally in freshwater and marine environments, for example, in shallow, warm waters with low flow and increased O₂ decay through microbial metabolism (Diaz and Breitburg. 2009). Hypoxia may occur on a scale ranging from acute (short-term) to chronic (long-term), and in levels ranging from mild to severe. The major challenge for organisms relying on aerobic metabolism is the maintenance of sufficient O₂ supply to the tissues and organs. This challenge is countered by adaptations that involve mechanisms to reduce the O₂ demand through metabolic depression and concomitant ionic and pH disturbances (Bickler and Buck 2007), anatomical modifications to structures involved in O₂ uptake (e.g. changes to gill surface area, Chapman et al., 2007) and behavioural changes that allow escape from hypoxic zones (Kramer 1987). Within fish, hypoxia tolerance is highly varied and is often habitat and life style dependent (Chapman et al. 2002).

In fish, two major strategies to cope with hypoxia have evolved: either O₂ regulation (maintenance of metabolic rate) or O₂ conformation (reduction of metabolic rate; Marvin and Heath 1968). Hypoxia tolerance has classically been closely linked to the critical O₂ tension for metabolic rate (P_{crit}), where O₂ regulation is no longer sustained (aerobic scope = 0) with declining partial pressures of O₂ (PO₂) and a transition from regulation to conformation occurs (Yeager and Ultsch 1989). Hence, more hypoxia tolerant species are thought to have a lower P_{crit} . While not all fish are strictly O₂ regulators or conformers, there appears to be a correlation between lifestyle and P_{crit} (hypoxia tolerance), where

sedentary, relatively inactive species tend to be conformers and pelagic, active fish tend to be regulators (Burggren and Randall 1978; Marvin and Heath 1968).

As described previously, hypoxia reduces the aerobic scope (Fig. 1.1) of a species and high water temperatures may lead to functional hypoxia (hypoxemia) as a result of the Q_{10} -effect⁵ on metabolic rate (Schmidt-Nielsen 1997). Hence, thermally induced hypoxemia results from a mismatch between the capacity to supply O_2 and the O_2 demand (both being temperature dependent processes, Pörtner and Lannig 2009), that needs to be compensated for.

1.4. Generalised physiological response to hypoxia in adult fish

In the aquatic environment, O_2 availability is significantly lower than in air due to its lower O_2 capacitance and diffusivity (Dejours 1981). Additionally, ventilation of water across respiratory organs is more energetically demanding due to the higher viscosity of water compared with air.

Although adult fish are generally regarded as O_2 regulators, the metabolic hypoxic response has been well defined in fish and involves metabolic depression to various degrees that is induced at different critical thresholds (Boutilier et al. 1988; Fry 1971; Kazakov and Khalyapina 1981).

⁵ Q_{10} is the temperature coefficient; the factor by which the rate of a reaction increases for every 10°C rise in temperature.

$$Q_{10} = \left(\frac{R_2}{R_1} \right)^{\frac{10}{T_2 - T_1}},$$

where R_1 and R_2 are the corresponding rates at two different temperatures (T_1 and T_2).

In response to hypoxia, fish will hyperventilate. In rainbow trout (*Oncorhynchus mykiss*) for example, acute hypoxia elicits an increase in ventilatory volume (Smith and Davie 1984). Longer hypoxia exposure and gradual hypoxia increase ventilatory volume, anaerobic metabolism and bradycardia⁶ (Dunn and Hochachka 1986; Hopleton and Randall 1967; Hughes and Saunders 1970; Smith and Jones 1978). Acute as well as chronic hypoxia induces a stress response by elevating catecholamine secretion and the responsiveness to cholinergic stimulation (Montpetit and Perry 1998; Tetens and Christensen 1987).

Long-term hypoxia also affects hematological parameters by increasing blood O₂ carrying capacity through an increased haematocrit (Hc) and hemoglobin (Hb) concentration, as well as Hb-O₂ binding affinity (Lai et al. 2006; Nikinmaa 1990; Soivio et al. 1980; Wells 2009). Catecholamines such as epinephrine and norepinephrine in the blood circulation bind to receptors on the erythrocyte membrane (Nikinmaa and Huestis 1984), a unique feature of fish erythrocytes. This raises the intracellular pH and thus the Hb-O₂ affinity through the Bohr-effect⁷ (Fievet et al. 1988; Nickerson et al. 2003; Nikinmaa 1983; Nikinmaa et al. 1987) and also via the dissociation of ATP-Hb complexes through erythrocyte swelling (Nikinmaa 1983).

On a cellular level, the stress response to environmental challenge is characterised by an increased synthesis of heat shock proteins (Hsp, Schlesinger 1990; Feder and Hofmann 1999). Hsps are a highly conserved family of proteins (chaperons)

⁶ Bradycardia refers to a decrease in heart rate below normal

⁷ The Bohr-effect states that hemoglobin O₂ binding affinity is inversely related to acidity and CO₂ concentration. A decrease in blood pH or increase in CO₂ concentration results in the release of O₂ from hemoglobin and vice versa.

that are critical to the maintenance of cell homeostasis through protection from protein unfolding and damage. Hsps also appear to play a key role during early embryonic development (Deane and Woo 2011). While changes in temperature have significant and wide ranging effects on the expression of Hsps and therefore the maintenance of cellular function at suboptimal thermal conditions in fish (Basu et al. et al. 2002), the effects of hypoxia on Hsp expression has hardly been studied. Hypoxia or anoxia⁸ elicits a physiological stress response and alters Hsp expression, but its expression shows a high level of tissue, cell and temporal specificity (Kregel 2002).

On the molecular level, regulation of the hypoxic response in fish is mediated via the hypoxia-inducible factor 1- (HIF-1) pathway (Soitamo et al. 2001), with HIF-1 α being the key regulating factor. HIF-1 α is stabilized in hypoxia, dimerises with HIF-1 β and triggers the expression of hypoxia-inducible genes (Nikinmaa and Rees 2005; Soitamo et al. 2001). These, amongst other mechanisms, may lead to increased erythropoiesis (Lai et al. 2006; Semenza and Wang 1992) and an increase in vascular endothelial growth factor (VEGF, Vuori et al. 2004) that promotes angiogenesis (Lee et al. 2002). Both these processes are important components that may improve O₂ transport in hypoxia.

1.5. Physiological response to hypoxia during early development in fish

Hypoxia is a natural occurrence in spawning habitats of fish and therefore a critical factor during early developmental stages (Dhiyebi et al. 2012; Peterson and Quinn 1996; Rubin 1998; Whitman and Clark 1982). At embryonic stages,

⁸ Anoxia is defined as the complete lack of oxygen.

the chorion of the egg forms a diffusive barrier for gas exchange that restricts O₂ uptake (Ciuhandu et al. 2005; Ciuhandu et al. 2007; Miller et al. 2008; Rombough 1988a). Chronic hypoxia has profound consequences by reducing developmental rates, hatching success, altering metabolic rate, cardiac function and by increasing mortality (Alderdice et al. 1958; Hamor and Garside 1976; Miller et al. 2011; Rombough 1988b; Shumway et al. 1964; Sowden and Power 1985).

During embryonic and larval stages, fish and other vertebrates appear to be O₂ conformers rather than regulators (Pelster 1999). Rainbow trout embryos reduce metabolic rate during acute (50% saturation, 3 hours) as well as chronic hypoxia (50% saturation, 15 days) by approximately 50% (Miller et al. 2008). Early larval stages of the clawed frog (*Xenopus laevis*) have been reported to display a P_{crit} of 20 kPa when the external gills are not yet present (Hastings and Burggren 1995).

In fish the hypoxic cardiorespiratory response during development is less well defined. In zebrafish (*Danio rerio*), a hypoxia tolerant tropical species, acute as well as chronic hypoxia has been shown to elicit tachycardia during development (Jacob et al. 2002; Shang and Wu 2004) and Bagatto et al. (2005) described a decrease in heart rate in severe hypoxia. Barrionuevo and Burggren (1999) found no coupling of heart rate to a reduced metabolic rate in acute hypoxia during early development. HIF-1 α mRNA expression in zebrafish embryos is increased in hypoxia but genes encoding for erythropoietin and Hb show a reduced expression (Ton et al. 2003).

1.6. Temperature and teratogenic factors during fish incubation

Temperature is regarded as the ecological master factor for fish (Brett 1971). An increase in ambient temperature leads to a reduction of O₂ solubility, and within the range of thermal limits, increases metabolism, as a result of temperature dependence of activation energy for the whole organism (Fry 1971; Jones 1982; Pörtner 2002) and therefore the demand for O₂. Furthermore, the thermal optimum as well as the metabolic scope (the range of temperatures where metabolic efficiency is greatest) depends on the animals thermal history or acclimatization (Brett 1971; Jobling 1994; Pörtner and Farrell 2008). Variations in metabolism at similar acclimation temperatures are representative of changes to the thermal optimum (Atkins and Benfey 2008). Scott and Johnston (2012) have recently shown that embryonic temperature has persistent effects on thermal acclimation capacity in zebrafish. This included aerobic exercise performance, thermal sensitivity, variation in muscle fibre type composition and expression of genes involved in energy metabolism, angiogenesis, cell stress, muscle contraction remodelling and apoptosis.

Incubation temperature in egg and larval development has also been determined as an etiological factor for deformities of skeletal structures and organ systems in Atlantic salmon (*Salmo salar*) and other fish species. As deformities pose a significant economic as well as ethical challenge to the aquaculture industry, efforts have been made to optimize rearing practices. These conditions include vertebral deformities, reduced operculae, torsions of the swim bladder as well as aplasia of septum transversum (Kvellestad et al. 2000; Poppe et al. 1997; Takle et al. 2005; Vagsholm and Djupvik 1998). Embryos are generally more vulnerable

to temperature induced deformities (Hansen and Falk-Petersen 2001; Koo and Johnston 1978; Ornsrud et al. 2004a; Ornsrud et al. 2004b; Sato et al. 1983; Wang and Tsai 2000; Wiegand et al. 1989) and the prevalence of these conditions appear to be more severe the earlier the stressor occurs during egg development. Other teratogenic factors that induce deformities are nutrition (Cahu et al. 2003), infections (Madsen et al. 2001), antibiotics (Toften and Jobling 1996), genetics (Sadler et al. 2001) as well as hypoxia, especially during early development as it may induce apoptosis and disrupt organogenesis, a key process of growth (Castro Sanchez et al. 2011; Shang and Wu 2004). The optimal incubation temperature for Atlantic salmon eggs and the yolk-sac stage has been determined to be 8°C or lower, where growth is maximised at minimal potential for teratogenic effects (Baeverfjord et al. 2009). A better understanding of the physiological responses to these teratogenic factors can thus assist in preventing their occurrence in the hatchery or the natural environment.

1.7. Study-specific background

This thesis focussed on the physiological response to hypoxia and changes in ambient temperature of developing Atlantic salmon, *S. salar*. Salmonids are generally regarded as highly aerobic, hypoxia intolerant species inhabiting temperate, high O₂ environments (Holeton and Randall 1967; Smith and Heath 1980).

Current knowledge of the respiratory physiology of vertebrates during development lags far behind that of adults. Previous work on fish and amphibian embryos has suggested that the egg chorion poses a significant diffusive barrier

for O₂ towards the embryo. Also, a constraint on O₂ supply slows development in vertebrates and may influence hatching (Adolph 1979; Alderdice et al. 1958; Bradford and Seymour 1988; Czerkies et al. 2001; Mills and Barnhart 1999; Warkentin 2007). These studies include precise quantitative estimates of O₂ exchange across the egg shell and the magnitude of metabolic activity. The “*bigger is worse during incubation hypothesis*” was established more than half a century ago and suggests that embryos from smaller eggs will have selection advantages under hypoxic conditions due to a better surface area to volume ratio (Hendry et al. 2001; Kinnison et al. 2001; Krogh 1959). Attempts have been made to estimate O₂ diffusion coefficients across salmonid eggs (Rombough 1989) and scaling coefficients of metabolic rate and egg surface area indicate that surface area increases at a greater rate than metabolic rate (Einum et al. 2002), despite the long standing belief that embryos in smaller eggs have an advantage of obtaining O₂ from the environment. Even though embryos from larger eggs in a study by Einum et al. (2002) show increased survival compared with embryos from smaller eggs in hypoxia, a study by Rombough (2007) could not verify differences in hypoxia tolerance of different sized eggs. However, these studies did not further investigate the maternal effects of egg size difference on metabolic rate and O₂ exchange across the egg chorion.

In extension of studying the response of hypoxia and maternal effects in embryos, the effects of hypoxia on developmental stages post hatching were investigated. Environmental hypoxia, a common occurrence in fish habitats and of major concern to the aquaculture industry is a challenge for a species' survival and fitness, which relies on aerobic metabolism (Roussel 2007; Shang and Wu 2004).

Extensive work has been conducted to improve the understanding of the biological effects of acute and chronic hypoxia and temperature and has been shown to have profound effects on the respiratory physiology of adult and developing fish (Bickler and Buck 2007; Richards 2010; Rombough 1988b and citations within). In comparison, there is less information available on the physiological response to environmental hyperoxia⁹. The hypoxic and hyperoxic respiratory response in fish varies largely inter- and intraspecifically and does not allow an overarching description. Acute or chronic exposure to different levels of O₂ elicits different responses that are further confounded by acute or chronic temperature changes. For example, acute hypoxia leads to a reduction in metabolic rate and heart rate in adult rainbow trout, a hypoxia intolerant species (Holeton 1971; McDonald and McMahon 1977), whereas their larvae lack a bradycardic response to acute hypoxia with decreasing metabolic rate but display bradycardia after chronic hypoxia exposure (Miller et al. 2011). In zebrafish on the other hand, a tropical, hypoxia tolerant species, cardiac output is increased in chronic hypoxia after hatching (Jacob et al. 2002). However, inter-study comparisons are usually difficult because of different experimental designs, methodological approaches and the severity of the environmental challenges. Therefore, in an integrative approach, the metabolic and cardiorespiratory responses and adaptations to acute and chronically changing environments (hypoxia, hyperoxia and temperature) during early development in *S. salar* were investigated to disentangle the physiological effects of the individual components at this critical life stage.

⁹ Hyperoxia refers to levels of O₂ above normal

In addition to environmental factors and how they influence developmental physiology, intrinsic (genetic) factors were then taken into consideration. Growth hormone (GH) transgenesis is a recent, innovative molecular tool to increase growth rates in animals. Biologically, a rapid growth rate should be beneficial to an animal because the time spent during vulnerable, early life stages is reduced and chances of survival are increased (Stearns 1992). Similarly, faster growth through genetic modification is of economic interest to the aquaculture industry, as fish production could be increased at a lower cost. Physiological consequences of GH transgenesis have been described in adult salmonids. Here, GH-transgenesis leads to accelerated growth, elevated metabolism and improvements to O₂ delivery. This occurs as a consequence of the increased O₂ demand that encompasses morphological as well as biochemical modifications to the heart, gills, blood as well as metabolic enzyme activity (Deitch et al. 2006; Devlin et al. 1994; Herbert et al. 2001; Martinez et al. 1999; Shao Jun et al. 1992; Stevens and Sutterlin 1999; Stevens et al. 1998; Stokstad 2002). Information on the cellular stress response in fish to hypoxia is very limited and no information is available on the differential expression of Hsps as a consequence of GH-transgenesis. However, hypoxia does not appear to be an indicator for hypoxic stress in juvenile Atlantic salmon (Zarate and Bradley 2003). Similar information on larval stages is missing and since respiratory mechanisms during early larval development are substantially different to adult stages (Rombough 1988a), the responses during development are also very likely to differ.

Another intrinsic factor, triploidy, is a natural trait in some fish and is artificially induced in the aquaculture environment to produce sterile offspring that are

thought to have higher growth potential and better flesh quality. Physiologically, polyploidy is mainly associated with changes to cell size (increase in cell volume) and gonadal development (Benfey 1999). However, the benefits of growing triploid fish have been very controversial in recent years (Maxime 2008) as clear evidence for improved performance in triploids is lacking. In contrast, triploids appear to be more severely affected by adverse environmental conditions (like hypoxia) than diploids (Maxime 2008). Furthermore, knowledge on the physiological consequences of triploidy during larval development is sparse. How the combination of GH-transgenesis and polyploidy affect metabolism and cardiorespiratory function remains fundamentally unexplored.

1.8. Objectives and hypotheses

The objectives of this thesis were to investigate the metabolic, cardiorespiratory and cellular stress response to acute and chronic environmental challenges of normal as well as genetically modified Atlantic salmon at early developmental stages. (1) In particular, differences in metabolic rate and O₂ exchange across the chorion of different sized eggs (from maiden and repeat spawning females), a result of the maternal effect of female body size, and how this influences hypoxia induced hatching were elucidated. (2) The plasticity of metabolic rate and cardiorespiratory function (heart- and ventilation rate) to acute and chronically changing environments (thermal, hypoxic as well as hyperoxic changes) were investigated to better understand the interactive effects of these variables. (3) Finally the effects of accelerated growth rates on these parameters through GH-transgenesis in combination with triploidization during early development were

analysed. In the course of this thesis, novel methodological approaches to respirometry were developed that are applicable to small aquatic animals.

(1) It is hypothesised that repeat spawning females have larger eggs with a larger surface area in respect to their metabolic demand; despite smaller eggs from maiden spawning females having an advantageous surface area to volume ratio. This, in turn, will affect their hypoxia tolerance, displayed through differences in hypoxia induced hatching.

(2) It is further hypothesised that hypoxic acclimation results in a delay in developmental rate and decreased metabolic rates due to lower O₂ availability. The presence of hypoxic cardiorespiratory control mechanisms will allow changes in heart- and ventilatory rates in hypoxia to alleviate the reduced O₂ supply. In addition, hyperoxic acclimation will result in accelerated growth but is not associated with a chronically increased demand for O₂ since the high abundance of O₂ does not necessitate improvements to O₂ delivery.

(3) Because faster growth through GH- transgenesis is generally associated with increased anabolic processes and a higher aerobic demand, increased metabolic rates in alevins are suggested, which are exacerbated by triploidy. Furthermore, hypoxia will augment metabolic and cardiorespiratory responses to GH- transgenesis, that are also reflected in the cellular stress response, because with faster growth under low O₂ availability, metabolic energy is diverted to maintain aerobic function and will impact on cellular homeostasis more severely.

Atlantic salmon is a suitable species to use to address these questions, as it is particularly sensitive to environmental perturbations and the transparency of its body (see Appendix 4) at early alevin stages allows easy, non-invasive measurement of cardiorespiratory function. Furthermore, Atlantic salmon is becoming a model organism for fish biology with extensive research being conducted in particular in the context of aquaculture practices such as genetic modification and selective breeding and the current attempt to sequence the salmon genome (Davidson et al. 2010).

2. Methods: Measurement of metabolic rate, heart rate, ventilation rate and heat shock protein expression

2.1. Egg, geometry and embryonic and yolk-sac alevin mass and morphometry

Egg and yolk-sac alevin (alevin) dimensions were measured using a horizontal dissecting microscope (Nikon SMZ645) with an integrated ocular micrometer. The internal (r_i) and external egg radii (r_o) were measured at 4 locations of the capsule (90° apart) to account for deviations in sphericity of the egg (see Fig. 2.1). Despite the comparatively thin egg capsules of *S. salar* eggs, adjusting the focal point of the microscope to the middle plane enables an optical estimation of capsule thickness ($L = r_o - r_i$) by discerning the capsule from the actual embryo and yolk-sac (Fig. 2.1).

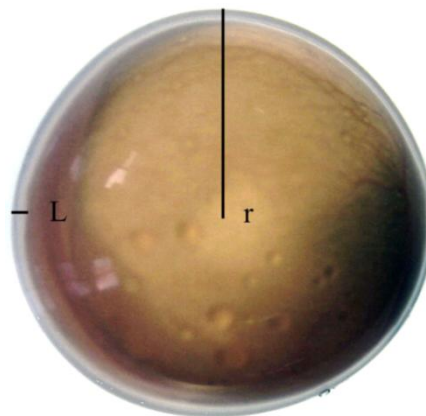


Figure 2.1. *S.salar* egg under a dissecting microscope. Measurement of the radius (r) and capsule thickness ($r_o - r_i$, L) of the egg are indicated by the black lines.

Embryos and yolk-sac alevins were killed after an experiment. Eggs were weighed, and then the embryo (with yolk-sac) was removed from the egg capsule with micro-operating scissors. Total wet body mass in embryos and yolk-sac alevins was weighed, then the yolk-sac (Appendix 4) was separated from the

alevin body with micro-operating scissors to measure wet yolk-free body and wet yolk mass. Body length (mm) and number of caudal fin rays (n, as a proxy for developmental stage) were measured under a dissecting microscope.

2.2. Measurement of metabolic rate in eggs and alevins

The rate of oxygen consumption (\cong metabolic rate = $\dot{M}O_2$) in eggs and alevins was measured using fluorescence-based closed system respirometry. A single egg or alevin was placed in an individual well in a polystyrene multiwell plate (24 wells x 3.3 ml, Oxodish[®], PreSens, Regensburg, Germany) that was filled with air-equilibrated water from the rearing trays (partial pressure of O_2 , $PO_2 = 21$ 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 egg or alevin did not contact the optode (Fig. 2.2). 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 cover slip 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 (2mm Ø) stainless steel ball around the perimeter at the bottom of each well ensuring the water in each well was gently mixed; the eggs or alevins remained motionless on the mesh. The PO_2 in each well was measured at 2 min intervals starting from 21 kPa conditions until the PO_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} M cobalt chloride ($\text{PO}_2 = 0$ kPa) and air-equilibrated water ($\text{PO}_2 = 21$ kPa).

The $\dot{\text{M}}\text{O}_2$ ($\mu\text{mol O}_2 \text{ min}^{-1}$) for each egg or alevin was calculated from the decline in PO_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 chapter 2.3. for details).

Mass-specific $\dot{\text{M}}\text{O}_2$ was adjusted for yolk-free body mass (g) to account for differences in body mass between 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 1g/ml, the stainless steel ball bearing and mesh.

2.3. Correcting for diffusion and validating the use of plastic multiwell plates with integrated optodes

Respirometry is a well-established method for the measurement of the rate of O_2 consumption (R , mmol min^{-1}) of cells and organisms. Classically, in either open-flow or closed respirometry systems R is derived from changes in oxygen concentration ($[\text{O}_2]$, mmol L^{-1}), which is the product of the capacitance coefficient for O_2 (βO_2 , $\text{mmol L}^{-1} \text{ kPa}^{-1}$, which is dependent on temperature) and partial pressure (PO_2 , kPa), the latter being an easily measured variable. In open-flow systems, R is the product of flow (L min^{-1}), βO_2 and the difference in PO_2 between the inflowing and outflowing air or water from the chamber containing the cell or organism. In well-mixed closed systems, R is the product of the decline

in PO_2 over time, βO_2 and the volume (V) of the sealed chamber (see Lighton 2008 for more details on each method). In recent years, the use of integrated luminescence based optochemical O_2 sensors (optodes) to measure PO_2 have gained favour over formerly used Clarke-type electrochemical sensors in respirometric studies because they do not consume O_2 , require low maintenance and provide more stable measurements. In closed system designs, the simultaneous measurement of replicates using polystyrene multiwell plates as well as glass vials with integrated optodes are increasingly used to measure R in small animals and cells (Abaci et al. 2010; Beckers et al. 2010; Deshpande and Heinzle 2004; Köster et al. 2008; White et al. 2011). Ideally, respirometry chambers should be non-diffusible for gases. It is known that polystyrene is O_2 -permeable (Arain et al. 2005) so that O_2 will diffuse into the polystyrene well if the PO_2 within a well is reduced below the PO_2 of the surrounding, usually normoxic, atmosphere. Despite an increase in the use of polystyrene-based multiwell plates as respirometers, gas diffusion through the walls of the plates and their accurate implementation into respirometric studies has not been sufficiently evaluated. To make precise measurements of R of cells, tissues or whole animals using this system, the O_2 diffusion into the sealed wells needs to be accounted for while the O_2 within each well is reduced by R.

For this purpose, (i) an equation to determine the instantaneous rate of O_2 consumption that takes into consideration diffusion of O_2 when using a polystyrene-based well as a closed respirometer is provided, (ii) the rate of O_2 diffusion across the walls of a polystyrene-based well at different temperatures

was measured, and (iii) a novel method using an ‘O₂ scavenger’ to calibrate small closed-well respirometers is described.

2.3.1. The polystyrene-based multiwell aquatic respirometer

The OxoDish[®] (PreSens) is an inexpensive modified 24 well polystyrene cell culture plate (Cellstar[®], greiner bio-one) with central integrated optodes in the bottom of each well. The volume of each cylindrical well is 3.3 ml (depth = 16.5 mm, Ø ≈ 16 mm), ideal for aquatic respirometry in small animals or eggs. To ensure the water in each well is thoroughly mixed a 2 mm (Ø) stainless steel ball was placed within each well, the ball moving around the perimeter of the well and around the optode when the plate is positioned on an orbital mixer. A mesh situated above the ball enables small animals or eggs to be placed in the well and avoid contact with the optode and/or ball. Each well is individually sealed with a glass cover slip (Fig. 2.2).

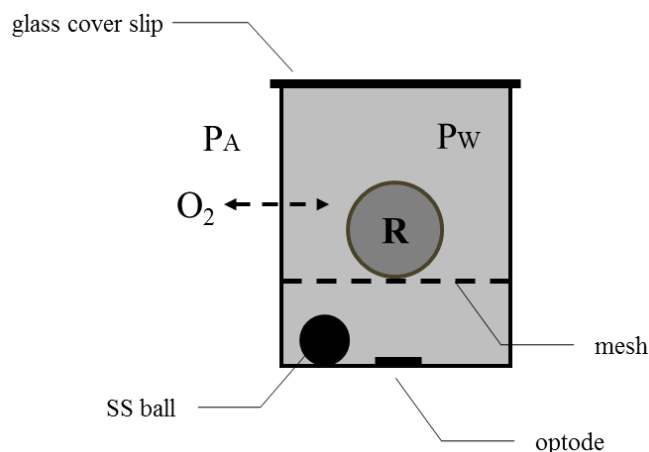


Figure 2.2. A polystyrene-based well used for aquatic respirometry. O₂ can diffuse across the walls of the respirometer, the top sealed with a glass cover-slip. A centrally integrated optode is situated on the bottom of the well. A stainless steel (SS) ball moves around the perimeter to ensure the water is thoroughly mixed. The egg (or animal) to be measured is separated from the optode and ball by a mesh. R, the rate of O₂ consumed by the egg; P_W, the partial pressure of O₂ in the water within the respirometer; P_A, the partial pressure of oxygen in the atmosphere surrounding the well.

2.3.1.i. Determining the instantaneous rate of O₂ consumption

The egg depicted in the respirometer in Fig. 2.2 consumes O₂ at an instantaneous rate R(t). That is, in the infinitesimal time interval [t, t+δt], the egg consumes a number of moles of O₂, R(t)δt. Assume that the medium inside and outside the well are thoroughly mixed, and let P_W(t) denote the PO₂ inside the well at time t, and P_A the (constant) PO₂ outside the well.

By Fick's law, the diffusive flux is proportional to the gradient (P_A - P_W(t)) across the surface, and conservation of O₂ requires that the change in moles of O₂ in the well balances the moles of O₂ replenished or lost through diffusion and the moles of O₂ consumed

$$V \cdot \beta_{O_2} \frac{dP_W}{dt} = kV \cdot \beta_{O_2} (P_A - P_W(t)) - R(t) \quad \text{eqn. 2}$$

Here V is the volume of the well and k is the diffusion constant. This differential equation has solution (Boyce and DiPrima 2012)

$$P_W(t) = P_A + (P_W(0) - P_A)e^{-kt} - \frac{\beta_{O_2}}{V} \int_0^t R(\tau) e^{-k(t-\tau)} d\tau \quad \text{eqn. 3}$$

where τ is a dummy variable of integration and e^{-k(t-τ)} is an integrating factor.

In the absence of the organism, R(t)=0 and

$$P_W(t) = P_A + (P_W(0) - P_A)e^{-kt} \quad \text{eqn. 4}$$

so that

$$\log(|P_W(t) - P_A|) = \log(|P_W(0) - P_A|) - kt \quad \text{eqn. 5}$$

And k can be determined from the slope of a plot of $\log(|P_W(t) - P_A|)$ against time. Then given observations of both $P_W(t)$ and dP_W/dt , $R(t)$ can be estimated as

$$R(t) = V \cdot \beta_{O_2} \left(k (P_A - P_W(t)) - \frac{dP_W}{dt} \right) \quad \text{eqn. 6}$$

2.3.1.ii. Oxygen diffusion across the walls of a polystyrene-based multiwell plate

To evaluate the rate of O_2 diffusion across the surface of the polystyrene wells, 12 wells within a 24-well calibrated Oxodish (PreSens) were filled with air-equilibrated pure water ($PO_2 = 21$ kPa) inside a temperature controlled room at constant ($\pm 0.5^\circ\text{C}$) temperatures of 8, 22.5, 37°C and placed on a SensorDishReader (SDR, PreSens). Four wells containing a PO_2 zero solution (1% sodium sulphite solution, an O_2 scavenger) were used as controls to account for any drift of the optode signal. The individual wells were then sealed under the exclusion of air bubbles with glass cover slips and the Oxodish placed within an anoxic environment; achieved by flowing nitrogen (50 ml min^{-1}) into a sealed plastic bag with a small leak that ensured the bag remained inflated under slight positive pressure (~ 0.1 kPa). The entire setup was placed on an orbital mixer (70 rpm) to continuously move the stainless steel ball around the perimeter of each well. The PO_2 within each well was measured with the integrated optode every minute using the appropriate data acquisition system (SDR_v38, PreSens) until a

PO₂ of ~5 kPa had been reached. As a control, where no diffusion of O₂ can occur across the surface of each well, the experiment was repeated with a custom built hermetically sealed 24-well aluminium plate with glass bottom and top, the wells with equivalent dimensions to the Oxodish and each well with an optode sensor spot (PSt5, PreSens).

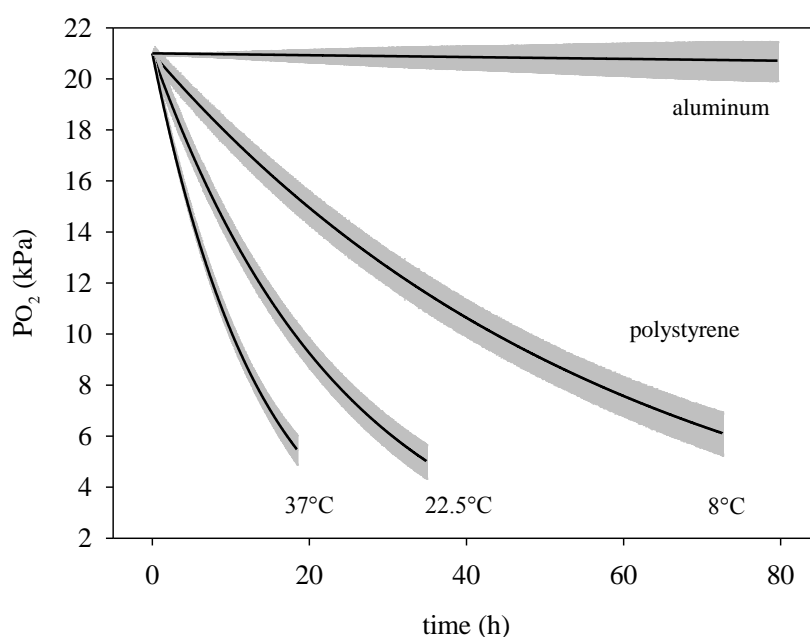


Figure 2.3. Temperature dependent O₂ diffusion through a polystyrene-based well and custom built aluminium (non-diffusible) well over time. The PO₂ in the polystyrene wells decreased exponentially with time, the rate of diffusion depending on temperature; shaded areas are the SEM.

Over time, in keeping with Fick's law of diffusion, O₂ diffused across the wall of the Oxodish and the PO₂ in the water in the wells decreased exponentially, with the change in PO₂ over time being greater the warmer the temperature (Fig. 2.3). In comparison, the PO₂ in the aluminium (non-diffusible) wells remained constant over the same time course. A plot of $\log(|P_W(t) - P_A|)$ against time, where on this occasion $P_A = 0$, yields straight lines and the slopes of which are the k values for

each temperature (Fig. 2.4). The inset panel (Fig. 2.4) reveals the linear relationship that exists between k and temperature; hence, given temperature, k can be predicted for the Oxodish polystyrene-based multiwell plate.

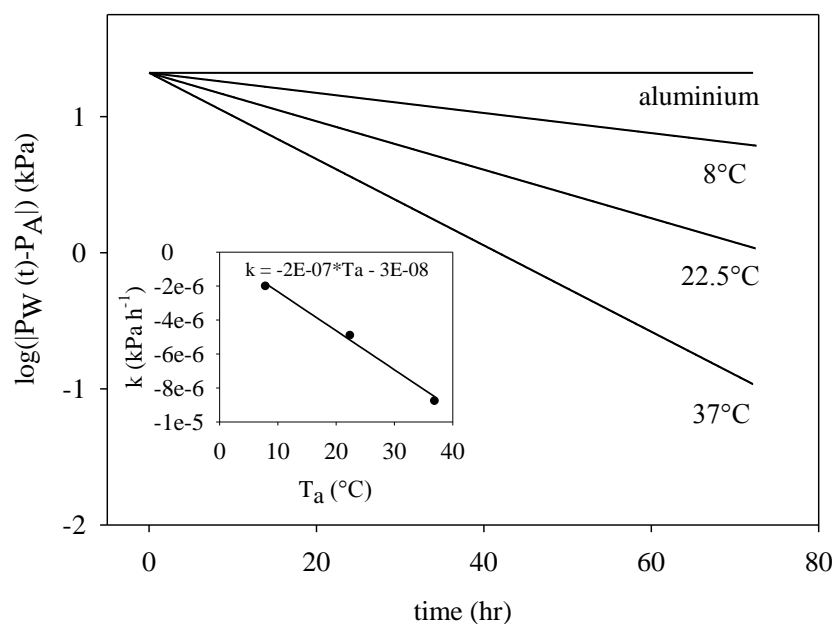
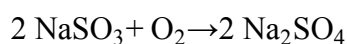


Figure 2.4. $\log(|P_W(t) - P_A|)$ against time for a polystyrene-based well at select temperatures and for a custom built aluminium well (non-diffusible). Inset: the slope of the relationship, k , for each of the polystyrene-based wells against temperature.

2.3.1.iii. Calibration of a small closed-well respirometer

In order to account for the O_2 diffusion occurring across the chambers, the respirometric data obtained needs to be appropriately calibrated. This was achieved by comparing the rates of O_2 consumption in the polystyrene and aluminium well plates using an O_2 scavenger (sodium sulphite) delivered by micro-osmotic pumps.

The chemical reaction for the scavenging of O_2 by sodium sulphite is defined as:



At 37°C the solubility of O₂ in aerated fresh water is 209 µmol L⁻¹, therefore each 3.3 ml well is calculated to contain 0.79 µmol O₂. Given the molecular weight of NaSO₃ is 126 g and for O₂ 32 g, approximately 7.8 parts of NaSO₃ are required to consume 1 part of O₂; hence, 5.4 µmol of NaSO₃ are required to consume all the O₂ in 3.3 ml of water.

A micro-osmotic pump in which the rate of delivery is controlled by the influx of water through a semipermeable membrane, which in turn provides the driving pressure to expel the contents of a collapsable reservoir, was used to deliver NaSO₃ at a controlled rate within a closed-well respirometer. Each micro-osmotic pump (Alzet[®] model 1003D, reservoir volume 90 µl, pumping rate 1.0 µl hr⁻¹, displacement volume 0.5 ml) was filled with 0.1 M NaSO₃ solution (total 9 µmol NaSO₃), approximately 1.66 x that required to extract all the O₂ in 3.3 ml of water at 37°C. As each micro-osmotic pump delivers 1.0 µl hr⁻¹ and 7.8 parts of NaSO₃ are required to consume each part of O₂, the rate at which O₂ will be consumed will theoretically equal 0.01270 µmol hr⁻¹, equivalent to 0.00021 µmol min⁻¹.

Five micro-osmotic pumps were filled and each pump was placed in a well filled with water. Four other wells were filled with PO₂ zero solution (1.0% sodium sulphite) and four with air-equilibrated pure water (PO₂ = 21 kPa). All wells were sealed with glass cover-slips. Experiments were conducted using an Oxodish and the 24-well aluminium plate, preparation and measurement were done in air at 37°C and the rate of O₂ decay in the water of each well was measured as described in section (ii). For comparison, the experiments were repeated at 8°C

using fertilised eggs (368 degree days, 46 days at 8°C since fertilisation) from Atlantic salmon (*Salmo salar*).

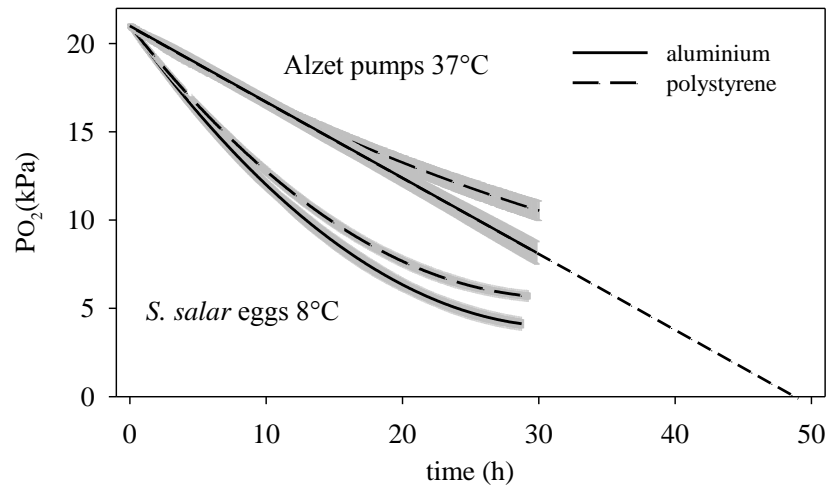


Figure 2.5. Change in PO₂ over time in both aluminium non-diffusible and polystyrene-based wells that hold either Alzet micro-osmotic pumps at 37°C containing a 0.1 M sodium sulphite (O₂ scavenging) solution or *S. salar* eggs at 8°C. The short dashed line indicates the theoretically calculated decrease in PO₂ for Alzet pumps in a sealed system; shaded areas are the SEM.

The decrease in PO₂ over time in the non-diffusible aluminium wells was linear for the Alzet pump, reflecting a constant rate of O₂ consumption (Fig. 2.5), whereas in the absence of O₂ diffusion, the curve-linear nature of the *S. salar* eggs in the aluminium wells reflected a declining rate of O₂ consumption in the egg with declining PO₂. Diffusion of O₂ in to the Oxodish prevents such straightforward conclusions from the O₂ decay curves. R(t) was determined for each well using eqn. (5), k and βO₂ (Dejours 1981) at the appropriate temperature and the volume of the well corrected for the presence of the mesh, stainless steel ball and either the micro-osmotic pump or the egg. By accounting for O₂ diffusion, R_{determined} from measurements made in the Oxodish was not discernably different from R_{calculated} using the non-diffusible multiwell

aluminium plate, for either the Alzet pumps or eggs respectively (Fig. 2.6). The calculated R for the Alzet pump was equivalent to the value theoretically determined.

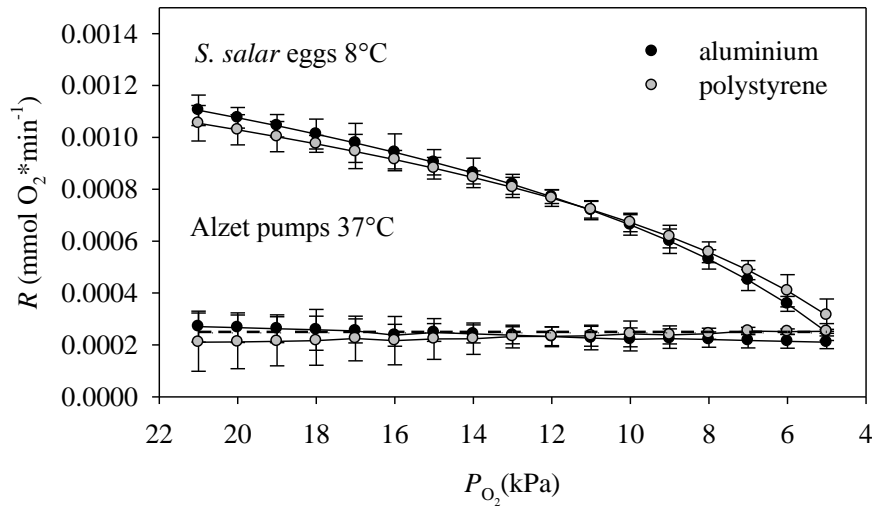


Figure 2.6. Calculated rates of O_2 consumption (R) for either Alzet micro-osmotic pumps at 37°C containing a 0.1 M sodium sulphite (O_2 scavenging) solution or *S. salar* eggs at 8°C. Measurements were made in either aluminium non-diffusible (solid symbol) or polystyrene-based (grey symbol) wells. The dashed line indicates the theoretically calculated R for Alzet pumps delivering $1 \mu\text{l h}^{-1}$ of 0.1 M sodium sulphite solution. There was no difference in measured or theoretical values for R calculated for the Alzet pump, or for measured values calculated for eggs using either the aluminium or polystyrene-based wells.

2.4. Simultaneous measurement of metabolic rate, heart rate and ventilation rate in individual alevins

Simultaneous measurements of metabolic rate ($\dot{M}O_2$), heart rate (f_H) and ventilation rate (f_V) were performed on individual alevins using a combined optophysiological and respirometry system (Fig 2.7). Individual yolk-sac 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, accuracy $\pm 0.025^\circ\text{C}$) at the respective

temperature. Water temperature in the chamber was measured with a thermocouple (accuracy $\pm 0.1^\circ\text{C}$, traceable to a National Standard). Normoxic water ($\text{PO}_2 = 21 \text{ kPa}$) was pumped through the chamber at a constant flow rate of 1.5 ml min^{-1} using a peristaltic pump (Living Systems Instrumentation, Burlington, Vermont), the water first passing through a heat exchanger in the water bath to maintain the required temperature.

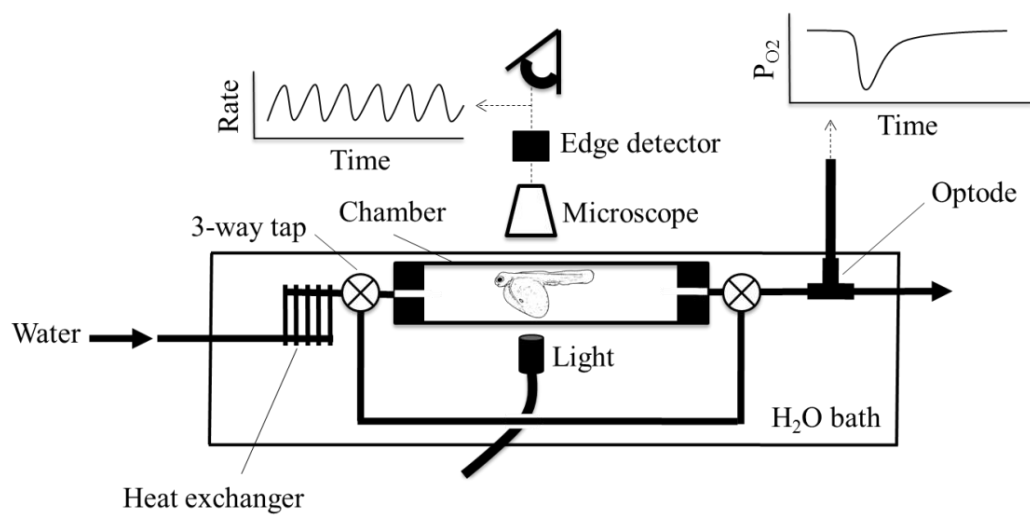


Figure 2.7. Diagram of the respirometry setup in combination with the optophysiological system to simultaneously measure MO_2 , f_H and f_V in individual *S.salar* alevins at different levels of PO_2 . In principle, the respirometry chamber containing the alevin was sealed for a period of $\sim 30 \text{ min}$ while f_H and f_V were recorded, each recorded at a separate time. MO_2 was calculated from the time integral of the gas concentration curve, derived from the PO_2 recording, after flushing the chamber.

To minimise stress that may have been caused by handling, alevins were left to rest within the chamber under constant flow of normoxic water for 1 hr before measurement. The chamber was then sealed for approximately 30 min prior to flushing with water at the original incoming PO_2 until the PO_2 within the chamber had returned to the original PO_2 (approx. 8 min). The process of sealing and flushing was repeated for each experiment, where the desired incoming PO_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, USA) and was checked with and optochemical O₂ sensor (Oxygen meter, HQ10, Hach, 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 O₂ sensor in a flow-through cell (FTC-PSt3, PreSens) in combination with an O₂ analyser (Fibox, PreSens) was used. This was connected in series downstream to the respirometry chamber and maintained at the same ambient temperature (Fig. 2.7). In the present closed but intermittently flushed respirometry system, the rates of O₂ consumption ($\dot{M}O_2$, $\mu\text{mol min}^{-1}$) were calculated, as previously described (Clark et al., 2005). Following a period where the chamber is sealed, it is then flushed with fresh water and $\dot{M}O_2$ determined from the time integral of the resulting PO₂ curve ($\int PO_2$, $\text{kPa}\cdot\text{min}^{-1}$) multiplied by the oxygen capacitance ($\beta_{wO_2}^{10}$, $\mu\text{mol}\cdot\text{L}^{-1}\cdot\text{kPa}^{-1}$), the flow of water (\dot{V} , $\text{ml}\cdot\text{min}^{-1}$) used to flush the chamber, and the reciprocal of the time (t, min) during which the compartment was sealed (eqn. 7). Mass-specific $\dot{M}O_2$ was adjusted for wet yolk-free body mass (g).

$$\dot{M}O_2 = \beta_{wO_2} \cdot \int PO_2 \cdot \frac{\dot{V}}{t} \quad \text{eqn. 7}$$

During periods when the chamber was sealed, heart rate (f_H) and ventilation rate (f_V) were measured using optophysiological methods. For this purpose, a

¹⁰ β_{wO_2} depends on salinity (here assumed 0) and temperature

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. 2.7). Video information was processed by a video dimension analyser (V94, Living Systems Instrumentation, Burlington, Vermont) 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). Separate measurements of f_H and f_V were performed for each level of PO_2 within the first 5 min of the chamber being sealed. Within this timeframe the PO_2 within the sealed chamber did not drop lower than 1.25 kPa from the initial value. This was verified by measuring the PO_2 within the chamber using a microoptode. Therefore, the measurements for f_H and f_V are representative of the desired PO_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.

2.5. 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 PO_2 within the chamber declined as a result of oxygen consumption, flushing the chamber with fresh water will result in an exponential rise of the PO_2 in the outflowing water as it approaches the incoming PO_2 (noting that oxygen consumption is continually occurring but with a

high enough flow of water the difference between inflowing and outflowing PO_2 is effectively zero (Fig. 2.8).

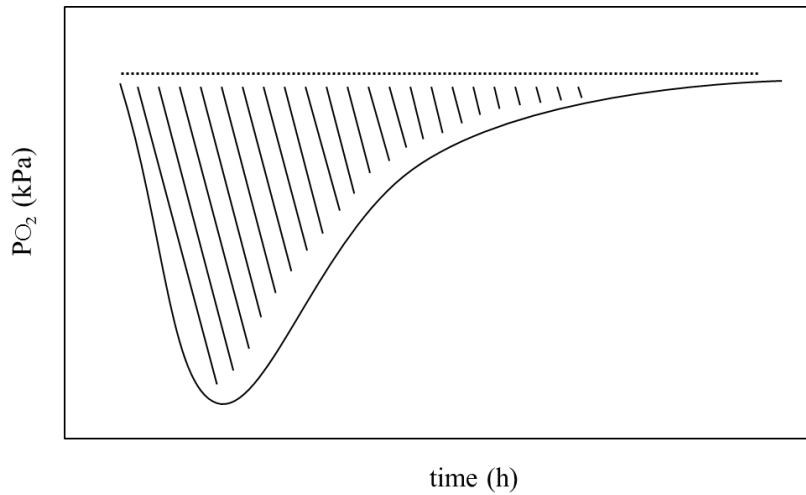


Figure 2.8. Example of the PO_2 profile of outflowing water leaving the respirometry chamber against time that occurs when the chamber, previously sealed and in which O_2 is depleted by the oxygen consumption of the animal inside, is flushed with fresh incoming water (dotted line). The dashed area represents the time integral that is used to calculate MO_2 .

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 PO_2 (21, 13 or 5 kPa) flowed at a constant rate (1.5 ml min^{-1}). From the time integral of the resulting PO_2 curve, multiplied by the O_2 capacitance (βw_{O_2} , $\text{mmol L}^{-1} \text{ kPa}^{-1}$) and flow of water (vol time^{-1}), the number of moles of O_2 injected can be determined (M, Fig. 2.9). All tests were performed in triplicate and averaged.

The accuracy of the system was calculated by comparing between theoretical (the number of moles replaced in chamber water of a given PO_2 following injection of a known volume of anoxic water) and calculated amount 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 PO_2 to ~ 4 kPa, this being similar to that seen when flushing the chamber during experiments.

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

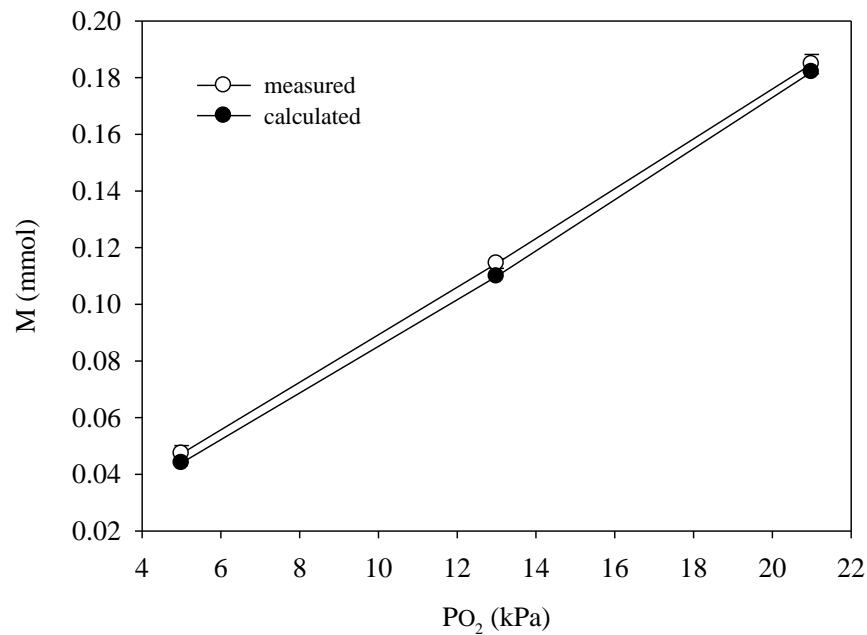


Figure 2.9. Comparison between theoretical and calculated M when injecting anoxic water into the respirometer. Data show means \pm SE.

2.6. Determination of $P_{crit-hatch}$ in eggs

$P_{crit-hatch}$ (ambient PO_2 where hatching was triggered) for embryos that hatched during the measurements of $\dot{M}O_2$ in the closed system respirometers was determined by examining the relationship between $\dot{M}O_2$ and PO_2 . The point where the normally decreasing curve-linear relationship between $\dot{M}O_2$ and PO_2 for eggs no longer occurred (either an increase or no reduction in $\dot{M}O_2$ with decreasing PO_2 was observed) was determined to be $P_{crit-hatch}$ for that embryo.

2.7. Measurement of perivitelline PO₂ (PO_{2(in)}) in eggs

PO_{2(in)} (the PO₂ inside the egg, that the embryo is exposed to) was measured in eggs in normoxia (PO_{2(out)} = 21 kPa), followed by gradual (~10 min) sequential exposure to 15, 10 and 5 kPa. After the measurement at 5 kPa, PO_{2(out)} was reset to normoxia, where another measurement was conducted (to compare the initial and final values for normoxia). The egg was then exposed to an hyperoxic environment of 30 kPa.

For the measurement of PO_{2(in)}, individual eggs were placed in 8°C air equilibrated water within an open well of a modified flow-through respirometer (similar to the previously described respirometer, Fig. 2.7) with an integrated temperature and O₂ sensor in a flow through cell (FTC, Sensor Type PSt3, Presens). The FTC was placed in series, downstream to the well to measure PO_{2(out)} of the water as it left the respirometer. The well was partially submerged in a waterbath to allow accurate temperature control. Water flow through the open well was set to a constant 1 ml min⁻¹. Each egg was allowed 30 min to recover from handling in normoxic water within the chamber prior to measurement. PO_{2(in)} was then determined using a fibreoptic microoptode (Type-PSt1, <50 µm tip, PreSens) connected to an O₂ analyser (Oxy-10 micro, PreSens, Regensburg, Germany).

To aid in inserting the micro-optode through the egg capsule, magnification was provided by a horizontal dissecting microscope and the connecting cable to the micro-optode was housed within a 16G needle and mounted to a micromanipulator. The micro-optode was carefully inserted through the capsule

in decrements <1 mm, until the capsule was pierced with the tip, and then left in position for the entire measurement period. *S. salar* embryos fill the entire egg capsule, leaving very little fluid-filled perivitelline space (pvs). To ensure the optode measured $PO_{2(in)}$, the light emitted at the tip of the optode was maintained visible at all times; inserting the optode past the pvs into embryonic tissue concealed the light. At each $PO_{2(out)}$, $PO_{2(in)}$ was measured for a period of at least 5 min after $PO_{2(in)}$ was stabilised. At times embryo movement was observed inside the egg when the needle penetrated the capsule, however during data collection the embryo seldom moved.

Before each measurement, the microoptode was calibrated with air saturated and anoxic water containing sodium sulphite (as described section 2.4). $PO_{2(out)}$ was adjusted by bubbling a mixture of O_2 and N_2 through the water using a high precision low flow gas blender (V10040A, Bird).

2.8. Calculation of egg capsule oxygen conductance GO_2

O_2 exchange across the egg capsule determines the O_2 environment the embryo is exposed to and can be described by the Fick equation;

$$\dot{M}O_2 = G_{O_2} \cdot (P_{O_{2(out)}} - P_{O_{2(in)}}) \quad \text{eqn. 8}$$

where the rate of O_2 flux, or metabolic rate, ($\dot{M}O_2$, $\mu\text{mol h}^{-1}$) is dependent on the egg capsule O_2 conductance (G_{O_2} , $\text{nmol h}^{-1} \text{ kPa}^{-1}$) and the O_2 partial pressure

difference (PO_2 , kPa) between the outside ($PO_{2(out)}$) and the inside ($PO_{2(in)}$) of the egg capsule.

GO_2 can be estimated by taking into account O_2 permeability, described by Krogh's diffusion coefficient (GO_2 ; $nmol\ mm^{-1}\ h^{-1}\ kPa^{-1}$), and the O_2 conductance of the egg, such that:

$$GO_2 = \frac{4 \cdot \pi \cdot r_o \cdot r_i \cdot KO_2}{r_o - r_i} \quad \text{eqn. 9}$$

Where $4 \cdot \pi \cdot r_o \cdot r_i$ represents the effective surface area (ESA, mm^2) of the (assumed spherical) egg (r_o = outer radius; r_i = inner radius, and $r_o - r_i$ the thickness of the capsule, mm). Krogh's diffusion coefficient is calculated as:

$$KO_2 = \frac{\dot{M}_{O_2}}{sa \cdot (PO_{2(out)} - PO_{2(in)}) \cdot L^{-1}} \quad \text{eqn.10}$$

where, sa is the surface area (mm^2) of the egg, and L is the capsule thickness (mm).

2.9. Heat shock protein (Hsp) immunodetection

Whole alevins were ground in liquid nitrogen and soluble protein was extracted as in Fowler et al. (2009). Briefly, 25-35 mg of ground sample was homogenized in tissue homogenization buffer (50 mM Tris, Sigma-Aldrich; 2% SDS, Bio-Rad; 0.1 mM protease inhibitor cocktail, Sigma-Aldrich) using a PowerGen 125 polytron (Fisher Scientific). Samples were then pushed through a 27G needle

several times and spun at 14000 g for 10 minutes 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 rainbow trout (Hsp70) were loaded onto each gel to allow direct comparison between gels. For immunodetection, rabbit salmonid-specific primary antibodies against Hsp70 (AS05061), Hsc 70 (AS05062) and Hsp 90 (AS05063) were used at a final concentration of 1:50 000 (Agrisera). The Hsp 70 antibody detects only the inducible form of Hsp 70 and does not cross-react with the constitutively expressed isoform (Hsc 70), while the Hsp 90 antibody does not distinguish between the Hsp 90 α , Hsp 90 β a and Hsp 90 β b isoforms of the protein (Rendell et al. 2006). A goat anti-rabbit antibody (SAB-300; Stressmarq) was used at a concentration of 1:50 000 as secondary antibody.

Protein bands were visualized using the ELC Advance Western Blotting Detection Kit (GE Healthcare) and the Versadoc Imaging System (Bio-Rad). Bands were then quantified using ImageLab software (Bio-Rad) and the band

density of each sample was divided with the band density of the appropriate standard to obtain the relative band density. After each round of immunoblotting antibodies were stripped of the membranes using acid stripping buffer (0.4 M glycine, Sigma-Aldrich; 0.07 M SDS, Bio-Rad; 0.018 M Tween 20, Sigma-Aldrich; pH 2.2) for 2 X 30 minutes, followed by 3 X 5 minute washes in PBS (1.37 M NaCl, 27 mM KCl, 43 mM Na₂HPO₄·7H₂O, 14 mM KH₂PO₄; Sigma-Aldrich) and membranes were re-probed using a different antibody.

3. The maternal effect of differences in egg size influence metabolic rate and hypoxia induced hatching in Atlantic salmon (*Salmo salar*) embryos: implications for respiratory gas exchange across the egg capsule

3.1. Abstract

Hypoxia affects developmental rates, respiration and may trigger premature hatching in vertebrate embryos. Here, the metabolic response to hypoxia of mature Atlantic salmon (*S. salar*) embryos and newly hatched yolk-sac alevins under consideration of differences in egg size, a maternal effect dependent on whether females are maiden or repeat spawners was investigated. In addition, how and when metabolic rate in the different phenotypes is altered during hypoxia induced hatching was studied and insight into how this may be affected by gas exchange across the egg capsule is given.

In embryos and yolk-sac alevins, acute hypoxia induced metabolic depression became greater as the severity of hypoxia increased. The mass-specific metabolic rate of yolk-sac alevins remained proportionally higher (~2x on average) than embryos at all partial pressures of oxygen (PO_2). Critical PO_2 s for hatching were higher in embryos from maiden spawners (17 kPa) compared with embryos from repeat spawners (13 kPa). In embryos from maiden and repeat spawners metabolic rate at hatching was similar, despite differences in critical PO_2 for hatching. The metabolic rate of newly hatched yolk-sac alevins remained constant, at the same rate as of hatching; thereby reducing the difference in metabolic rate in comparison to yolk-sac alevins. While the PO_2 within the egg capsule of eggs from maiden spawners was substantially lower than the ambient

PO_2 and decreased exponentially with hypoxia, for a given ambient PO_2 , the internal PO_2 was higher, the bigger the volume of the egg. It was hypothesised that the egg capsule poses a significant barrier to oxygen exchange for the mature embryo that is more severe in eggs from maiden spawners than in eggs from repeat spawners. This was shown by the lower critical PO_2 value for hatching and a more advantageous egg surface area to metabolic rate ratio (0.52 vs. 0.57) in the larger eggs of the repeat spawners. Thus, in contrast to the “*bigger is worse during incubation*” hypothesis, larger embryos from repeat spawners will tolerate more severe levels of hypoxia until hypoxia induced hatching occurs.

3.2. Introduction

During development, aquatic embryos and larvae are highly susceptible to changes in environmental O₂. Low environmental O₂ (hypoxia) impairs growth, reduces respiration rates and affects hatching in salmonids (Alderdice et al. 1958; Garside 1959; Gruber and Wieser 1983; Hamor and Garside 1976). Delayed hatching occurs in fish embryos exposed to hypoxia at early stages of development, while premature hatching may be triggered in advanced embryos exposed to hypoxia (Alderdice et al. 1958; Dimichele and Taylor 1980; Latham and Just 1989).

The Atlantic salmon (*Salmon salar*) embryo resides within an egg capsule and is surrounded by perivitelline fluid. Together, the capsule and perivitelline fluid form a physical barrier for the diffusion of O₂ towards the vitelline membrane of the egg which is the major site of gas exchange across the embryo (Fu et al. 2010; Rombough 1988a; Wells and Pinder 1996a, b).

Studies investigating the P_{O₂} across the egg capsule of fish, together with the observation of an increase in respiration rates after hatching, indicate that the capsule is indeed a barrier to O₂ transfer into the egg (Rombough 1988a). Despite suggestions that the egg capsule may not limit O₂ uptake when comparing body mass of chorionated (embryos with egg capsule) and dechorionated trout embryos (Ciuhandu et al. 2005), recent evidence in rainbow trout eggs showed a significant decrease in dissolved O₂ across the egg capsule as well as across the perivitelline fluid under normoxic conditions (Ciuhandu et al. 2007). Rainbow trout embryos exposed to chronic hypoxia display a similar O₂ gradient across the

chorion as embryos kept in normoxia (Miller et al. 2008). Further, the P_{O_2} in the perivitelline fluid ($PO_{2(in)}$) of both the normoxic and chronically hypoxic eggs was higher than in normoxic eggs exposed to acute hypoxia at 29 days post-fertilisation. This observation was accompanied by a reduction in growth rate and metabolic rate ($\dot{M}O_2$) after chronic hypoxia, suggesting an adaptive response to chronic hypoxia resulting in a decreased demand for O_2 (Miller et al. 2008).

In salmonids, the increase in respiration during hatching in normoxia has also been associated with an increase in activity that facilitates the exodus from the egg capsule (Ninness et al. 2006a). The process of hatching appears to be a response to the decrease in PO_2 within the egg that develops as the metabolic demand increases during embryonic development (Rombough 1988a). While the decrease in PO_2 should increase the driving force for O_2 diffusion across the capsule, a PO_2 threshold for hatching must be reached where O_2 supply cannot meet the O_2 demand. It is likely that hypoxia then triggers hatching in order to facilitate gas exchange (Rombough 1988b). However, the mechanisms underlying hypoxia induced hatching have not been resolved.

Hypoxia reduces growth rate, through a reduction in $\dot{M}O_2$, therefore diminishing the need for O_2 (Shumway et al. 1964; Hamor and Garside 1976; Spicer and Burggren 2003; Miller et al. 2008). Furthermore, egg size affects $\dot{M}O_2$ since the diffusion of O_2 is dependent on the O_2 conductance (GO_2), which in turn is influenced by the geometry of the egg and its capsule properties. Fish egg size is determined by maternal body size and age, where bigger/ older females yield larger eggs (Belding 1940; Kazakov 1981). In Chinook salmon (*Oncorhynchus*

tshawytscha) for example, the mean egg size within a population can vary up to 300% (Rombough 1985). Juveniles from larger eggs show increased survival rates, growth and a delayed emergence with larger amounts of residual yolk (Mousseau and Fox 1998; Roff 1992). Survival rates of Brown trout embryos in normoxia were independent of egg size while embryos from smaller eggs in hypoxia had lower survival rates (Einum et al. 2002). Larger eggs have a smaller surface area to volume ratio and theoretically a reduced conductance relative to their size. It has therefore been hypothesised that larger eggs, due to a proportional increase in $\dot{M}O_2$ with egg volume or mass, will have more difficulties in meeting their higher metabolic demand and therefore a selection disadvantage (Hendry et al. 2001; Sargent et al. 1987). This has led to the “*bigger is worse during incubation*” hypothesis. Recently, this hypothesis has been challenged by Einum et al. (2002). This study demonstrated that in Atlantic salmon the metabolic demand increases at a slower rate relative to egg mass, but a similar finding did not translate into an increase in hypoxia tolerance indicated by P_{crit} or P_{50}^{11} values in Chinook salmon (Rombough 2007). These studies however, have not taken into consideration maternal or genetic effects that may influence O_2 uptake.

In the present study, the $\dot{M}O_2$ of mature *S. salar* embryos and newly hatched alevins and their response to acute, progressive hypoxia (21 → 5 kPa) was measured. In addition, the transition from egg to alevin was examined by continuously measuring $\dot{M}O_2$ during hypoxia induced hatching. Furthermore, the maternal effects of differences in egg size on $\dot{M}O_2$ and hypoxia induced hatching

¹¹ The PO_2 at which the hemoglobin (Hb) is 50% saturated with O_2 that serves as an indicator of Hb- O_2 affinity

were investigated, by comparing eggs from maiden and repeat spawning females. This strategy allowed the examination of the effects of egg size on metabolic parameters.

It was hypothesised that differences in egg size that are due to maternal body size, will be reflected in differences in metabolic rate of the embryos. This assumption is based on the notion that the oxygen exchange across the egg capsule will be facilitated in bigger eggs due to an advantageous metabolic rate to surface area ratio. Therefore, embryos from bigger eggs from repeat spawning females will have an advantage to obtain oxygen from the environment and show signs of increased hypoxia tolerance.

3.3. Experimental protocol

3.3.1. *Fish husbandry and maintenance*

Mature eyed embryos from commercial mass spawnings (1 week prior to expected hatching date, 456 degree days, 57 days at 8°C since fertilisation), were collected from maiden (first spawning, 2+ year old fish) and repeat (second spawning, 3+ year old fish) spawning Atlantic salmon (*Salmo salar*). Embryos were incubated since fertilization in continuous-flow incubation trays at 8°C under normoxic conditions. On transfer to the laboratory, they were maintained at 8°C in well-aerated spring water in the dark. Embryos were left undisturbed for at least 24h prior to experimentation.

3.3.2. *Treatments and measurements*

\dot{M}_{O_2} at 8°C was measured using closed system respirometry (as described in section 2.2) in mature embryos during the final week before the expected hatching date (Stage I and Stage II for embryos during hatching). Similarly, newly hatched yolk-sac alevins were measured within 3 days after peak hatching occurred (Stage III), noting that hatching in the laboratory in embryos from maiden and repeat spawners occurred over a 4 day window. Egg geometry and embryo and alevin morphometry and mass was determined according to section 2.1. Determination of $P_{crit-hatch}$ and the measurement of perivitelline PO_2 ($PO_{2(in)}$) are elaborated in section 2.6 and 2.7 respectively.

3.3.3. Statistical analysis

Sample sizes (n) for each measurement are provided in figures accompanying the results. Statistical comparisons of egg geometry and embryo mass parameters between the two phenotypes (maiden and repeat spawners) were compared using one-way analysis of variance (ANOVA). $\dot{M}O_2$ and mass-specific $\dot{M}O_2$ in embryos and alevins were compared using two-way repeated measure ANOVA with PO_2 and developmental stage (Stage I, Stage II, Stage III) as factors, whereas phenotypic differences were compared within developmental stages using phenotype (maidens vs repeats) and P_{O_2} as factors. Differences in $PO_{2(in)}$ were determined using one-way repeated measure ANOVA with $PO_{2(out)}$ as a factor. To compare capsule thickness and $PO_{2(in)}$ as a function of egg volume, least squares linear regressions analysis was performed. In all cases, the level of significance was set to $P = 0.05$. All pairwise multiple comparisons procedures were performed using Bonferroni post hoc t-tests.

3.4. Results

3.4.1. Egg geometry and embryo and alevin morphometry

The radius (r), surface area (sa) and volume (v) of eggs from repeat spawners were bigger than the eggs from maiden spawners (Table 3.2). Embryos from repeat spawners had a wet body mass that was 1.4x and a yolk-free body mass that was 1.2x greater than that of embryos from maiden spawners. Capsule thickness (L) in eggs from maiden spawners was 0.133 ± 0.02 mm. Capsule thickness did not change with an increase in egg volume in eggs from maiden spawners ($P = 0.75$, Fig. 3.1). The total wet mass of an alevin from repeat spawners was greater than that of alevins from maiden spawners, as was yolk-free alevin body mass.

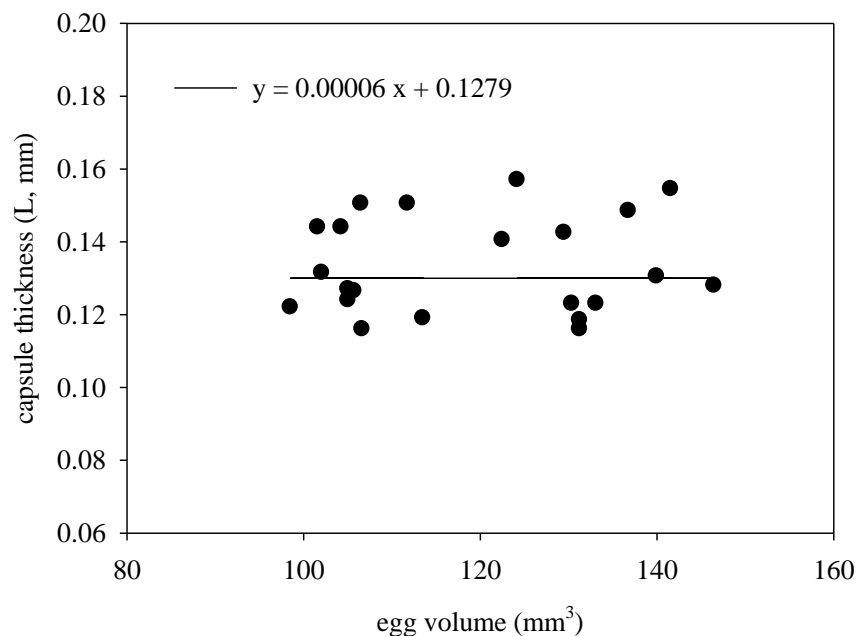


Figure 3.1. Relationship between egg capsule thickness (mm) and egg volume (mm³) of eggs from maiden spawners (n = 23). This relationship is described by a linear equation in the form of: $y = 0.00006x + 0.1279$, $r^2 = 0.05$.

3.4.2. Mass-specific metabolic rate and hatching

Eggs from maiden spawners: In normoxia, $\dot{M}O_2/g$ in alevins (Stage III) from maiden spawners was ~40% higher than in eggs (Stage I, $P < 0.001$, Fig 3.2). With progressive hypoxia, $\dot{M}O_2/g$ decreased continuously in both Stage I eggs and Stage III alevins; $\dot{M}O_2/g$ in Stage III alevins remained significantly higher at all PO_2 levels ($P < 0.001$) compared to unhatched Stage I embryos. In Stage I eggs, this decrease was of exponential nature with a statistically significant reduction in $\dot{M}O_2/g$ with at least every 2 kPa (PO_2 , one-way repeated measures ANOVA, $P < 0.001$, see Appendix 1). Overall, 44% of all eggs measured, hatched during the experiments (Stage II). Relative to the decrease in $\dot{M}O_2/g$ of unhatched Stage I embryos with hypoxia, the $\dot{M}O_2$ of hatching Stage II embryos remained constant at their hatching $\dot{M}O_2/g$, with decreasing PO_2 , until it equalled the $\dot{M}O_2/g$ of Stage III alevins ($PO_2 \sim 8$ kPa). With further decreasing PO_2 , $\dot{M}O_2/g$ of newly hatched Stage II alevins decreased in tandem with Stage III alevins (Fig. 3.2).

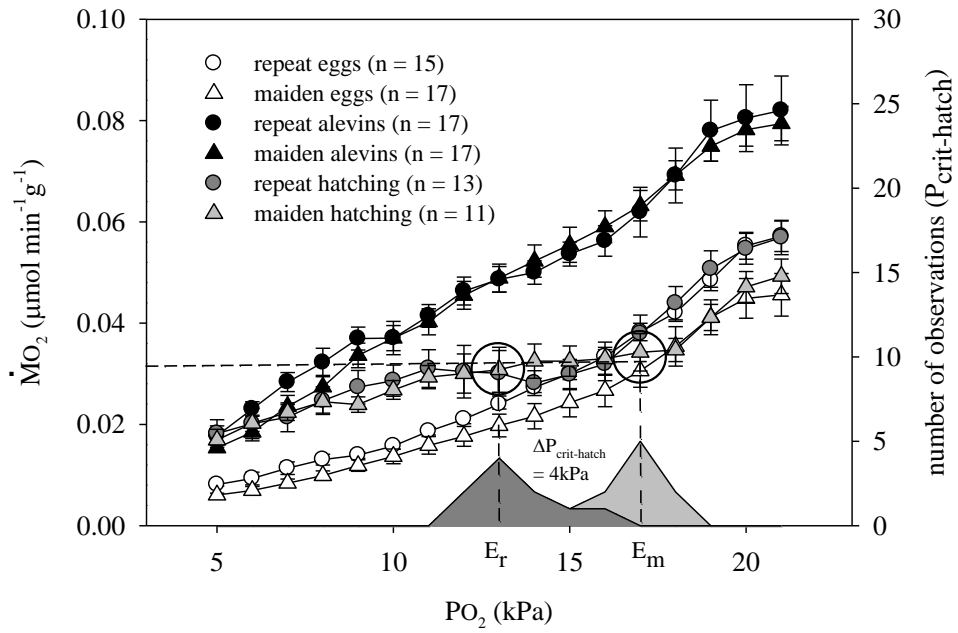


Figure 3.2. Mass-specific metabolic rate ($\dot{M}O_2$) of mature *S. salar* eggs (white), eggs during hatching (grey) and alevins (black) from maiden (triangles) and repeat (circles) spawners in normoxia and after progressive, acute hypoxia using closed system respirometry. Dotted lines indicate the median $P_{crit-hatch}$ for different phenotypes (E_r = embryos from repeat spawners; E_m = embryos from maiden spawners) and $\dot{M}O_2$ at hatching. Data show mean \pm SE. Shaded areas indicate number of observations/ distribution for $P_{crit-hatch}$ values in maiden and repeat eggs.

Eggs from repeat spawners: A similar pattern as reported for eggs from maiden spawners was observed for eggs from repeat spawners. However, $\dot{M}O_2/g$ of Stage I embryos from repeat spawners were significantly higher compared to Stage I embryos from maiden spawners in normoxia (21-18 kPa, $P = 0.04$). As the PO_2 decreased beyond these levels, the difference in $\dot{M}O_2/g$ diminished between embryos of the different phenotypes.

Hatching: Hatching of Stage II embryos from maiden spawners occurred at a median $P_{crit-hatch}$ of 17 kPa (range: 19-14 kPa) while in Stage II embryos from repeat spawners hatching occurred at a median $P_{crit-hatch}$ of 13 kPa (range: 17-9 kPa; Fig. 3.2, Table 1). Hatching in Stage II embryos from either phenotype occurred at a similar $\dot{M}O_2/g$ (on average $0.030 \pm 0.003 \mu mol h^{-1} g^{-1}$). This level of

metabolism was maintained in both phenotypes during severe hypoxia before decreasing in a similar manner as the Stage III alevins at severe hypoxia (below 9 kPa). There was no difference in $\dot{M}O_2/g$ between Stage II alevins from either phenotype once the embryos hatched ($P = 0.055$). The $\dot{M}O_2/g$ of Stage III alevins originating from maiden and repeat spawners was similar at all levels of PO_2 ($P = 1.0$).

3.4.3. Perivitelline PO_2 ($PO_{2(in)}$) and GO_2

These measurements were conducted only in Stage I eggs from maiden spawners. For eggs in normoxic water ($PO_{2(out)} = 21$ kPa) the perivitelline PO_2 ($PO_{2(in)}$) was $\sim 4.46 \pm 0.4$ kPa. Accompanying a stepwise decline in $PO_{2(out)}$ was a decline in $PO_{2(in)}$ (Fig. 3.3a), significant between steps except 15 kPa and 10 kPa ($P = 0.433$). After returning the $PO_{2(out)}$ from 5 kPa to 21 kPa, $PO_{2(in)}$ increased to values similar to the initial measurement at 21 kPa (4.20 ± 0.7 vs. initial value: 4.46 ± 0.4 kPa, $P = 0.712$). When the Stage I egg was then exposed to hyperoxic water ($PO_{2(out)} = 30$ kPa) the $PO_{2(in)}$ increased to 8.2 ± 0.9 kPa, above the value observed in normoxia. Overall, a decline in $PO_{2(out)}$ was accompanied by an exponential decline in $PO_{2(in)}$ ($y = 0.7528e^{0.0822x}$, $r^2 = 0.96$, Fig. 3.3 a).

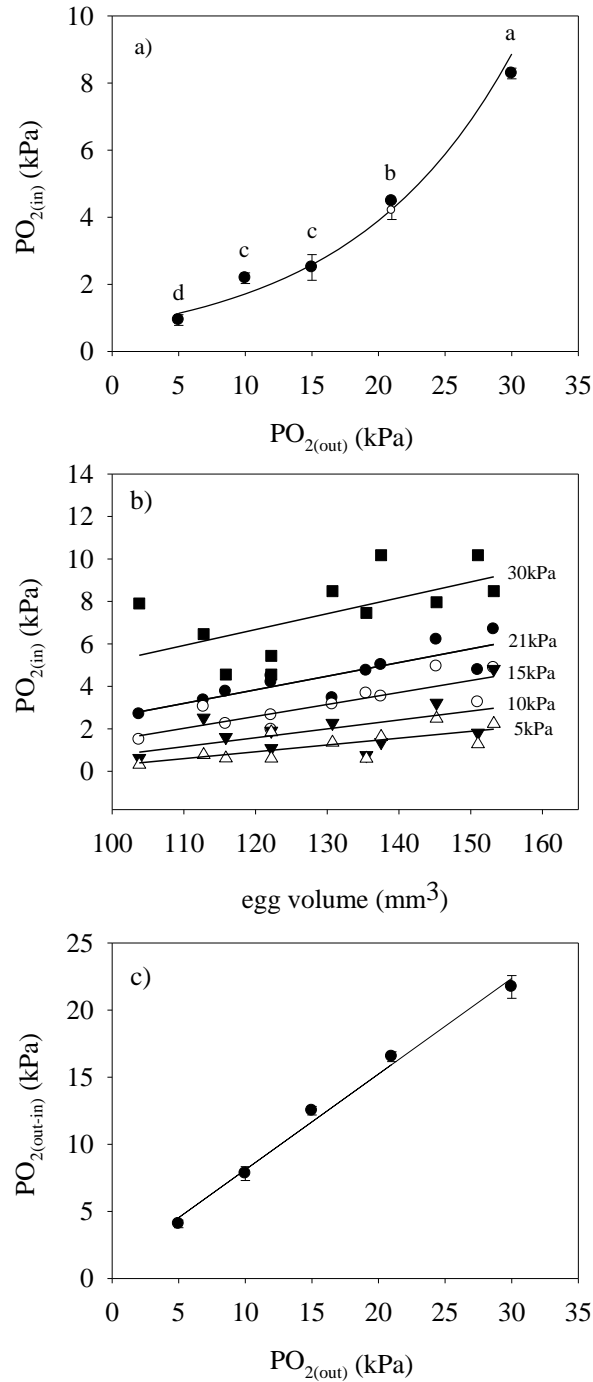


Figure 3.3. Perivitteline P_{O_2} ($P_{O_{2(in)}}$) for eggs from maiden spawners. a) Changes in mean \pm SE $P_{O_{2(in)}}$ of eggs ($n = 11$) with a stepwise decrease in $P_{O_{2(out)}}$ from 21 kPa to 5 kPa (filled circles) and after reoxygenation to 21 kPa (unfilled circle) and hyperoxia at 30 kPa. Statistical differences between different $P_{O_{2(out)}}$ are indicated by different letters; line indicates an exponential relationship: $y = 0.7528e^{0.0822x}$, $r^2 = 0.96$. b) $P_{O_{2(in)}}$ as a function of egg volume at different $P_{O_{2(out)}}$. Lines indicate linear regressions at different $P_{O_{2(out)}}$ determined by least squares regression. At all $P_{O_{2(out)}}$ values, regressions were significantly different from 0 ($P = 0.032$) but the slope of the regressions curves (m) decreased with increasing hypoxia (see Table 3.1). c) Changes in $P_{O_{2(out-in)}}$ with a stepwise decrease in $P_{O_{2(out)}}$ from 21 kPa to 5 kPa; linear regression: $y = 0.7128x + 0.9754$; $r^2 = 0.99$.

As a function of egg volume, $PO_{2(in)}$ in normoxia increased in a linear fashion (Fig. 3.3b). This relationship was maintained under hypoxic and hyperoxic conditions. As egg volume correlates with egg size, bigger eggs therefore tend to have a greater $PO_{2(in)}$ at the same $PO_{2(out)}$. The slopes of the linear regressions decreased with decreasing $PO_{2(out)}$ s, which suggests that the gradient between $PO_{2(out)}$ and $PO_{2(in)}$ becomes smaller the lower the $PO_{2(out)}$. This is demonstrated when plotting $PO_{2(out)} - PO_{2(in)}$ against $PO_{2(out)}$ (Fig. 3.3. c) where $PO_{2(out)} - PO_{2(in)}$ increases in a linear fashion with increasing $PO_{2(out)}$.

Table 3.1. Linear regression equations of the form $y = mx + b$ for $PO_{2(out)}$ against egg volume at different $PO_{2(out)}$. Asterisks indicate linear regressions where the slope (m) is significantly different to 0 ($P < 0.05$).

$PO_{2(out)}$ (kPa)	Linear equation ($y = mx + b$)	r^2
30	$y = 0.0748x - 3.4246^*$	0.38
21	$y = 0.0642x - 4.8301^*$	0.74
15	$y = 0.0562x - 4.9984^*$	0.70
10	$y = 0.0419x - 4.0770^*$	0.33
5	$y = 0.0321x - 3.4154^*$	0.51

Gas exchange across the egg capsule was modelled using the $PO_{2(in)}$ measurements for eggs from maiden spawners. Krogh's diffusion coefficient (KO_2) for maiden spawner eggs was $0.015 \text{ nmol mm}^{-1} \text{ h}^{-1} \text{ kPa}^{-1}$ and the conductance across the egg capsule (GO_2) was calculated as $14.13 \text{ nmol h}^{-1} \text{ kPa}^{-1}$. GO_2 and $PO_{2(in)}$ is estimated to increase to $17.22 \text{ nmol h}^{-1} \text{ kPa}^{-1}$ and 5.15 kPa (respectively) for the larger eggs from repeat spawners (Table 3.2).

Table 3.2. Comparative egg geometry alevin mass (wet) and metabolic parameters relevant to O₂ exchange across the egg capsule of eggs and alevins from maiden and repeat spawning *S. Salar*. * indicates statistically significant differences ($P < 0.05$) between phenotypes (maiden vs repeat) using one-way ANOVA. ^ derived using L from maiden spawners. Data show means \pm SE.

eggs (Stage I & II)			
parameter	maiden	repeat	<i>P</i> -value
total wet mass (g)	0.124 \pm 0.004	0.169 \pm 0.007*	< 0.001
yolk-free wet body mass (g)	0.036 \pm 0.001	0.044 \pm 0.0004*	< 0.001
radius (r, mm)	3.14 \pm 0.02	3.50 \pm 0.05*	< 0.001
r _{out} - r _{in} (L, mm)	0.133 \pm 0.01	-	-
surface area (sa, mm ²)	121.6 \pm 2.01	154.8 \pm 5.1*	< 0.001
volume (v, mm ³)	130.37 \pm 3.13	182.3 \pm 7.9*	< 0.001
$\dot{M}O_2$ (nmol h ⁻¹) at 21 kPa	233.47 \pm 8.61	273.11 \pm 11.90*	0.010
mass-specific $\dot{M}O_2$ (μ mol min ⁻¹ g ⁻¹) at 21 kPa	0.045 \pm 0.004	0.057 \pm 0.003*	0.010
GO ₂ (nmol h ⁻¹ kPa ⁻¹)	14.13	17.22^	-
KO ₂ (nmol mm ⁻¹ kPa ⁻¹)	0.015	0.015^	-
PO _{2(in)} in normoxia	4.46 \pm 0.36	5.15^	-
sa/v ratio	0.93 \pm 0.06	0.85 \pm 0.04*	< 0.001
sa/ $\dot{M}O_2$ ratio	0.52 \pm 0.01	0.57 \pm 0.02*	0.035
mean P _{crit-hatch} (kPa)	16.0 \pm 0.9	13.5 \pm 0.7*	0.006
$\dot{M}O_2$ /g (μ mol min ⁻¹ g ⁻¹) at P _{crit-hatch}	0.032 \pm 0.003	0.028 \pm 0.002	0.231
alevins (Stage III)			
total wet mass (g)	0.112 \pm 0.004	0.151 \pm 0.003*	< 0.001
yolk-free wet body mass (g)	0.054 \pm 0.002	0.060 \pm 0.002*	0.270
mass-specific $\dot{M}O_2$ (μ mol min ⁻¹ g ⁻¹) at 21 kPa	0.079 \pm 0.004	0.084 \pm 0.003	0.204

GO₂ = O₂ conductance, $\dot{M}O_2$ /g = metabolic rate, KO₂ = Krogh's diffusion coefficient, P_{crit-hatch} = critical O₂ tension for hatching, PO_{2(in)} = perivitelline PO₂

3.5. Discussion

3.5.1. Mass-specific metabolic rate and hatching

The acute response to hypoxia, irrespective of the level, in mature Stage I & II embryos and Stage III alevins of *S. salar* was metabolic depression. Hypometabolism is a common response of vertebrates to acute hypoxia (Richards 2009). It enables the organism to survive, despite the inhibition of aerobic ATP production during hypoxia, which depends on the animal's ability to compensate for the lack of O₂ via O₂ independent pathways of ATP production. This suggests that hypometabolism is compensated through anaerobic pathways. If this was the case, it would be associated with an O₂ debt, paid back on return to normoxia. Whether the $\dot{M}O_2$ in the case of *S. salar* eggs or alevins is actively down-regulated or is a result of a transport limitation for O₂ uptake is yet to be assessed. However, it is generally considered that the mechanisms underlying metabolic rate suppression in vertebrates are regulated and involve the arrest of ion movement, protein synthesis, transcription and other anabolic pathways in the cell (Buck and Hochachka 1993; Hochachka et al. 1996; Lewis et al. 2007; Richards et al. 2007; Wieser and Krumschnabel 2001). Evidence for an active downregulation of $\dot{M}O_2$ in hypoxia was also provided by Frappell et al. (1991) where kittens (*Felis catus*) showed little anaerobic compensation in hypoxia. The fact that $\dot{M}O_2/g$ of embryos decreases with little variation in $PO_{2(out)}$ however, and that $PO_{2(in)}$ decreases exponentially with decreasing $PO_{2(in)}$ (see Appendix 1), tends to suggest that embryos are O₂ transport limited.

The higher $\dot{M}O_2/g$ in Stage III newly hatched alevins compared to mature Stage I embryos and the increase in $\dot{M}O_2/g$ of the Stage II hatching embryo relative to Stage I embryos (during hypoxia) is also indicative of a transport limitation of O_2 to the embryo within the egg shell. As the embryo hatches, the diffusive barrier for O_2 created by the egg is lost and O_2 supply is no longer limited. The O_2 uptake in a constant O_2 environment is then facilitated through direct diffusion of O_2 across the vitelline membrane, hence an increase in $\dot{M}O_2$ is possible. On the other hand, the increased $\dot{M}O_2/g$ observed during hatching could have resulted from an increase in energetic processes such as activity (facilitated by the loss of the capsule), ventilation or ion cycling. Because O_2 uptake is almost entirely cutaneous at this developmental stage (Wells and Pinder 1996a, b), it is questionable whether this elevated O_2 consumption is facilitated through an increase in heart- or ventilation rates. Indeed, heart rate in rainbow trout remained unchanged before and just after hatching (Miller et al. 2011) and Atlantic salmon heart rate at 8°C appears to be higher in embryos just before hatching (Klinkhardt et al. 1987), suggesting that heart rate and $\dot{M}O_2$ are not coupled.

The increase in metabolic rate during the transition from the Stage I embryo to Stage III yolk-sac alevin relative to the Stage I embryo could equally be due to the exposure of the embryo to a hyperoxic environment relative to the conditions within the egg capsule. The exposure to acute hyperoxia can increase the rate of oxygen consumption, particularly in animals following development in relative hypoxic environments (see Chapter 4 and 5).

Once the $\dot{M}O_2/g$ of the Stage II hatching embryo equalled the $\dot{M}O_2/g$ of the Stage III alevin under the same hypoxic conditions, $\dot{M}O_2/g$ decreased in the Stage II hatchlings following the same pattern of metabolic depression under increasing hypoxia as in the Stage III alevins. Therefore, despite maintaining O_2 uptake during and immediately after hatching, there is no indication for an additional increase in $\dot{M}O_2/g$ above the level seen in the Stage III alevin. Equally, an increase in $\dot{M}O_2/g$ during hatching has been associated with an increase in activity in rainbow trout (Ninness et al. 2006a). A transient increase in $\dot{M}O_2/g$ was not observed, rather a continuous increase relative to the Stage I unhatched embryo until the $\dot{M}O_2/g$ of Stage III alevins was matched. Together, this suggests that hatching is not associated with an O_2 debt that is a result of anerobic activity. Of importance is the fact that $\dot{M}O_2/g$ in a Stage II hatching alevin could be maintained during increasing hypoxia, suggesting that once hatched the Stage III alevin is not transport limited, at least not until the point where hypoxia is relatively severe and $\dot{M}O_2/g$ declines in the newly hatched Stage II alevin as it does for a 2-3 day old Stage III alevin.

About 44% of all embryos measured in this study hatched when exposed to hypoxic conditions during the measurements of $\dot{M}O_2$. All embryos from this study were fertilised in a mass spawning at a similar time and reared under identical conditions. Nevertheless, there are many factors during incubation that might impact on the maturation process of an embryo and influence the hatching process. Therefore, small differences in maturity could have resulted in the observed hypoxia induced hatching patterns. Oppen-Berntsen et al. (1990) report that in *S. salar* (2 days before natural hatching) in hypoxia, 100% of embryos

hatched within 2 hours. In contrast the approach in this study, they subjected the embryos to chronic, almost anoxic conditions (0.67 kPa). These data indicate that at less severe hypoxia, induced hatching does not necessarily occur in all embryos from a single fertilisation event. It is demonstrated that there is at least an embryo size/ maternal influence on the hypoxia induced hatching trigger, and predicted that there are several additional maternal and microenvironmental factors that influence hatching. It is further suggested that there is a hypoxic threshold-range, within which the fully developed embryo has the aerobic capacity to hatch. As the PO_2 falls below this level, the embryo may not have the capacity to meet the O_2 demand to hatch and therefore hypoxia induced hatching might not occur. Unless the embryo hatched at close to anoxic conditions (< 5 kPa), hypoxia could potentially be lethal, as the embryo would not be able to escape the hypoxic zone. Overall, the percentage of animals that were found dead after measurements was about 3%. This was not different to the number of dead alevins found in the holding trays in normoxia and were not part of the experiments.

Repeat-spawning females produced larger eggs with bigger embryos (Table.3.2), which had a higher normoxic $\dot{M}O_2/g$ than embryos from maiden spawners. $\dot{M}O_2/g$ remained higher Stage I embryos from repeat spawners compared with Stage I embryos from maiden spawners down to a PO_2 of 18 kPa. An increased $\dot{M}O_2/g$ could be an adaptive characteristic of repeat spawner embryos. However, this increase could also be related to differences in gas exchange limitation across the capsule, allowing an elevated $\dot{M}O_2/g$ in repeat spawner embryos through an absolute bigger surface area for gas exchange. This is supported by the fact that Stage III alevins from repeat spawners, despite having greater yolk-free body

mass, did not show differences in mass-specific metabolic rate compared to alevins from maiden spawners ($P = 0.569$, Table 3.2). The lower $\dot{M}O_2/g$ in Stage I maiden spawner embryos in normoxia and mild hypoxia is most likely a physical consequence of O_2 transport limitation across the egg capsule rather than a phenotypic difference. The lower $P_{crit-hatch}$ in eggs from repeat spawners indicates a higher tolerance to hypoxia, but as $\dot{M}O_2/g$ is maintained at similar levels between Stage II embryos from maiden and repeat spawners at hatching, this suggests improved capability to obtain O_2 in embryos from repeat spawners.

3.5.2. Perivitelline PO_2 ($PO_{2(in)}$) and oxygen exchange

For a given egg, $PO_{2(in)}$ as measured in eggs from maiden spawners was lower than $PO_{2(out)}$. This suggests that the egg capsule exhibits a resistance for O_2 diffusion towards the embryo, which consumes the O_2 in the perivitelline space and so establishes the low $PO_{2(in)}$. The $PO_{2(in)}$ measured in this study (4.46 kPa) is in the same range to reported values from normoxic rainbow trout eggs measured at 10°C (~3.41 kPa, graphically determined using UNSCAN-IT 6.1. software, Ciuhandu et al. 2007). The exponential decrease in $PO_{2(in)}$ during stepwise decreases in $PO_{2(out)}$ (Fig. 3.3a), suggests that with a reduction in $\dot{M}O_2$ with increasing hypoxia, the O_2 gradient across the egg capsule decreases as well. In agreement with this, the decrease in embryonic $\dot{M}O_2$ during progressive hypoxia was less severe at lower $PO_{2(out)}$ and equally shows a curve linear relationship (see Appendix 1). This adds further support to the idea that O_2 transport is limited by physical constraint through the egg capsule because a smaller decrease in $\dot{M}O_2$ with increasing hypoxia results in a less severe decrease in $PO_{2(in)}$ and O_2 uptake can therefore be more easily sustained. This finding is more clearly illustrated

when looking at the relationship between $PO_{2(out)} - (in)$ over $PO_{2(out)}$ (Fig. 3.3 c), where with increasing hypoxia, the gradient across the capsule is reduced.

In eggs from maiden spawners, $PO_{2(in)}$ positively correlates with egg volume and therefore egg size, irrespective of the $PO_{2(out)}$. The slope of the regressions (m) however, decreases with decreasing $PO_{2(out)}$ (Table 3.3b), consistent with the previous observations that $\dot{M}O_2$ decreases at a slower rate with progressive hypoxia (Appendix 1). Hence, O_2 exchange across the capsule is facilitated in larger eggs enabling higher $PO_{2(in)}$. If larger eggs have a higher $PO_{2(in)}$ at a given $PO_{2(out)}$ compared with smaller eggs, the embryos will tolerate lower $PO_{2(out)}$ s before critical O_2 levels to maintain aerobic metabolism are reached. It is predicted that the larger eggs from a repeat spawner would therefore have a higher $\dot{G}O_2$ compared with eggs from a maiden spawner. Despite the smaller maiden spawner eggs having an advantageous surface area to volume ratio of 0.96 compared with 0.85 in eggs from repeat spawners, the critical ratio to consider here is that of surface area to $\dot{M}O_2$, which theoretically is 0.57 in eggs from repeat spawners compared with 0.52 measured in eggs from maiden spawners (Table. 3.2). This concept is schematically illustrated in Fig. 3.4 where $PO_{2(in)}$ is compared to $PO_{2(out)}$ in the different phenotypes. The assumptions made in this model however, disregard the potential for other maternal effects on physiological or structural properties, such as on capsule thickness, that might influence the O_2 uptake.

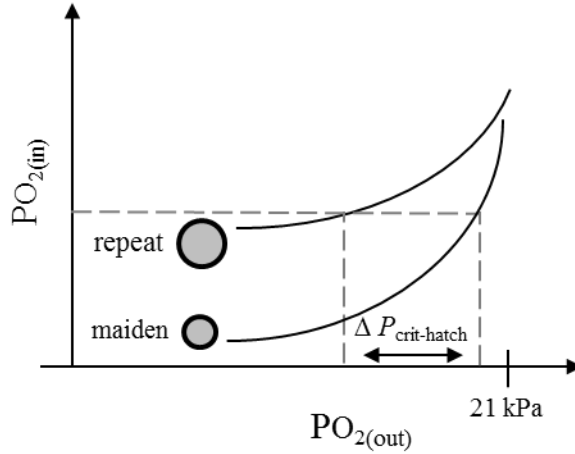


Figure 3.4. Theoretical model of changes in perivitelline PO_2 ($PO_{2(in)}$) in maiden and repeat *S. salar* eggs with increasing hypoxia. In accordance with eqn. 8 and 9, assuming that egg shell thickness (L), Krogh's diffusion coefficient (K_{O_2}) and metabolic rate ($\dot{M}O_2$, as measured here) is constant, any differences in $PO_{2(in)}$ can only be explained by differences in oxygen conductance (G_{O_2}) at a given $PO_{2(out)}$. As $PO_{2(in)}$ increases with egg volume at a given $PO_{2(out)}$, this suggests that larger eggs have an increased ability to obtain oxygen from the surrounding water, despite a smaller surface area to volume ratio. Hence, it is suspected that repeat eggs, being bigger than maiden eggs, will display higher $PO_{2(in)}$ in comparison to maiden eggs and a slower decrease in $PO_{2(in)}$ in hypoxia. As a result, the critical $PO_{2(out)}$ for hatching ($P_{crit-hatch}$) in repeat eggs is lower (indicated by dashed lines) compared to maiden eggs.

This model is supported by the fact that embryos from repeat spawners hatch at a lower $PO_{2(out)}$ (i.e. lower $P_{crit-hatch}$). If indeed, G_{O_2} was higher in eggs from repeat spawners and hatching is induced by internal hypoxia ($PO_{2(in)}$), these embryos will be able to sustain O_2 uptake and thus $\dot{M}O_2$ to a lower $PO_{2(out)}$ as suggested in this study.

This model is confounded by the assumption that the shell thickness (L) as well as K_{O_2} are similar in both phenotypes and could be a potential source of error and misinterpretation. However, Rombough (2007) suggests that egg shell thickness is unlikely to vary within a species. There is no evidence in the literature suggesting that either variable is different between the phenotypes. Shell thickness (L) in amphibian eggs generally appears to increase with an increase in

egg size (Seymour and Bradford 1995). In maiden *S. salar* eggs measured here, the slope of the linear regression of L as a function of volume was not different to zero, suggesting that L does not change with increasing egg size (Fig. 3.1). The value for L in this study is similar to brown trout (0.133 mm, Bonislawski et al. 2001) but different to other values for Atlantic salmon (0.04 mm, Hayes et al. 1951) or other salmonid species (0.043mm on average, Rombough 1989). In the latter studies, measurements of L were performed using electron microscopy techniques and embryos were incubated at various incubation temperatures. On the other hand, changes to the capsule tension or structure have also been suggested to occur with an increase in egg size as larger eggs will require higher capsule integrity to ensure physical protection. How these variables affect GO_2 is not clear and further studies will be required to address this matter. As KO_2 is a function of surface area and assuming that L is constant with an increase in egg size or between phenotypes, the theoretical GO_2 is calculated to be higher in the larger eggs from repeat spawners at $17.22 \text{ nmol h}^{-1} \text{ kPa}^{-1}$ compared with $14.13 \text{ nmol h}^{-1} \text{ kPa}^{-1}$ in eggs from maiden spawners. This results in a theoretical $\text{PO}_{2(\text{in})}$ of 5.15 kPa in eggs from repeat spawners in comparison to the measured 4.48 kPa in eggs from maiden spawners.

Einum et al. (2002) reported that in contrast to the conventional belief, survival of Atlantic salmon embryos in hypoxia is lower in smaller eggs, which is indicative of a lower hypoxia tolerance. Also, in their study, metabolic rate scales positively with egg size. The “*bigger is worse during incubation*” hypothesis had been put forward previously (Hendry et al. 2001; Sargent et al. 1987) and assumes that bigger eggs will have more difficulties meeting the higher O_2 demand under the

constraint of the egg capsule. Similar assumptions have been made for amphibians where capsule $\dot{V}O_2$ limits $\dot{M}O_2$ in larger eggs (Seymour and Bradford 1995). The data here do not indicate a disadvantage for O_2 exchange resulting from an increase in egg size in *S. salar* and is contradictory to the “*bigger is worse during incubation*” hypothesis. In fact, bigger eggs in hypoxia are at an advantage due to a larger surface area with respect to $\dot{M}O_2$, which appears to be more beneficial to obtain sufficient O_2 from the environment and thus survive in conditions of low O_2 availability. Unlike in Chinook salmon, where the greater surface area of the egg relative to the metabolic demand was not reflected in an improved hypoxia tolerance (Rombough, 2007), the data in the present study suggest that in mature Atlantic salmon eggs, larger embryos obtain more O_2 from the environment and that maternal effects of egg size impact on hypoxia induced hatching, that is dependent on the ability of the embryo to obtain sufficient O_2 . Taking these findings into consideration, embryos in larger eggs from repeat spawners will have a selection advantage over embryos in smaller eggs from maiden spawners.

Conclusions and perspectives

It was demonstrated that the maternal effect of differences in egg size have a significant impact on aerobic metabolism in early stage developing salmon. In addition to previous work which highlights that the egg capsule in salmonid embryos poses a physical barrier to O_2 exchange and that larger eggs have a larger surface area in regards to their metabolic demand, the maternal effect of egg size also plays a significant role in O_2 uptake, exemplified by differences in hypoxia induced hatching patterns. Embryos of larger eggs indeed appear to have

an advantage in O₂ uptake over embryos from small eggs and might therefore tolerate more severe hypoxia before hatching occurs. This in turn is likely to impact on survival and fitness of these embryos and favour offspring from bigger/older females.

4. Hypoxic acclimation leads to metabolic compensation after re-exposure to normoxia and increased cardiorespiratory sensitivity to acute hypoxia in Atlantic salmon (*Salmo salar*) yolk-sac alevins

4.1. Abstract

Low environmental O₂ (hypoxia) is a common condition in the aquatic environment. Prolonged hypoxia can have substantial effects on developmental rates, aerobic metabolism and survival in fish.

In order to investigate the effects of acute and chronic hypoxia exposure at a range of measurement (4, 8 and 12°C) and incubation temperatures (4°C and 8°C), metabolic rate and cardiorespiratory function (heart rate and ventilation rate) were studied in Atlantic salmon (*Salmo salar*) yolk-sac alevins after normoxic (PO₂ = 21 kPa) incubation or following acclimation to hypoxia (15 days at PO₂ = 10.5 kPa).

Morphometric data on alevins acclimated to hypoxia indicate a developmental delay through slower yolk absorption, compared with normoxia incubated animals. The general response to acute, progressive hypoxia (21 → 5 kPa) in all treatment groups was metabolic depression (~60%). Alevins acclimated to hypoxia had higher metabolic rates when measured in normoxia than alevins acclimated to normoxia. Metabolic rates here were elevated to the same degree irrespective of measurement temperature. Under severe, acute hypoxia (~5 kPa) and irrespective of measurement temperature or acclimation history, alevins were oxygen transport limited as metabolic rates were similar. While heart and ventilation rate were insensitive to acute hypoxia in normoxia acclimated fish, hypoxic acclimation led to cardiorespiratory regulation, indicating earlier onset of cardiorespiratory control mechanisms and therefore developmental plasticity. The

metabolic rate at the respective oxygen level during acclimation was similar in normoxia and hypoxia acclimated alevins. This is indicative of metabolic compensation to an intrinsic metabolic rate in hypoxia acclimated alevins after re-exposure to normoxia.

Together these observations suggest that hypoxic acclimation results in physiological adaptation through elevated metabolic rates that are not achieved by increased oxygen delivery. This implies that higher oxygen demand must be met by enhanced oxygen extraction via functional or structural mechanisms.

4.2. Introduction

Hypometabolism is a widespread response to hypoxia throughout the animal kingdom (Mortola et al. 2012; Pelster 2003; Richards 2009) and chronic hypoxia has been shown to delay developmental rates in fish and other vertebrates (Bagatto 2005; Ciuhandu et al. 2005; Hamor and Garside 1976; Miller et al. 2008; Shumway et al. 1964; Spicer and Burggren 2003). This is generally regarded as an “adaptive” response to reduce the O_2 demand (Miller et al. 2008) and results in reduced ATP consumption when O_2 dependent ATP production cannot be sustained (Richards 2010). While adult fish generally have the capacity to sustain metabolism through anaerobic pathways, the compensatory anaerobic capacity of salmonid embryos and larvae appears to be small (Gnaiger et al. 1981; Ninness et al. 2006a). Similarly, hypoxia reared zebrafish during early development (*Danio rerio*) show no differences in lactate concentrations to normoxia reared individuals (Barrionuevo et al. 2010) and thus it can be expected that the capacity to maintain metabolism via anaerobic pathways in larval fish in hypoxia is limited.

In order to meet the O_2 demand in acute hypoxia, adult salmonids decrease heart rate (f_H , bradycardia) while cardiac output (\dot{Q}) is maintained through an increase in cardiac stroke volume (Holeton 1971; Holeton and Randall 1967; Randall 1982). In contrast to this, salmonid larvae have been reported to either show elevated heart rates (tachycardia) or lack a response to acute hypoxia (Holeton 1971; McDonald and McMahon 1977). The absence of a bradycardic response to acute hypoxia could originate from the lack of cardio-inhibitory control at this developmental stage or an uncoupling of metabolic rate ($\dot{M}O_2$) and f_H . A recent

study provided evidence that cardio-inhibitory control is initiated just after hatching in rainbow trout (*Oncorhynchus mykiss*, Miller et al. 2011). In addition, they found that chronic hypoxia elicits bradycardia before hatching, elevates the adrenergic tone and delays the onset of cholinergic control.

In early zebrafish embryos (a hypoxia tolerant, tropical species), $\dot{M}O_2$ decreases without altering f_H under acute hypoxia, suggesting substantial resistance of the heart to hypoxia (Barrionuevo and Burggren 1999). In contrast, zebrafish larvae (1 day after hatching) exhibited increased f_H and \dot{Q} when raised in chronic hypoxia (10 kPa, Jacob et al. 2002) and more severe hypoxia (3 kPa) abated f_H and developmental rate (Bagatto 2005). In the latter study, the onset of the cardiac adrenergic response was advanced in animals that were developmentally delayed under chronic hypoxia, compared with normoxia. In developing zebrafish, the disruption of hemoglobin oxygen transport also does not impact on key processes involved in oxygen delivery like f_H or ventricular pressure and did not alter rates of O_2 consumption (Perster and Burggren 1996). This adds further support that aerobic processes are independent of hemoglobin despite the presence of blood circulation.

In anuran larvae, neither f_H or \dot{Q} change with chronic hypoxia during development (Territo and Altimiras 1998). Similarly, hypoxia has only a minor effect on cardiac activity in the chicken embryo and $\dot{M}O_2$ is not coupled to f_H (Mortola et al. 2009). Cardiovascular convection therefore does not contribute to hypoxia-induced metabolic depression in the chicken embryo.

In hypoxia, the chorioallantoic membrane capillarisation in chicken (*Gallus domesticus*) embryos is increased (Dusseau and Hutchins 1988; Hudlicka et al. 1992) while in larval amphibians growth of respiratory surfaces have been found after hypoxia exposure (Burggren and Mwalukoma 1983; Rogge and Warkentin 2008). In fish, developmental plasticity of the gills (gill size, gene expression) in response to hypoxia and ionic disturbance has also been described (Craig et al. 2007; Crispo and Chapman 2010). These modifications suggest that an altered O_2 environment might also trigger modification to the cardiovascular system. Taken together, this suggests that hypoxia may elicit structural improvements to favour O_2 delivery, hence permitting to some degree maintenance of \dot{M}_{O_2} , or to protect against a severe drop in \dot{M}_{O_2} should more severe hypoxia be encountered.

In most adult teleost fish, exposure to hypoxia elicits hyperventilation through an increase in ventilation frequency (f_V , Dunn and Hochachka 1986; Perry et al. 2009; Smith and Davie 1984). This leads to respiratory alkalosis through a lowered arterial partial pressure of CO_2 (P_{aCO_2}), that in turn facilitates O_2 uptake due to the Bohr effect (Jensen 2004; Riggs 1988). During early larval development in amphibians, acute hypoxia also increases the frequency of gill irrigation (Burggren and Doyle 1986; Feder and Wassersug 1984; Jia and Burggren 1997a; McKenzie and Taylor 1996; Orlando and Pinder 1995; West and Burggren 1982). In contrast, gill ventilation amplitude was unaltered in *Xenopus laevis* larvae in acute hypoxia whereas chronic, moderate hypoxia attenuated the increase in irrigation frequency in moderate acute hypoxia (Pan and Burggren 2010).

The hyperventilatory response to hypoxia in zebrafish appears to develop well before 14 dpf, suggesting the presence of an O₂ sensing mechanism before complete gill development (Jacob et al. 2002; Jonz and Nurse 2005). In larval amphibians and fish, O₂ chemoreception occurs in gill neuroepithelial cells (NECs) that are involved in the hypoxic ventilatory response (Burleson and Milsom 1993; Burleson and Smatresk 1990; Jia and Burggren 1997b; Jonz and Nurse 2006; Milsom and Burleson 2007; Straus et al. 2001). In zebrafish, NECs are present shortly after fertilisation (5 dpf) and at this stage appear sensitive to a hypoxic stimulus (Jonz and Nurse 2006). Recently Shakarchi et al. (2013) explored the neurochemical mechanisms of O₂ sensing in zebrafish and provide proof that the hyperventilatory response to hypoxia is controlled by a serotonergic mechanism at early developmental stages before the cholinergic system develops. This indicates developmental plasticity of the neurochemical control of ventilation which might concur with the transition from cutaneous to gill respiration. However, in bullfrogs and zebrafish the development of hypoxic ventilatory control is not altered by a chronic hypoxic pre-exposure (Simard et al. 2003; Vulesevic and Perry 2006). In contrast, chronic hypoxia *per se* blunts ventilatory responses to acute hypoxia in neonatal mammals (Sterni et al. 1999).

The interplay between hypoxia, temperature and their effects on development and performance has received significant attention over recent years (Munday, 2012; Pörtner 2002; Pörtner and Farrell 2008). Generally, an increase in ambient temperature leads to a Q₁₀ driven increase in metabolism (Fry 1971; Jones 1982; Pörtner 2002) and therefore in the demand for O₂. The rate of development in a range of taxa, which depends on the $\dot{M}O_2$, is therefore also dependent on

temperature and increases proportionally with increasing temperature (Gillooly and Dodson 2000).

In adults, cardiorespiratory function also increases with temperature to meet the elevated O_2 demand, however a tight coupling between cardiac activity and metabolic requirements at early developmental stages, where the nervous control is not fully established, is currently debated (Pelster 1999). During embryonic and larval development of salmonids, $\dot{M}O_2$ and f_H increase proportionally with temperature while \dot{Q} remains unchanged (Mirkovic and Rombough 1998; Rombough 1988a), suggesting that f_H can be used as a predictor of $\dot{M}O_2$ (Klinkhardt et al. 1987; Benfey and Bennett 2009). This assumption is somewhat surprising as cutaneous O_2 uptake is thought to predominate at this developmental stage and O_2 convection is not considered critical for O_2 delivery to the tissues (Burggren 2004).

Changes in temperature beyond the thermal optimum of an animal causes a reduction in aerobic scope (the limits within which aerobic metabolism can be sustained). Hence, an elevation in temperature with accompanying reduction in environmental O_2 , will exacerbate the reduction in aerobic scope through the increased demand in O_2 and put additional pressure on the ability of the animal to sustain metabolism. Furthermore, the thermal optimum of an animal as well as its metabolic scope depends on the animal's thermal history or acclimatization (Brett 1971; Jobling 1994; Pörtner and Farrell 2008). Variations in metabolism at similar acclimation temperatures are representative of changes to the thermal optimum (Atkins and Benfey 2008).

This background clearly demonstrates the developmental plasticity that acute vs. chronic changes in O₂ levels and temperature can cause in regards to metabolism and cardiorespiratory function. Taking an integrative approach, *S. salar* eggs were incubated at 4°C and 8°C and the effects of acute (21 kPa → 5 kPa) and chronic hypoxia (10.5 kPa for 15 days immediately post-hatching) exposure and ambient temperature (4, 8, 12°C) on $\dot{M}O_2$ and cardiorespiratory function (f_H and f_V) in newly hatched yolk-sac alevins were investigated.

It was hypothesised that acute hypoxia (by aggravating adequate O₂ supply to meet the demand) would result in a decrease in metabolism, reflected in alterations to f_H and f_V , since the preconditions for O₂ sensing and chronotropic cardiorespiratory reflexes appear to be functional at early larval stages of fish. Despite the increasing evidence that cardiovascular convection is not critical to sustain aerobic metabolism at early life stages where O₂ uptake is cutaneous, a certain hypoxic threshold must be reached, where alterations to f_H and f_V will become present. Secondly, it was hypothesised that chronic hypoxia will result in a lower O₂ demand due to delayed developmental rates and that the responses to hypoxia (either acute or chronic) would be amplified at both higher experimental temperatures and incubation temperatures due to the increased demand for O₂.

4.3 Experimental protocol

4.3.1. Fish husbandry and maintenance

Salmo salar eyed eggs from commercial mass spawnings were collected from the SALTAS Wayatinah Hatchery (Tasmania, Australia) where they had been incubated in continuous-flow incubation trays at 8°C (standard hatchery temperature) and 4°C (standard temperature to delay embryonic development for late input smolt and production backup). Eggs acclimated to 8°C were collected at 312 degree days (39 days at 8°C post fertilisation) and eggs acclimated to 4°C at 380 degree days (95 days at 4°C post fertilisation). In the laboratory they were kept separately similar to hatchery conditions and maintained in well aerated spring water at 8°C and 4°C ($\pm 1^\circ\text{C}$) in the dark, respectively. Peak hatching at 8°C occurred at ~448 degree days (56 days at 8°C post fertilisation) and in 4°C eggs at ~428 degree days (107 days at 4°C post fertilisation). Immediately after hatching, alevins were separated into two treatment groups for each incubation temperature. One group was kept under normoxic conditions while the other was exposed to chronic hypoxia (10.5 kPa) for 15 days by bubbling an air/ nitrogen mixture into the covered holding tray using a high precision gas mixer (GF-3/MP, gas mixing flowmeter, Cameron Instruments, Texas, USA).

4.3.2. Treatments and measurements

After the 15 day acclimation period, $\dot{M}\text{O}_2$ in normoxia and in response to progressive hypoxia (21 \rightarrow 5 kPa) was measured in alevins at experimental temperatures of 4, 8 and 12°C using closed system respirometry (for details see chapter 2.2). At each temperature, the sample size was $n = 40$ for normoxic

alevins and $n = 20$ for hypoxia acclimated alevins. The resulting treatment groups were denoted: $4/8_{\text{norm}}$, $8/8_{\text{norm}}$, $12/8_{\text{norm}}$, $4/4_{\text{norm}}$, $8/4_{\text{norm}}$, $12/4_{\text{norm}}$ and $4/8_{\text{hypo}}$, $8/8_{\text{hypo}}$, $12/8_{\text{hypo}}$, $4/4_{\text{hypo}}$, $8/4_{\text{hypo}}$ and $12/4_{\text{hypo}}$ where the numerator and denominator represent measurement (T_a) and incubation temperature respectively and the subscript indicates normoxic (norm) or hypoxic (hypo) acclimation (Fig. 4.1.).

The cardiorespiratory responses ($\dot{M}O_2$, f_H and f_V) of eight experimental groups ($4/8_{\text{norm}}$, $8/8_{\text{norm}}$, $4/4_{\text{norm}}$, $8/4_{\text{norm}}$, $4/8_{\text{hypo}}$, $8/8_{\text{hypo}}$, $4/4_{\text{hypo}}$, $8/4_{\text{hypo}}$) to a stepwise challenge of hypoxia ($21 \rightarrow 15 \rightarrow 10 \rightarrow 5$ kPa) were measured (for details see chapter 2.4). Here, sample sizes were $n = 8$ for normoxic animals and $n = 6$ for hypoxia acclimated groups. These latter experiments were repeated in a control group at 8°C where the PO_2 was kept at normoxic levels and served as a control to preclude temporal artefacts on the parameters measured. Alevin morphometry and mass was measured as described in chapter 2.1. $\dot{M}O_2$ in this chapter refers to mass-specific $\dot{M}O_2$ which was calculated on the basis of yolk-free body mass.

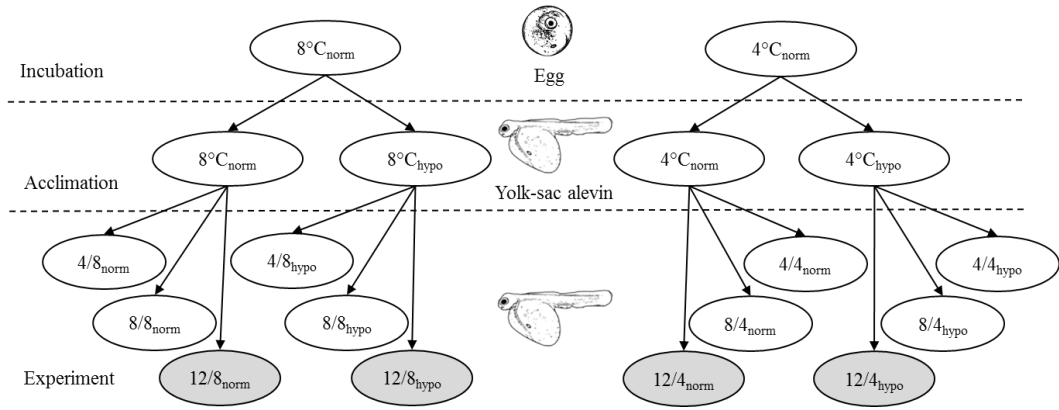


Figure 4.1. Schematic diagram of the experimental groups. Fertilised eggs were incubated at 4°C and 8°C in normoxia. At hatching, yolk-sac alevins were separated into acclimation groups at the respective incubation temperature and kept in normoxic (21 kPa) and hypoxic (10.5 kPa) conditions for 15 days. After the acclimation period, \dot{M}_{O_2} in response to acute, progressive hypoxia was measured in all acclimation groups at three ambient temperatures (4, 8 and 12°C), and simultaneous measurement of \dot{M}_{O_2} , f_H and f_V in response to acute, stepwise hypoxia were performed in all experimental groups except at ambient temperatures of 12°C (groups shaded in grey).

4.3.3. Statistical analysis

Statistical comparisons of mass and morphometry parameters between treatment groups (4_{norm}, 4_{hypo}, 8_{norm}, 8_{hypo}, the number here denotes the acclimation temperature) were made using one-way analysis of variance (ANOVA). Mass-specific \dot{M}_{O_2} , f_H and f_V in alevins were compared by two-way repeated measures ANOVA using PO_2 and treatment (normoxia vs. hypoxia) as factors within incubation groups and PO_2 and incubation temperature (8°C vs. 4°C) as factors between treatment groups. In all cases, the level of significance was set to $P = 0.05$. All pairwise multiple comparisons procedures were performed using Bonferroni post hoc t-tests.

4.4. Results

4.4.1. Body mass and morphometry

15 days post-hatch, hypoxia-acclimated alevins yolk-free body mass was lower at both incubation temperatures (19% at 4°C; 7% at 8°C) compared to normoxia acclimated alevins. Total mass however, was higher in alevins incubated at 8°C (7%) in comparison to 4°C and yolk mass was higher in hypoxia reared alevins in both groups (22% at 4°C; 17% at 8°C, Table 4.1) compared with normoxia reared ones. In 8°C incubated alevins, body length and number of caudal fin rays were significantly higher under normoxia compared with hypoxia acclimated individuals.

Table 4.1. Morphometry and body mass (wet) parameters of *S. salar* alevins incubated at 4 and 8°C with acclimation to normoxic or hypoxic conditions. Statistical differences between acclimation groups are indicated by differing superscript letters (One way ANOVA; $P < 0.05$). Data are mean \pm SE.

incubation temperature	4°C (508 degree days)		8°C (548 degree days)	
treatment	normoxia	hypoxia	normoxia	hypoxia
total mass (g)	0.107 \pm 0.001 ^a	0.114 \pm 0.002 ^a	0.122 \pm 0.001 ^a	0.132 \pm 0.003 ^b
yolk-free body mass (g)	0.053 \pm 0.001 ^a	0.043 \pm 0.001 ^b	0.055 \pm 0.001 ^a	0.049 \pm 0.001 ^b
yolk mass (g)	0.055 \pm 0.001 ^a	0.071 \pm 0.02 ^b	0.068 \pm 0.001 ^a	0.082 \pm 0.002 ^b
length (mm)	-	-	222 \pm 2 ^a	209 \pm 2 ^b
caudal fins (n)	-	-	16.0 \pm 0.2 ^a	14.7 \pm 0.7 ^b

4.4.2. Mass-specific metabolic rate

Alevins incubated at 8°C: The mean $\dot{M}O_2/g$ at a PO_2 of 21 kPa of the 8/8_{norm} and 12/8_{norm} treatments were ~1.5 and ~2 times higher than that of the 4/8_{norm} group (Fig. 4.2a). In normoxia, hypoxic acclimation led to a similar absolute increase in $\dot{M}O_2/g$ at all measurement temperatures (on average $0.060 \pm 0.006 \mu\text{mol min}^{-1} \text{g}^{-1}$).

With a progressive decline in P_{O_2} from 21 kPa to 5 kPa, $\dot{M}O_2/g$ decreased in all treatment groups. This metabolic depression was greatest at higher T_a such that the absolute $\dot{M}O_2/g$ in the 12/8_{norm} treatment fell more severely than in 8/8_{norm} and in 8/8_{norm} more severely than 4/8_{norm}. However, the proportional decrease in $\dot{M}O_2/g$ at each T_a (comparing $\dot{M}O_2/g$ at 21 kPa with 5 kPa) was similar (~60%). At PO_2 levels below 8 kPa there were no differences in $\dot{M}O_2/g$ among the normoxic treatment groups incubated at 8°C ($P = 0.46$; Fig. 4.2a). In hypoxia-acclimated alevins, $\dot{M}O_2/g$ of the 12/8_{hypo} and 8/8_{hypo} groups differed above a PO_2 of 8 kPa ($P = 0.015$) while both differed from 4/8_{hypo} at all PO_2 . At each T_a , $\dot{M}O_2/g$ differences between acclimation groups (norm vs. hypo) reduced with progressive hypoxia. At T_a of 4°C and 12°C, no differences in $\dot{M}O_2/g$ were detectable below PO_2 levels of 8 ($P = 0.083$) and 7 ($P = 0.165$) kPa respectively whereas at a T_a of 8°C, differences in $\dot{M}O_2/g$ were detectable throughout the PO_2 range ($P = 0.01$).

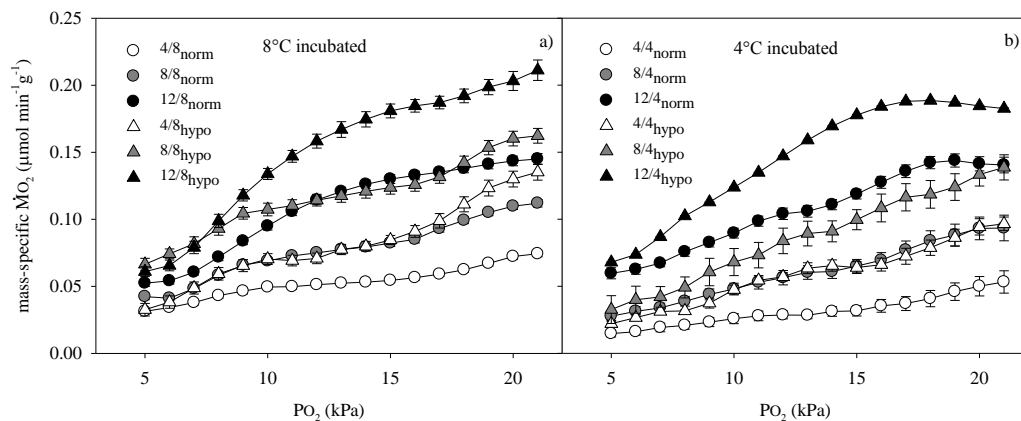


Figure 4.2. Changes in mean \pm S.E. mass-specific $\dot{M}O_2$ in response to a progressive decline in PO_2 at 4, 8 and 12°C of *S. salar* alevins incubated at a) 8°C and b) 4°C with acclimation to normoxic (norm) or hypoxic (hypo) conditions.

Alevins incubated at 4°C: In alevins incubated at 4°C, the overall $\dot{M}O_2/g$ response was similar to that exhibited by those incubated at 8°C (Fig. 4.2b). At a PO_2 of 21 kPa, the mean $\dot{M}O_2/g$ of the 8/4_{norm} and 12/4_{norm} treatments were ~1.8 and ~2.6 times higher than that of the 4/4_{norm} group. At a PO_2 of 21 kPa, the hypoxia-induced increase in $\dot{M}O_2/g$ was the same at all T_a (on average $0.043 \pm 0.001 \mu\text{mol min}^{-1} \text{g}^{-1}$). Once again, hypometabolism in response to falling PO_2 was greatest at higher T_a such that $\dot{M}O_2/g$ in the 12/4_{norm} treatment fell more severely than 8/4_{norm} and 8/4_{norm} more severely than 4/4_{norm}. However, in this instance, the proportional decrease (comparing $\dot{M}O_2/g$ at 21 kPa and 5 kPa) was not similar at all T_a in that $\dot{M}O_2/g$ at 12°C fell only ~57% compared to ~70% at 4 and 8°C. At a T_a of 4°C, $\dot{M}O_2$ differences among acclimation treatments (norm vs. hypo) did not persist below a PO_2 level of 8 kPa ($P = 0.17$). Corresponding figures for T_a of 8°C and 12°C were 9 ($P = 0.2$) and 7 ($P = 0.24$) kPa respectively (Fig. 4.2b). For both acclimation treatments $\dot{M}O_2/g$ measured at 12°C were higher than those measured at 4°C ($P < 0.001$) and 8°C ($P = 0.001$) throughout the PO_2 range whereas differences among 4°C and 8°C T_a groups did not persist below a PO_2 of 7 kPa ($P = 0.17$).

$\dot{M}O_2/g$ at the respective acclimation PO_2 in hypoxia (10.5 kPa) and normoxia (21 kPa) acclimated alevins within both incubation temperatures was similar (Fig. 4.3a and b).

The overall Q_{10} for $\dot{M}O_2/g$ at a PO_2 of 21 kPa for alevins incubated at 8°C was 2.3 and for alevins incubated at 4°C was 3.0.

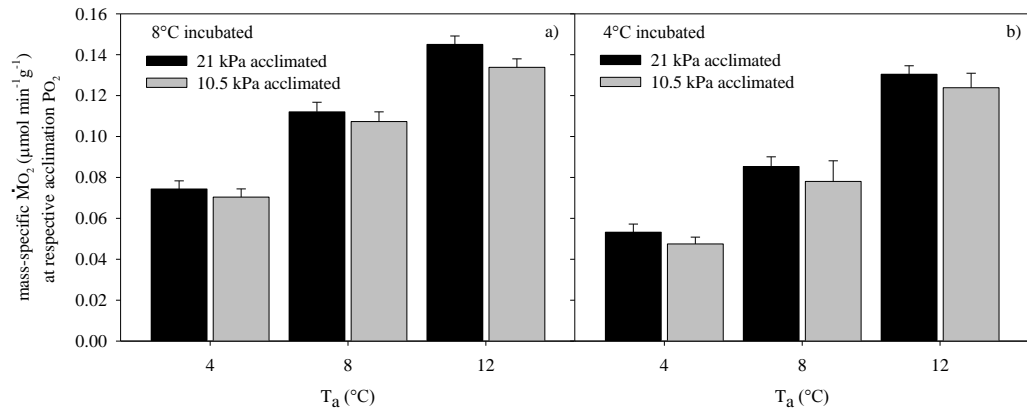


Figure 4.3. Mass-specific $\dot{M}O_2$ at different ambient temperatures (4, 8, 12°C) of yolk-sac alevins measured at their acclimation PO_2 , either normoxia (21 kPa) or hypoxia (10.5 kPa) for each incubation temperature of a) 8°C and b) 4°C. Hypoxia acclimated alevins raised their $\dot{M}O_2$ in hypoxia to the same level of a normoxia acclimated alevin in normoxia. Data shown are means \pm S.E.

4.4.3. Simultaneous measurement of mass-specific metabolic rate, heart- and ventilation rate

$\dot{M}O_2/\text{g}$ measurements made in the flow-through respirometry chamber were ~ 2.5 times higher than those made in the multiwell system (compare Fig. 4.4a and b, with Fig. 4.2a and b). The responses to progressive hypoxia and changes in temperature however, were similar to those reported in the multiwell system. That is, the stepwise, acute, hypoxic challenge led to metabolic depression, irrespective of measurement T_a or incubation temperature and the magnitude of the metabolic depression within the treatment groups was similar ($\sim 60\%$).

Hypoxic acclimation was also associated with an increase in $\dot{M}O_2/\text{g}$ measured at 21 kPa, irrespective of incubation temperature (on average $0.196 \pm 0.004 \mu\text{mol min}^{-1} \text{g}^{-1}$ at $T_a = 8^\circ\text{C}$ and $0.189 \pm 0.005 \mu\text{mol min}^{-1} \text{g}^{-1}$ at $T_a = 4^\circ\text{C}$). At a PO_2 above 5 kPa, $\dot{M}O_2/\text{g}$ of hypoxia- and normoxia-acclimated groups were

significantly different at both T_a in alevins incubated at 8°C and 4°C. The Q_{10} for $\dot{M}O_2/g$ was 1.7 for 8°C incubated and 3.1 for 4°C incubated alevins.

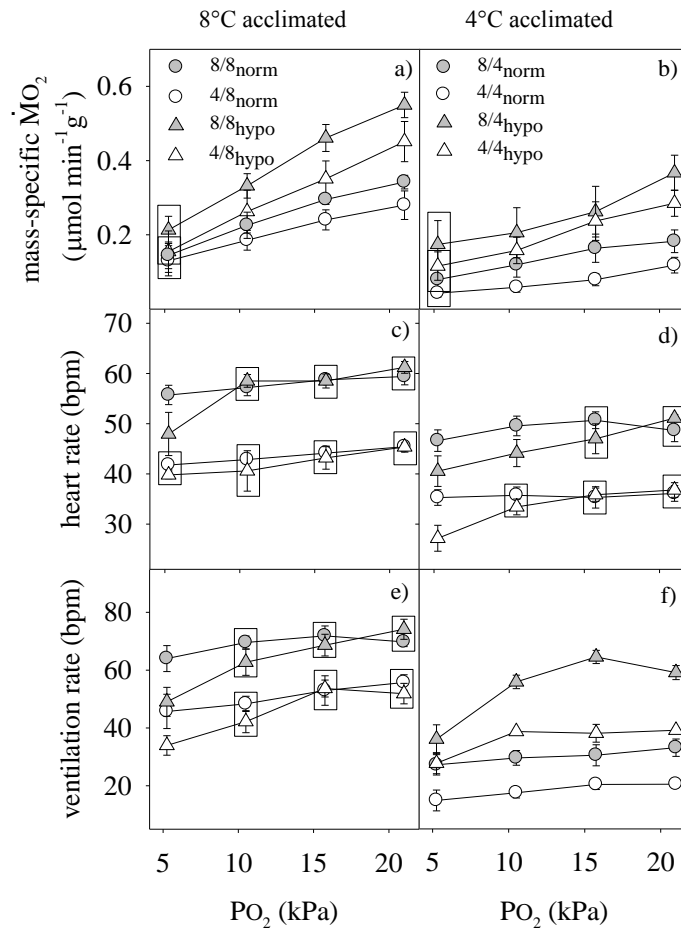


Figure 4.4. Changes in a) and b) mass-specific $\dot{M}O_2$, c) and d) heart rate and e) and f) ventilation rate of yolk-sac alevins acclimated to normoxic (norm) and hypoxic (hypo; $PO_2 = 10.5$ kPa, 15 days) conditions, incubated at 8°C and 4°C and measured at 4°C and 8°C (T_a) in response to a stepwise, acute hypoxic challenge ($PO_2 = 21 \rightarrow 15 \rightarrow 10 \rightarrow 5$ kPa). Boxes enclose statistically identical means within T_a groups. Data shown are means \pm S.E.

In alevins incubated at 8°C in normoxia, f_H at a T_a of 8°C (8/8_norm, 60 ± 2 bpm) was significantly higher ($P < 0.001$) than at a T_a of 4°C (4/8_norm, 45 ± 1 bpm, Fig. 4.4c). Hypoxic acclimation had no effect on f_H at most PO_2 levels. However at a PO_2 of 5 kPa, hypoxic acclimation was associated with a reduced f_H (47 ± 4 bpm) in 8/8_hypo alevins ($P = 0.02$).

In 4°C incubated alevins, again, hypoxic challenge did not elicit bradycardia in alevins incubated under normoxia (Fig. 4.4d) where f_H was 49 ± 2 bpm at T_a of 8°C and 36 ± 1 bpm at 4°C. Hypoxic acclimation was associated with significant reductions in f_H at PO_2 levels of 5 kPa (27 ± 3 bpm, $P = 0.012$) in 4/4_{hypo} alevins and at 10 kPa (44 ± 3 bpm, $P = 0.03$) and 5 kPa (40 ± 3 bpm, $P = 0.035$) in 8/4_{hypo} alevins. The Q_{10} for f_H at was 2.1, irrespective of acclimation temperature.

In 8°C incubated alevins, f_V at a T_a of 4°C (56 ± 3 bpm) was associated with a significant increase ($P = 0.006$) in f_V of ~11 bpm relative to a T_a of 8°C (70 ± 2 bpm, Fig. 4.4e). At this incubation temperature there was no change in f_V in response to the acute hypoxic challenge but f_V was significantly reduced at a PO_2 of 5 kPa in hypoxia-acclimated alevins at both T_a 's (8/8_{hypo} = 49 ± 5 bpm ($P < 0.001$) and 4/8_{hypo} = 34 ± 3 bpm, $P < 0.001$).

At an incubation temperature of 4°C, f_V was increased after hypoxic acclimation (4/4_{norm} = 20 ± 2 bpm vs. 4/4_{hypo} = 39 ± 1 bpm and 8/4_{norm} = 33 ± 3 bpm ($P < 0.01$) vs. 8/4_{hypo} = 59 ± 2 bpm, ($P < 0.01$) Fig. 4.4f). In normoxia acclimated alevins, f_V did not decline in response to acute hypoxia. At a PO_2 of 5 kPa on the other hand, after hypoxic acclimation at an incubation temperature of 4°C, the f_V was significantly reduced at both T_a (36 ± 2 bpm at 8°C, $P < 0.001$ and 27 ± 3 bpm at 4°C, $P = 0.009$). The Q_{10} for f_V at 8°C was 1.7 while that for incubation at 4°C was 3.3.

Generally, $\dot{M}O_2/g$, f_H and f_V appear to increase with an increase in acclimation temperature from 4°C to 8°C. For example, $\dot{M}O_2/g$ in 8/8_{norm} (0.34 ± 0.02 μmol

$\text{min}^{-1}\text{g}^{-1}$) was higher than in $8/4_{\text{norm}}$ ($0.18 \pm 0.03 \mu\text{mol min}^{-1}\text{g}^{-1}$). The same applied for f_{H} (49 ± 2 bpm in $8/4_{\text{norm}}$ and 60 ± 2 bpm in $8/8_{\text{norm}}$) and f_{V} (33 ± 3 bpm in $8/4_{\text{norm}}$ to 70 ± 2 bpm in $8/8_{\text{norm}}$). While this could be a result of the increase in incubation temperature, the age difference (~ 40 degree days) between the acclimation groups does not allow a clear distinction between the effects of incubation temperature and developmental stage and will not be further described here.

$\dot{\text{M}}\text{O}_2/\text{g}$, f_{H} as well as f_{V} in control animals that were not exposed to the hypoxic challenge remained constant over the entire measurement period (see Appendix 2), indicating that changes observed in each variable over time are not related to the measurement procedure.

4.5. Discussion

4.5.1. *Body mass and morphometry*

Body mass in alevins of *S. salar* acclimated to hypoxic conditions indicated a retardation of development, hence alevins had more yolk but a lower yolk-free body mass. This finding is consistent with the shorter body length and lower number of caudal fin rays observed in hypoxia-acclimated alevins at 8°C and is in agreement with previous reports of hypoxia-related developmental delays in salmonids (Alderdice et al. 1958; Ciuhandu et al. 2005; Hamor and Garside 1976; Miller et al. 2011; Miller et al. 2008; Shumway et al. 1964).

4.5.2. *Mass-specific metabolic rate*

The observed Q_{10} driven increase in $\dot{M}O_2/g$ with increasing T_a is common in ectothermic vertebrates (Scholander et al. 1953). The Q_{10} values for $\dot{M}O_2/g$ in this study are similar to what has been reported in other salmonid embryos and larvae (1.8-4.9, Rombough 1988b) including *S. salar* (Hamor and Garside 1977). In contrast, an increase in $\dot{M}O_2/g$ in normoxia, after hypoxic acclimation is unexpected. The developmental delay evidenced by differences in body mass, coupled with the general understanding that $\dot{M}O_2$ increases proportionally with developmental rates in salmonids (Mirkovic and Rombough 1998; Rombough 1988a) would tend to suggest that $\dot{M}O_2$ should be reduced following hypoxic acclimation. The present observation is indicative of hypoxia-triggered physiological modifications that enhance mechanisms of O_2 uptake when the animal is returned to normoxic conditions. Even though development might be

delayed under chronic hypoxia, the hypoxic exposure could have caused morphological or physiological changes. For instance, a reduction in cutaneous thickness (to facilitate O₂ diffusion), gill surface area, circulation or ventilation as well as O₂ carrying capacity or hemoglobin binding characteristics may lead to improved O₂ uptake when alevins are re-exposed to normoxia (relative hyperoxia). Morphological changes to respiratory structures have previously been reported in chicken embryos (Dusseau and Hutchins 1988; Hudlicka et al. 1992), amphibians (Burggren and Mwalukoma 1983; Rogge and Warkentin 2008) and fish larvae in response to hypoxia and ionic disturbance (Craig et al. 2007; Crispo and Chapman 2010).

The level of acute hypoxia-induced metabolic depression relative to $\dot{M}O_2/g$ in normoxia was similar at different T_a (60%) and $\dot{M}O_2/g$ was similar in severe hypoxia (5 kPa) in most treatment groups. Therefore, it is likely that O₂ uptake under severe, acute hypoxia (5 kPa) is transport limited at this developmental stage. That said, in 4°C incubated alevins, the metabolic depression (comparing values at 21 and 5 kPa) observed at a T_a of 12°C was less marked than those at 4°C and 8°C. Also, the $\dot{M}O_2/g$ under severe hypoxia at a T_a of 12°C was relatively high in both normoxia- and hypoxia-acclimated groups at an incubation temperature of 4°C (12/4_{norm} and 12/4_{hypo}) and in hypoxia acclimated alevins, incubated to 8°C (12/8_{hypo}). It appears that at 5 kPa at a T_a of 12°C O₂ transport might not have reached its full limitation, despite the elevated O₂ demand.

Iuchi and Yamagami (1969) identified two polymorph hemoglobins (Hb) during ontogenic development of rainbow trout. The embryonic form (Hb_E) possessed

higher affinity for O₂ than the adult form (Hb_A). More recently, Bianchini (2012) reported that Hb concentration, erythrocyte number and haematocrit were significantly reduced in hypoxia-reared rainbow trout embryos, suggesting a reduction in overall metabolism. Furthermore, due to a delay in the developmental transition from Hb_E to Hb_A, hypoxia-reared animals displayed higher concentrations of Hb_E mRNA and this Hb_E also had higher O₂ affinity (P₅₀) and cooperativity (Hill coefficient, n_H). In addition, there was a concomitant delay in the morphological change from embryonic to adult erythrocytes. If hypoxia-reared *S. salar* alevins possess more, high affinity Hb_E compared with those raised in normoxia, the higher $\dot{M}O_2/g$ observed in this study might be partly explained. It remains to be investigated whether Hb_E loads and delivers more O₂ than Hb_A. Meanwhile, Rombough and Drader (2009) have concluded that Hb assists in O₂ uptake during extreme hypoxia of zebrafish larvae, as hemoglobin poisoning with carbon monoxide resulted in elevated levels of O₂ during respiration experiments.

Another possibility is that due to their relatively smaller yolk-free body size, hypoxia incubated alevins have a more advantageous surface area to volume ratio and cutaneous O₂ exchange will be facilitated. It should also be noted that hypoxia-acclimated alevins were subsequently measured under normoxic conditions. The associated exposure to a “relative hyperoxic” environment may have influenced $\dot{M}O_2$. As shown in chapter 5, acute hyperoxia in fact causes hypermetabolism at similar developmental stages.

In this context, the concept of developmental trajectories might become relevant (for details see Burggren and Reyna 2011). The increased $\dot{M}O_2$ after normoxia re-exposure might be a first indication for a compensatory mechanism resulting from the abnormal environment previously experienced. It has been shown that hypoxia reared chickens for example, have the ability to compensate for the delay in development during hypoxic periods and “catch up” with normoxia reared animals in development (Villamor et al. 2004; Zoer et al. 2009).

The fact the hypoxic acclimation induced increase in $\dot{M}O_2/g$ in normoxia is similar irrespective of T_a is interesting. It suggests that there is a set increase in $\dot{M}O_2/g$ associated with hypoxic acclimation that matches the required amount to reset the $\dot{M}O_2$ to an intrinsic level (Fig. 4.2). More importantly, $\dot{M}O_2/g$ for alevins measured at the acclimation PO_2 levels are similar at measured T_a within an incubation temperature group (Fig. 4.3). This is an interesting outcome and highlights that hypoxic acclimation indeed maintains the “normal“ $\dot{M}O_2$ set-point; i.e. $\dot{M}O_2/g$ is no different for a given temperature between different acclimation PO_2 . This strengthens the idea that hypoxic acclimation leads to improvements of O_2 uptake to return $\dot{M}O_2/g$ to an intrinsic level observed in normoxia.

A further possibility may be that the smaller body mass in hypoxia acclimated alevins is a result of compensatory mechanisms, displayed by higher $\dot{M}O_2/g$ after normoxic re-exposure. The increase in $\dot{M}O_2/g$ may be required to compensate for the lack of O_2 . Hence, the cost of the metabolic compensation is paid at the expense of a reduced body mass and is a possible reason why development is delayed in hypoxia.

A similar situation was seen in daphnia, where chronic hypoxia was associated with an attempt to return $\dot{M}O_2$ to normoxic levels, achieved with improved O_2 uptake as a result of increased hemoglobin levels (Wiggins and Frappell 2000). Clearly, maintaining metabolic rate is paramount to ensure maintenance of normal function and in the case of developing animals, growth. This becomes particularly relevant when the capacity for anaerobic metabolism is limited, as suggested for early life stages in fish. The fact that hypoxia acclimated animals are smaller probably is a reflection on the time taken within the acclimation period for improvements in O_2 delivery to occur and $\dot{M}O_2/g$ to return towards normal, following the hypometabolism associated with acute hypoxia.

Intraspecific variation in $\dot{M}O_2$ has been widely studied and factors known to contribute to it include body mass, temperature, age, sex, season and reproductive state (Burton et al. 2011). Differences in $\dot{M}O_2$ (up to threefold) have even been observed between siblings (Johnston et al. 2007; Metcalfe et al. 1995; Steyermark et al. 2005) but their origins and whether they are present during early development are unknown. Variation in hypoxia tolerance of the same localised Atlantic salmon population as used in this study has also been described (Barnes et al. 2011) and could be the result of developmental plasticity that occurred during critical windows during development. As shown in this study, chronic hypoxia affects $\dot{M}O_2$ shortly after hatching. If animals within a population, are exposed to different environmental perturbations that affect $\dot{M}O_2$ during development and these are carried on to adulthood, changes to the aerobic scope

(the limits within which aerobic metabolism can be sustained) might occur that result in altered hypoxia tolerance.

4.5.3. Simultaneous measurement of mass-specific metabolic rate, heart rate and ventilation rate

Despite the differences in absolute $\dot{M}O_2/g$ between measurement systems, the $\dot{M}O_2/g$ response to a stepwise change in acute hypoxia was essentially consistent with that observed in response to progressive acute hypoxia (Section 4.5.2). The generally elevated $\dot{M}O_2/g$ associated with this flow-through respirometer may have been due to constrained nature of the animal within the chamber or the use of light to visualize the heart and operculum. For example, in the latter context, light intensity has been shown to elevate $\dot{M}O_2$ in fish (Hamor and Garside 1975; Rombough 1988b). Literature data on $\dot{M}O_2$ in alevins at this stage is highly variable and data presented here for the flow-through respirometer or multiwell system approach are within the range of published data (see Appendix 3). The Q_{10} -effect on $\dot{M}O_2/g$ overall, were similar between measurement systems.

In regard to O_2 delivery, the present study suggests that f_H and f_V are not coupled in a 1:1 relationship to $\dot{M}O_2$ in normoxia acclimated alevins as f_H and f_V were maintained at normoxic values while $\dot{M}O_2$ decreased with acute hypoxia. This indicates that O_2 convection is not strongly linked, if at all, to O_2 supply in yolk-sac alevins and adds further support to previous findings that the embryonic/larval heart serves other functions such as angiogenesis or hormone transport (Burggren 2004; Pelster 2003). In addition, it is concluded that f_H , at least for salmon alevins, is not an accurate predictor of $\dot{M}O_2$ in hypoxia. However, hypoxia

acclimated alevins that exhibited elevated $\dot{M}O_2/g$, displayed greater f_H and f_V sensitivity under severe hypoxia by decreasing rates compared to normoxic animals. This adds further support to the possibility that exposure to chronic hypoxia may lead to changes to cardiorespiratory control mechanisms. However, f_H or f_V were not generally increased with chronic hypoxia and alevins adapted to chronic hypoxia through other mechanisms to elevate $\dot{M}O_2/g$. It is likely that the onset of cholinergic regulation of heart rate as seen in zebrafish (Bagatto 2005) or rainbow trout (Miller et al. 2011) was modified and allowed the reduction in f_H in severe hypoxia.

Differences in the Q_{10} -effect on $\dot{M}O_2/g$ and f_V between acclimation temperatures were present. While it can not be discerned, whether these differences are due to developmental stage or caused by the different acclimation temperature, it is noteworthy that the Q_{10} for $\dot{M}O_2/g$ and f_V were similar at both incubation temperatures while the Q_{10} for f_H was different to $\dot{M}O_2/g$ and f_V . This could be an indicator that f_V , unlike f_H is linked to $\dot{M}O_2/g$, despite showing no coupling in hypoxia.

In alevins incubated at 4°C, hypoxic acclimation was associated with changes to f_V above the rates measured in normoxic animals. The Q_{10} for these animals was 3.3 for f_V , which corresponds to that of $\dot{M}O_2/g$ ($Q_{10} = 3.1$) again indicating a close relationship between $\dot{M}O_2/g$ and f_V . Incubation at 4°C could be associated with a more advanced development of respiratory control at the time measured. The increase in f_V with increasing $\dot{M}O_2/g$ suggests that a higher demand in O_2 in

normoxia, as a result of hypoxic acclimation is at least, partially accomplished by improved O_2 delivery through f_V .

In the past it has been proposed that in older *Xenopus laevis* larvae, reflex bradycardia in hypoxia, is indicative of a regulatory mechanism while in earlier larvae, a decrease in f_H is more likely due to the lack of O_2 to sustain cardiac metabolism (Pan and Burggren 2013). This, in turn, impairs changes to \dot{Q} through an increase in cardiac stroke volume (Fritsche and Burggren 1996; Orlando and Pinder 1995). Further, it is possible that the maintenance of f_H with decreasing $\dot{M}O_2/g$ in acute hypoxia was generally enabled through an increase or a diversion of the O_2 supply to the heart, which only accounts for a small fraction of total $\dot{M}O_2$ (Raddatz et al. 1992). This has been previously suggested for chicken embryos in hypothermia by Tazawa (1973). In the present study, if after hypoxic acclimation f_H and f_V were down-regulated under acute hypoxia, a sustained $\dot{M}O_2/g$ in excess of the limits of O_2 transport would have been expected owing to increases in \dot{Q} or ventilation volume. This could have been the case in the 12/4_{norm} and 12/4_{hypo} treatments in alevins incubated to 4°C, where $\dot{M}O_2$ was elevated in comparison to all other groups and even in severe hypoxia. Alternatively, the increase in O_2 demand after hypoxic acclimation in normoxia may have resulted in earlier onset of aerobic limits for cardiorespiratory function in subsequent hypoxia.

In fish such as rainbow trout, neural mechanisms for cardiorespiratory control appear to be present at the hatching stage (Miller et al. 2011; Perry et al. 2009). If this is also the case in *S. salar* and acute hypoxia does not trigger its down-

regulation with a decrease in $\dot{M}O_2$, the significance of f_H and f_V for O_2 supply to the tissues in normoxia is likely to be minimal and cutaneous diffusion of O_2 will suffice to meet O_2 demand. Consequently there is an absence of mechanisms to support O_2 delivery under adverse environmental conditions where animals become hypoxemic owing to their limited capacity to obtain environmental O_2 . Clearly, the present data suggest that alevins are transport limited under acute hypoxia as upon return to 21 kPa there is a substantial increase in $\dot{M}O_2/g$.

It is suggested that despite the expected decrease in developmental rates under chronic hypoxia (10.5 kPa), irrespective of incubation temperature, alevins adapt by improving mechanisms for O_2 uptake that clearly return $\dot{M}O_2/g$ (at 10.5 kPa) to levels similar to those seen in their normoxic counterparts. The ability to increase $\dot{M}O_2/g$ in hypoxia, and subsequently elevate it further when exposed to relative hyperoxia in return to 21 kPa must be associated with improvements in O_2 delivery independent of heart rates or ventilation rates. O_2 delivery is likely to be facilitated through modifications of hemoglobin concentration or morphological changes that are independent of aerobic metabolism. Increases in ambient temperature clearly lead to an increase in O_2 demand. However, this increase was not augmented by chronic hypoxia exposure and an increase in incubation temperature from 4°C to 8°C was not generally associated with differences in the acute or chronic hypoxic response with the exception of f_V in 4°C incubated alevins.

Conclusions and perspectives

Evidence that chronic environmental challenges like hypoxia may lead to structural or functional changes in O₂ delivery of Atlantic salmon alevins was found, that allows metabolic rate to return (increase) to “normal“ levels when re-exposed to normoxia. The question thus becomes whether despite the small setback in developmental rate with chronic hypoxia (possibly due to the initial acute hypometabolism), the animal has the ability to compensate when returned to a normal environment or continue on this developmental trajectory. It will be critical to assess, whether this developmental, phenotypic plasticity falls within a critical window during development that may alter the hypoxic response in later life stages. The exploration of this field could also be beneficial for the aquaculture industry to obtain more robust (e.g. hypoxia tolerant) commercial populations.

5. Acute but not chronic hyperoxia increases metabolic rate and does not alter the cardiorespiratory response in Atlantic salmon (*Salmo salar*) yolk-sac alevins at 4°C and 8°C

5.1. Abstract

Oxygenation of water in intensive fish farming may be required in circumstances when water supply is reduced or fish density is increased. In order to understand the effects of additional oxygen on respiration in fish, the effects of acute and chronic hyperoxia exposure on metabolic rate and cardiorespiratory function (heart rate and ventilation rate) in Atlantic salmon (*Salmo salar*) yolk-sac alevins incubated at 4°C, and how it is affected by an increase in ambient temperature (4°C and 8°C) were investigated. In addition, previous findings on chronic hypoxia exposure in alevins indicate that re-exposure to normoxic conditions after hypoxia exposure (relative hyperoxia) elevates metabolism above normal. Hence, it was investigated whether acute hyperoxia above normoxic (21 kPa) conditions also alters oxygen uptake.

Morphometric data on alevins acclimated to hyperoxia (15 days at 28 kPa) indicate advanced developmental rates compared with normoxia incubated animals. While acute hypoxia generally leads to metabolic depression (~70%, 21 kPa compared with 5 kPa), acute hyperoxia (28 kPa) causes hypermetabolism (~30% compared with normoxia at 4°C and ~20% at 8°C). Hyperoxic acclimation did not alter metabolic rate at 4°C or 8°C in acute hyperoxia, normoxia or hypoxia. Heart rates and ventilation rates were also unaltered with acute and were unaffected by chronic hyperoxia exposure.

It is shown that acute hyperoxia increases oxygen uptake above normoxic conditions but hyperoxic acclimation does not result in physiological adaptation

in regards to metabolic rate or oxygen delivery. Oxygen uptake is not limited by oxygen transport capacity but by oxygen availability at this developmental stage.

5.2. Introduction

Oxygenation of freshwater in commercial fish farming hatcheries is a common procedure to ensure optimal growth and survival (Caldwell and Hinshaw 1994; Edsall and Smith 1990). Oxygenation above normal partial pressures of O₂ (P_{O₂}, hyperoxia) has in some cases lead to increased growth rates (Dabrowski et al. 2004; Hosfeld et al. 2010; Lysne et al. 2006) while in others no effects on growth were reported (Edsall and Smith 1990; Foss et al. 2007; Foss et al. 2003; Ruyet et al. 2002). Behaviour in Atlantic salmon pre-smolt is affected by hyperoxia but the observed differences cease after 3 weeks, suggesting behavioural adaptation (Espmark and Baeverfjord 2009). In the same study the fish also displayed a reduction in hemoglobin concentration in hyperoxia that might be representative of a reduced need of O₂ binding proteins.

Due to the low solubility of CO₂ in water, ventilation in fish is generally driven by the concentration of O₂ in the water. Consequently, fish respond to environmental hyperoxia by hypoventilation (Gilmour 2001; Hosfeld et al. 2010; Perry et al. 2009; Randall and Jones 1973; Smith and Jones 1982; Wood and Jackson 1980). In contrast to the hypoxic (low environmental O₂) ventilatory response which leads to respiratory alkalosis through hyperventilation, hypoventilation leads to respiratory acidosis as CO₂ accumulates in the blood and is counteracted by an increase in plasma bicarbonate (Brauner et al. 2000; Perry and Gilmour 2006; Perry et al. 2009; Randall and Daxboeck 1984; Wood 1991). Brauner et al. (1999) have also reported reduced hypo-osmoregulatory ability of coho salmon after severe hyperoxia exposure (96 h, ~76 kPa).

Zebrafish chronically exposed to hyperoxia (50 kPa for first 7 days post fertilisation) have higher ventilation rates under conditions of rest at maturity, while adult zebrafish in hyperoxia (50 kPa; 7 day exposure during adulthood) showed no changes in ventilation patterns (Vulesevic and Perry 2006). Hyperoxia exposure during critical periods of development in mammals affects ventilation in adults (Mitchell and Johnson 2003) and the attenuation of the hypoxic ventilatory response through hyperoxic exposure has also been documented and extensively studied in the past (Bavis et al. 2011; Bavis et al. 2013; Teppema and Dahan 2013). During critical developmental periods, hyperoxia impairs the maturation of the carotid body chemoreceptors and may have prolonged effects on the size of the sinus nerve (Bamford et al. 1999). The result is a reduced response to hypoxia because afferent inputs from the carotid body and neurons in the carotid sinus nerve are blunted (Bisgard et al. 2005).

Hypermetabolism in response to chronic hyperoxia has been reported in fish (Foss et al. 2003), juvenile alligators that also exhibited lower breathing rates compared to normoxic alligators (Owerkowicz et al. 2009) and neonate mammals in acute hyperoxia (Frappell et al. 1992; Mortola and Tenney 1986) but not in chronically, hyperoxia raised *Xenopus laevis* larvae (Territo and Altimiras 1998). In that study *X. laevis* larvae also did not show differences in heart rates in either hypoxic or hyperoxic conditions.

In addition, hyperoxia has also been shown to result in increased levels of reactive oxygen species (ROS, e.g. superoxide, hydrogen peroxide or hydroxyl radicals) which are causatives of oxidative stress and therefore may cause damage

to tissues or cells (Cadenas and Davies 2000) and according to the free radical theory of aging, an increase in radical production could potentially decrease life span (Harman 1956, 1972). Therefore developmental hyperoxia might have detrimental effects in later life. However, the physiological response to acute or chronic hyperoxia during vertebrate development is poorly understood compared with adults.

Hosfeld et al. (2008) found no indications for impaired growth, mortality or seawater tolerance in *S. salar* smolt after chronic hyperoxia (26 kPa for 42 days) exposure. The physiological effects of hyperoxia in earlier life stages of development in *S. salar* have not been investigated so far.

Here, metabolic rate ($\dot{M}O_2$) and cardiorespiratory function (heart rate (f_H) and ventilation (f_V) rate) was measured in *S. salar* yolk-sac alevins (alevins) incubated at 4°C, (1) in response to acute hypoxia as well as hyperoxia at ambient temperatures of 4°C and 8°C, and (2) how this response is influenced after a chronic exposure to hyperoxia of 28 kPa for 15 days post hatching. As *S. salar* at this stage do not rely on gill respiration to obtain O_2 from the environment (Wells and Pinder 1996a, b), the hyperoxic respiratory response may largely vary from adult stages.

It was predicted that hyperoxia will result in accelerated growth compared to normoxia reared alevins due to the increased availability of O_2 . Since normoxia reared alevins have an increased $\dot{M}O_2$ at 8°C compared to 4°C and increase O_2 uptake in “relative hyperoxia“ after hypoxic acclimation (see, chapter 4), they are

not O_2 transport limited at $4^\circ C$ or $8^\circ C$ in “relative normoxia“ and have the capacity to raise their O_2 demand. Therefore it was hypothesised that alevins will display a higher $\dot{M}O_2$ but no changes in f_H or f_V in acute hyperoxia as at this stage, hypoxic hypometabolism is also not coupled to changes in f_H or f_V (see chapter 4). Furthermore, it was hypothesised that hyperoxia acclimated alevins will not alter $\dot{M}O_2$ in normoxia as the high O_2 environment they were reared in, does not necessitate changes to mechanisms for O_2 uptake or delivery that would enable an elevated O_2 demand post exposure.

5.3. Experimental protocol

5.3.1. Fish husbandry and maintenance

Eyed eggs from a commercial mass spawning event were collected from the SALTAS Wayatinah Hatchery (Tasmania, Australia) where they had been incubated in continuous-flow incubation trays at 4°C. In the laboratory, the fish were kept similar to hatchery conditions and maintained in well aerated spring water at 4°C ($\pm 1^\circ\text{C}$), in darkness. Just after hatching (432 degree days post fertilisation) occurred, newly hatched yolk-sac alevins were separated into two treatment groups where one was kept under normoxic conditions ($\text{PO}_2 = 21 \text{ kPa}$) and the other group under chronic hyperoxic conditions ($\text{PO}_2 = 28 \text{ kPa}$) for 15 days by bubbling an air/ nitrogen mixture into the covered holding tray using a gas mixer (GF-3/MP, gas mixing flowmeter, Cameron Instruments, Texas, USA).

5.3.2. Treatments and Measurements

After the 15 day acclimation period, $\dot{\text{M}}\text{O}_2$ in normoxia and in response to progressive hypoxia ($21 \rightarrow 5 \text{ kPa}$) was measured in each treatment group of alevins at an ambient temperature of 4°C and 8°C, using closed system respirometry (see Section 2.2). Sample sizes for both treatment groups at each measurement temperature were $n = 20$. Experimental groups will be referred to as 4_{norm} , 8_{norm} , and 4_{hyper} , 8_{hyper} where the number stands for measurement temperature (T_a) and the subscript indicates normoxic (norm) or hyperoxic (hyper) acclimation conditions. In a separate experiment, the cardiorespiratory response of alevins to two stepwise hypoxic challenges was investigated. Firstly from normoxia to hypoxia (referred to as Series I: $21 \rightarrow 15 \rightarrow 10 \rightarrow 5 \text{ kPa}$) in

normoxia acclimated alevins and secondly, from hyperoxia to hypoxia in normoxia and hyperoxia acclimated alevins by measuring $\dot{M}O_2$, f_H and f_V simultaneously (referred to as Series II: 28 → 20 → 10 kPa, for details on methodology see chapter 2.4). Sample sizes for each group here were $n = 8$. Alevin morphometry and mass was measured as described in chapter 2.1. $\dot{M}O_2$ in this chapter refers to mass-specific $\dot{M}O_2$ which was calculated on the basis of yolk-free body mass.

5.3.3. *Statistical analysis*

Statistical comparisons of mass and morphometry parameters between treatment groups (4°C normoxia or hyperoxia) were performed using one-way analysis of variance (ANOVA). $\dot{M}O_2/g$, f_H and f_V in alevins were compared using two-way repeated measures ANOVA with PO_2 and treatment (normoxia vs. hyperoxia) as factors within T_a groups and with PO_2 and measurement temperature (8°C vs. 4°C) between T_a groups. To test homogeneity of slopes and differences between intercepts to comparing linear regressions between measurement temperatures (4°C vs. 8°C) analysis of covariance (ANCOVA) were performed. In all cases, the level of significance was set to $P = 0.05$. All pairwise multiple comparisons procedures were performed using Bonferroni post hoc t-tests.

5.4. Results

5.4.1. Body mass and morphometry

Yolk-free body mass was smaller (11%) and yolk mass was higher (14%) in alevins raised under normoxic conditions in comparison to those acclimated to hyperoxia while total mass was similar between acclimation groups (Table 5.1). Body length was higher in hyperoxia acclimated alevins and the number of caudal fin rays was identical between both groups.

Table 5.1. Comparisons of morphometric and body mass (wet) parameters of yolk-sac alevins (*S. salar*) incubated at 4°C in normoxia and after chronic hyperoxia (PO₂= 28 kPa; 15 days) exposure. Statistical differences between treatment groups are indicated using asterisks (One way ANOVA; $P < 0.05$). Data show mean \pm SE, n = 20 per group.

incubation temperature	4°C (492 degree days)	
treatment	normoxia	hyperoxia
total mass (g)	0.153 \pm 0.009	0.146 \pm 0.006
yolk-free body mass (g)	0.062 \pm 0.003	0.070 \pm 0.002*
yolk mass (g)	0.091 \pm 0.005	0.076 \pm 0.004*
length (mm)	220 \pm 2	230 \pm 3*
caudal fin rays (n)	15.7 \pm 2.0	16.0 \pm 3.2

5.4.2. Mass-specific metabolic rate

$\dot{M}O_2/g$ was higher when measured at 8°C compared with 4°C in normoxia ($P < 0.001$) and hyperoxic acclimation had no effect on $\dot{M}O_2/g$ at either measurement temperature (Fig. 5.1). With increasing hypoxia (21 \rightarrow 5 kPa), $\dot{M}O_2/g$ in all treatment groups decreased. This metabolic depression was more severe the higher the T_a ($0.080 \pm 0.003 \mu\text{mol min}^{-1} \text{g}^{-1}$ at 4°C and $0.100 \pm 0.008 \mu\text{mol min}^{-1} \text{g}^{-1}$ at 8°C, $P = 0.013$), but proportionally, the decrease in $\dot{M}O_2/g$ (relative to

$\dot{M}O_2/g$ at 21 kPa at the respective T_a) was similar and on average ~71%. As the $\dot{M}O_2/g$ decreased more severely at 8°C compared to 4°C, the temperature induced difference diminished in acute hypoxic conditions. There was no difference in $\dot{M}O_2/g$ below a P_{O_2} of 8 kPa between T_a groups ($P = 0.23$; Fig. 5.1).

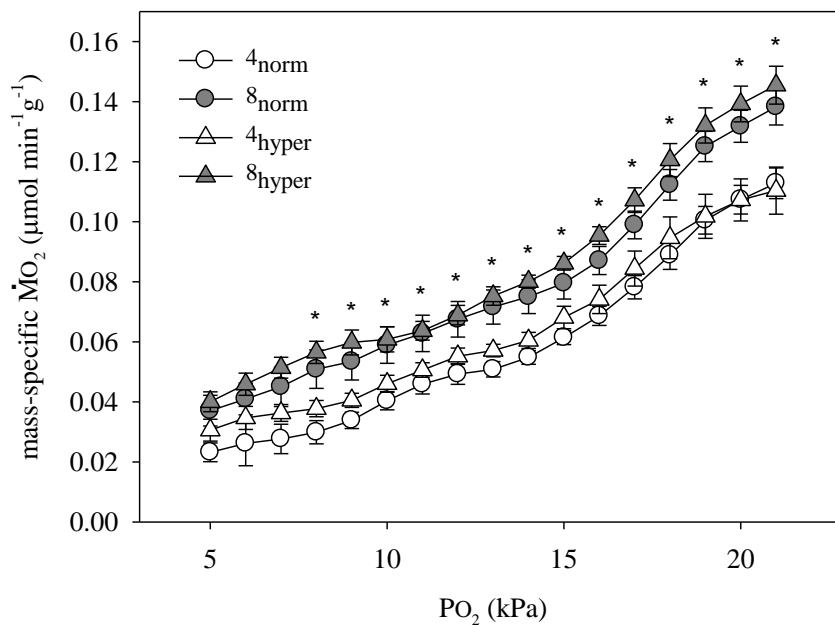


Figure 5.1. Changes in response to progressive, acute hypoxia (21 → 5 kPa) in mass-specific $\dot{M}O_2$ of *S. salar* yolk-sac alevins acclimated to normoxic (norm, $P_{O_2} = 21$ kPa) and hyperoxic (hyper; $P_{O_2} = 28$ kPa) conditions and measured at 4°C and 8°C using closed system respirometry (see chapter 2.2). Asterisks indicate differences in mass-specific $\dot{M}O_2$ between measurement temperatures at different P_{O_2} in normoxia acclimated alevins (one-way ANOVA, $P < 0.05$). Data shown are means \pm S.E.

5.4.3. Simultaneous measurement of mass-specific metabolic rate, heart- and ventilation rate

The stepwise, acute, hypoxic challenges (Series I and II) led to metabolic depression, irrespective of the T_a or initial P_{O_2} (Fig. 5.2a). The slopes of the linear regressions (for 28 to 10 kPa) within series were similar ($m: = 0.005$; $P = 0.017$, ANCOVA) indicating that there is no difference in $\dot{M}O_2/g$ whether or not

alevins were pre-exposed to hyperoxia. Therefore, the metabolic depression in absolute numbers within treatment groups was similar up to a PO_2 of 10 kPa. In Series I, $\dot{M}O_2/g$ at severe levels of hypoxia (5 kPa) was not statistically different ($P = 0.1$). $\dot{M}O_2/g$ measured at 4°C and 8°C in alevins that had been pre-exposed to hyperoxia were not statistically different to normoxia exposed ones. $\dot{M}O_2/g$ in acute hyperoxia (28 kPa) at 4°C and 8°C was increased above normoxic levels (30% and 20% respectively) but hyperoxic acclimation had no effect on $\dot{M}O_2/g$ irrespective of T_a .

Within each T_a and independent of acclimation regimes or PO_2 , f_H remained constant (Fig. 5.2b). f_H however did increase with increasing measurement temperature from 40 ± 3 bpm to 61 ± 2 bpm ($P < 0.01$). A similar result was found for f_V (Fig. 5.2c), which did not alter after chronic or acute hyperoxia or subsequent hypoxic exposure. f_V at 4°C was 40 ± 4 bpm and 56 ± 3 bpm at 8°C.

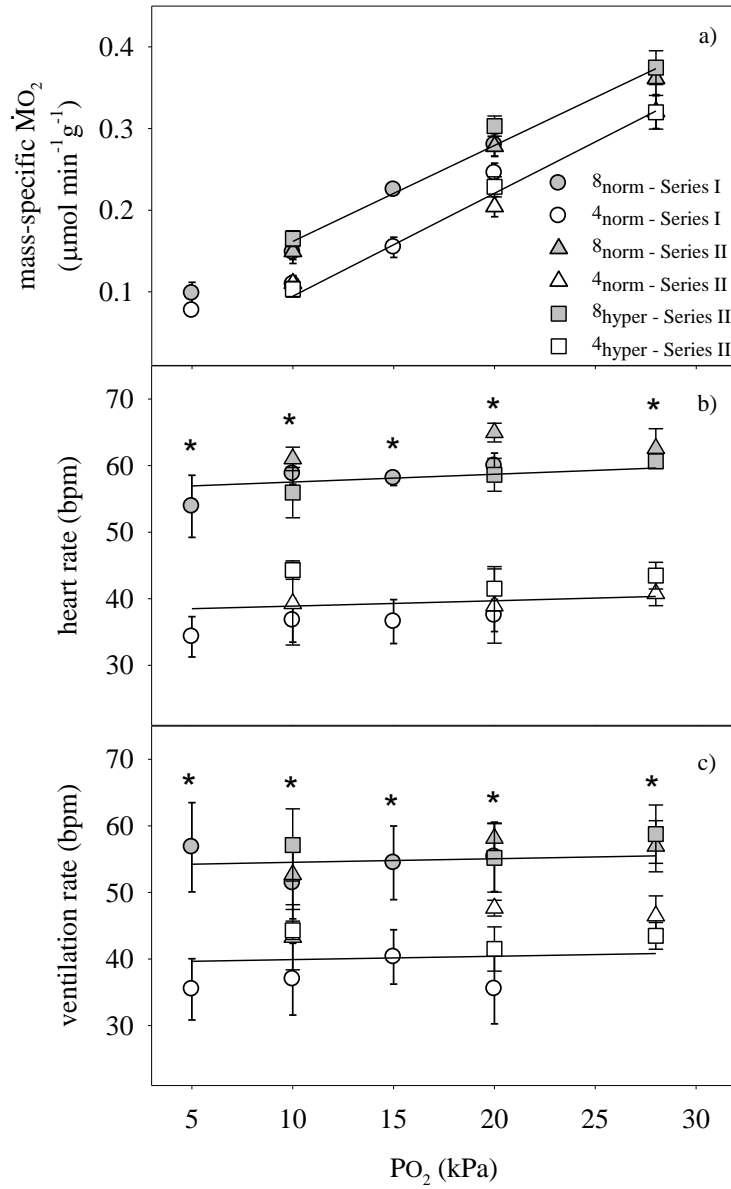


Figure 5.2. Changes in response to two stepwise challenges in a) mass-specific $\dot{M}O_2$, b) f_H and c) f_V of yolk-sac alevins acclimated to normoxic (norm) and hyperoxic (hyper; $PO_2 = 28$ kPa, 15 days) conditions and measured at 4°C and 8°C (T_a). Series I, acute hypoxic challenge ($PO_2 = 21 \rightarrow 15 \rightarrow 10 \rightarrow 5$ kPa) of normoxia acclimated alevins, Series II acute hyperoxic followed by acute hypoxic challenge ($PO_2 = 28 \rightarrow 20 \rightarrow 10$ kPa) in alevins acclimated to normoxic and hyperoxic conditions. Statistically significant differences between T_a groups are indicated using asterisks (one-way repeated measures ANOVA, $P < 0.05$). Data shown are means \pm S.E.

5.5. Discussion

5.5.1. *Body mass and morphometry*

The differences in body morphometry between alevins acclimated in hyperoxia compared with normoxia are indicative of faster growth through higher yolk absorption in hyperoxia, as suggested previously (Dabrowski et al. 2004; Hosfeld et al. 2010; Lysne et al. 2006), despite their similar age at measurement.

5.5.2. *Mass-specific metabolic rate, heart rate and ventilation rate*

$\dot{M}O_2/g$ after hyperoxic acclimation was unaltered when measured in normoxia ($PO_2 = 21$ kPa) and acute hypoxic conditions. This suggests that chronic hyperoxia, unlike chronic hypoxia (chapter 4) does not cause structural and/or functional modifications that affect O_2 uptake in normoxia or acute hypoxic conditions.

In acute hyperoxia however, $\dot{M}O_2/g$ is substantially elevated at 4°C and 8°C compared to measurements in normoxia ($PO_2 = 21$ kPa). This increase in $\dot{M}O_2/g$ occurred independent of acclimation conditions (normoxia vs. hyperoxia) or measurement temperature, hence, the O_2 demand increases with an increase in O_2 supply.

Neither the acute hyperoxia induced hypermetabolism, nor hyperoxic acclimation had an effect on f_H or f_V . Both f_H and f_V may be insensitive to hyperoxia at this developmental stage, despite showing increased sensitivity after acclimation to hypoxia (10.5 kPa) in severe acute hypoxia (see chapter 4).

Similar findings have been confirmed in amphibians. Although changes in stroke volumes having been reported in *X. laevis* when the blood is ablated from O₂ by carbon monoxide poisoning, cardiovascular parameters in hypoxia are not altered and therefore would only moderately contribute to O₂ uptake (Territo and Burggren 1998). It is suggested that the effects of severe levels of hyperoxia need to be further investigated to estimate the limitations of O₂ transport in hyperoxia.

Values for $\dot{M}O_2/g$ at 5 kPa in normoxia reared alevins at 4°C and 8°C was similar and indicates that alevins at these severe levels of hypoxia are O₂ transport limited. Therefore, despite the Q₁₀-driven increase in $\dot{M}O_2/g$ at a T_a of 8°C in normoxia, this higher O₂ demand can no longer be maintained in severe hypoxia. Whether animals pre-exposed to hyperoxia show differences in $\dot{M}O_2/g$ at severe levels of hypoxia in Series II can only be speculated, but since the hypoxic response and the linear regressions between normoxia and hyperoxia reared alevins are similar, it is suspected that equally, transport limitations will ultimately be reached in severe hypoxia (5 kPa). This is consistent with the previous experiments in this thesis, where $\dot{M}O_2/g$ was similar at severe levels of hypoxia (5 kPa, chapter 4) in normoxia and chronic hypoxia reared alevins. In conclusion, while chronic hypoxia increases O₂ uptake in normoxia, acutely, O₂ uptake is driven by O₂ availability.

Some of the increase in $\dot{M}O_2/g$ however, might also be related to potential challenges caused by reactive oxygen species (ROS) that have widely been reported to be deleterious to normal cell function, due to oxidative stress

(Cadenas and Davies 2000). Here, an increase of cell damage might lead to an increase in metabolism to supply the energy required for repair mechanisms.

Findings on O₂ delivery by Territo and Altimiras (1998) who used *X. laevis* larvae as their study model under hyperoxic acclimation (PO₂ = 35 kPa) conditions were confirmed. *X. laevis* larvae during early developmental stages, like salmonids do not rely on O₂ convection for the supply of O₂ to the tissues (Territo and Burggren 1998; Pelster 1999). It is proposed that in *S. salar*, at developmental stages where O₂ uptake is cutaneous, chronic hyperoxia does not lead to chronic hypermetabolism despite potential morphological changes to respiratory structures as they have been found in bullfrogs for example (Burggren and Mwalukoma 1983). In the avian embryo on the contrary, hyperoxic incubation induces an increase in $\dot{M}O_2$ (Ar et al. 1991; Stock et al. 1985). Hypermetabolism in severe (PO₂ = 100 kPa), acute hyperoxia has also been confirmed in neonatal rats (Frappell et al. 1992) and mice (Mortola and Tenney 1986) where $\dot{M}O_2$ is also limited by the supply of O₂. Chronic hyperoxia exposure alters the hypoxic ventilatory response in neonatal rats (Bavis et al. 2010) and perinatal hyperoxia (60 kPa) for the first month of life impairs the hypoxic ventilatory response in adults (Ling et al. 1996), but whether chronic hyperoxia leads to metabolic alterations in newborn mammals remains unknown.

The only study reporting on hypermetabolism in response to chronic hyperoxia (26 kPa) in fish shows a ~20% increase in mean, daily $\dot{M}O_2$ at 8°C compared to normoxic conditions (Foss et al. 2003). However, this finding is questionable due to the robustness of the methodological approach (authors reported but did not

quantify differences in activity between hyperoxic and normoxic animals and the aquatic respirometer was open to the atmosphere).

Espmark and Baeverfjord (2009) have reported behavioural changes in pre-smolt salmon in chronic hyperoxia, which were no longer present after 3 weeks of exposure. These fish also displayed lower hemoglobin and plasma chloride levels. This response indicates an adaptive mechanism to hyperoxia. $\dot{M}O_2/g$ in the alevins in this study is elevated in acute hyperoxia and possibly remains elevated throughout the exposure. This increased O_2 supply however, is unlikely to cause any structural or functional alterations that alter $\dot{M}O_2$ when re-exposed to normoxia (relative “hypoxia”).

Conclusions and perspectives

As hypothesised, the exposure of *S. salar* alevins to acute hyperoxia causes hypermetabolism without affecting heart rates or ventilation rates. Chronic hyperoxia exposure accelerates development but does not alter metabolic rate through modifications to O_2 uptake or delivery despite indications for faster growth. Taken together, under acute hypoxic or hyperoxic conditions the O_2 demand is determined by the supply of O_2 .

In regards to hatchery practices, hyperoxic water may increase $\dot{M}O_2$ acutely, but $\dot{M}O_2$ will most likely not be maintained elevated after re-exposure to normoxia and therefore, enhanced growth rates might not be sustained. Whether or not the hyperoxic exposure experienced in early life will have consequences at mature life stages should be part of future research efforts.

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

6.1. Abstract

Growth hormone (GH) transgenic Atlantic salmon (*Salmo salar*) display dramatically accelerated growth rates compared to non-transgenics. Recent research into the physiology of GH-transgenic fish has revealed cardiorespiratory and metabolic modifications that accompany the increased growth. An elevated routine metabolism has been described for pre-smolt as well as post-smolt GH-transgenic *S. salar* that in addition, display improvements in oxygen delivery to support the increase in aerobic demand.

The early ontogenic effects of GH-transgenesis on the cardiorespiratory and metabolic physiology, in particular during adverse environmental conditions (e.g. hypoxia) in *S. salar* and especially how it is affected by polyploidy, are unclear. In this study, the effects of GH-transgenesis and polyploidy on metabolic rate, heart rate and ventilation rate as well as heat shock protein (Hsp) expression, after exposure to acute hypoxia (21 → 5 kPa) in *S. salar* yolk-sac alevins shortly after hatching were investigated.

Metabolic rate decreased with decreasing partial pressures of oxygen (PO₂) in all genotypes. In normoxia, triploid transgenics displayed the highest mass specific metabolic rates in comparison to diploid transgenics and triploids, which again show higher rates than diploid non-transgenics. In hypoxia, at a PO₂ of 13 and 5 kPa, a lower mass-specific metabolic rate in diploid transgenics in comparison to all other genotypes was observed. Despite these differences in metabolism, no

evidence for improved O₂ uptake through heart- or ventilation rate was found. In fact, heart rate decreased in diploid non-transgenics while ventilation rate decreased in diploid non-transgenics and triploid transgenics at severe levels of hypoxia. Hsp70 was not expressed in alevins irrespective of their genotype or treatment, while after acute hypoxia, Hsc70 expression was decreased in transgenics and Hsp90 expression was decreased in all genotypes.

These data suggest that physiological changes through GH transgenesis and polyploidy are manifested at an early developmental stage in Atlantic salmon. Here, these changes encompass an increased metabolism due to transgenesis and polyploidy. Furthermore the cardiorespiratory response to hypoxia between diploids and triploid transgenics support the presence of genotypic differences. Evidence that the cellular stress response is altered by hypoxia and affected by GH-transgenesis is shown.

6.2. Introduction

Growth hormone (GH) transgenic fish have gained increasing attention in the aquaculture industry as a result of accelerated growth rates (~two to tenfold) when compared to 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 O₂ 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 addition, modifications to the cardiorespiratory physiology in GH transgenic Atlantic salmon (*Salmo salar*) have been documented that include an increase in heart size, cardiac output, a higher hemoglobin concentration, muscle aerobic enzyme activity and an increase in gill surface area (Blier et al. 2002; Cogswell et al. 2002; Deitch et al. 2006; Stevens and Sutterlin 1999).

In some fish 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, non-functional 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 chromosomes, nuclear size is increased in 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 higher growth due to the greater

abundance of genetic material as well as the diversion of growth from sexual maturation. The evidence is inconclusive (Tiwary et al. 2004), as in triploid fish, including Atlantic salmon, slower (Galbreath et al. 1994) as well as similar growth to diploids (McGeachy et al. 1995) has been reported. In the past, a reduced performance of triploid fish in comparison to diploids, especially under conditions of high O₂ demand has been suggested to be related to changes to their respiratory physiology and therefore hypoxia tolerance (Benfey 1999). Poor performance in terms of growth as well as deformity prevalence 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). Differences in hematological parameters, a reduction in gill surface area, as well as metabolism due to triploidy have been reported for some fish species but are lacking in others (Benfey 1999). Triploid Atlantic salmon display a reduction in O₂ carrying capacity (Graham et al. 1985), and triploid rainbow trout show higher rates for O₂ consumption (Oliva-Teles and Kaushik 1987), while triploid brook trout have lower rates of O₂ consumption in comparison to diploids (Stillwell and Benfey 1996). Clearly, more information of the physiological effects of triploidy is required.

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 Hsps) strategies (Breau et al. 2007; Barton 2002; Ackermann et al. 2000). The cellular stress response, characterised 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 Hsp throughout ontogeny has gained more

attention and it has been suggested that Hsps play a key role during early embryonic development (Deane and Woo 2011). Within the highly conserved Hsp families (e.g. Hsp70) constitutive (Hsc70) as well as stress-induced (Hsp70) isoforms have been isolated and throughout development, are differentially, tissue and stressor specifically expressed (Basu et al. 2002).

Given that GH-transgenic *S.salar* 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 O₂ availability. Up as well as downregulation of Hsp with low environmental O₂ levels in different species has been demonstrated and shows a high level of tissue, cell and temporal specificity (Araiksinen et al. 1998; Currie and Tufts 1997; Currie and Boutilier 2001; Golding et al. 2000; Ramaglia and Buck 2004).

A decreased mRNA expression of Hsp70 has been described in hepatic tissue of sparids after exogenous administration of growth hormone (Deane et al. 1999). Exercise, increasing metabolism and resulting in cellular oxidative stress, increases Hsp expression in various tissues of rats (Salo et al. 1991; Smolka et al. 2000).

The above findings suggest that in fish, GH transgenesis as well as triploidy, both part of current aquaculture practices, have significant impacts on aspects of the O₂ uptake, transport as well as utilization pathway. Further, these impacts might

be augmented under adverse environmental conditions and also reflected on a cellular level. The presence as well as the onset of alterations to the respiratory physiology and cellular stress response of fish caused by GH-transgenesis and particularly its interaction with polyploidy during early development, when fish are most sensitive to environmental perturbations, have not been investigated so far.

Respiration in *S. salar* yolk-sac alevins at hatching is primarily cutaneous under normoxic conditions (Wells and Pinder 1996a, b), and at this stage the larvae are exclusively dependent on the energy supply contained within the yolk-sac. Hence, the O₂ demand as well as the supply might be under different regulatory control to later stages of development or adulthood, and be impacted differently by ploidal level or GH-transgenesis.

In the present study, experiments to determine whether differences in metabolic rate, cardiorespiratory function and the expression of cellular stress proteins (Hsp70, Hsc70 and Hsp90) between diploid and triploid, transgenic and non-transgenic *S. salar* are present in newly hatched yolk-sac alevins and how these parameters are altered under conditions of reduced O₂ availability, were conducted.

It was hypothesised that growth hormone 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 O₂ delivery through cardiorespiratory modifications, especially when exposed to acute

hypoxia. Despite triploids appearing to be more susceptible to hypoxia, there is no clear evidence for that assumption. Therefore it is hypothesized that the O₂ demand is unlikely to differ from 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.

6.3. Materials and Methods

6.3.1. Experimental animals

Salmo salar yolk-sac alevins used in the experiments were reared under standard hatchery conditions (8°C, in darkness) at AquaBounty® farms (Fortune, Prince Edward Island, Canada) in heath stacks 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 *S. salar* 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-1 α lineage and exhibit a rapid growth phenotype (Aqua Bounty Technologies 2010). Triploidy in *S. salar* was induced by hydrostatic pressure shock after fertilisation of the egg, which prevents the extrusion of the second polar body during meiotic development and renders the animals sterile with an average effectiveness of 99%. The eggs for the experimental animals in this study hatched at ~400 degree days (50 days at 8°C since fertilisation).

6.3.2. Treatments and Measurements

Firstly, measurements of metabolic rate (\dot{M}_{O_2}) were performed at 8°C on 2n and 3n transgenic (2nTx, 3nTx) as well as 2n and 3n non-transgenic (2nNt, 3nNt) *S. salar* yolk-sac alevins (Series I; see chapter 2.2 for details on methodology). Subsequent measurements were conducted of \dot{M}_{O_2} , heart rate (f_H), as well as ventilation rate (f_V , Series II; for details see chapter 2.4). Alevin morphometry and mass was measured as described in chapter 2.1. \dot{M}_{O_2} in this chapter refers to mass-specific \dot{M}_{O_2} which was calculated on the basis of yolk-free body mass.

In another experiment (Series III) the intracellular stress response of alevins of the different genotypes (2nNt, 2nTx, 3nNt, 3nTx) to a similar hypoxic stress as in Series I was tested. Therefore, measurements of $\dot{M}O_2$, as described in Series I (for methodology see chapter 2.2) were performed in 2nNt, 2nTx, 3nNt, 3nTx ($n = 5$ respectively) 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 the closed system respirometry chambers. Experiments were performed until a PO_2 of 5 kPa was reached. In parallel, 10 alevins per genotype were kept under the same experimental conditions in normoxia for the same duration as the experimental group. For this purpose, the alevins were placed in similar multiwell plates, submerged in continuously, well aerated water with only a thin mesh separating the alevins from escaping into the surrounding water. After this procedure, experimental animals were transferred to normoxic water for 24 h to allow for the manifestation of the Hsp response. The control group was kept within the same container for the same duration after quick removal from the wells to mimic the handling stress that the experimental animals were exposed to. Subsequently all specimens were kept at -80°C until Hsp immunodetection (see chapter 2.9).

6.3.3. 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 PO_2 and genotype (2nNt, 2nTx, 3nNt, 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 transgenic (yes or no) as factors were performed. All pairwise multiple comparisons procedures were performed using Bonferroni post hoc t-tests.

6.4. Results

6.4.1. Body mass and morphometry

Total body and yolk mass was lower in 3nTx yolk-sac alevins, in comparison to all other genotypes (Table 6.1). Yolk-free body mass was highest in 2nNt and differed from 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 in animals used in Series I.

Table 6.1. Comparisons of morphometric parameters of newly hatched yolk-sac alevins of different genotypes (2nNt, 2nTx, 3nNt, 3nTx) raised at 8°C. Data show means \pm SE. Differing letters between groups indicate significant differences at $P < 0.05$.

	2nNt (n=28)	2nTx (n=30)	3nNt (n=30)	3nTx (n=29)
total body mass (g)	0.132 \pm 0.002	0.124 \pm 0.002	0.123 \pm 0.002	^a 0.112 \pm 0.002
yolk-free body mass (g)	^a 0.064 \pm 0.001	0.060 \pm 0.001	^b 0.058 \pm 0.001	^b 0.057 \pm 0.001
yolk mass (g)	0.068 \pm 0.002	0.064 \pm 0.002	0.065 \pm 0.002	^a 0.055 \pm 0.002
caudal fin rays (n)	17.2 \pm 1.7	16.9 \pm 1.7	17.0 \pm 1.6	16.6 \pm 1.6
total length (mm)	20.8 \pm 1.7	20.3 \pm 0.9	20.5 \pm 0.7	20.5 \pm 0.8

6.4.2. Series I: Mass-specific metabolic rate

Generally, $\dot{M}O_2/g$ decreased with increasing hypoxia. However, this decrease displayed a triphasic pattern. Within a PO_2 range of 15 to 11 kPa, values for $\dot{M}O_2/g$ within the groups decreased at a slower rate and did not change significantly (two-way-RM-ANOVA, $P = 0.1$). Therefore the data were separated into three sections (Fig. 6.1, dotted lines) and values for $\dot{M}O_2/g$ were pooled within a range of 3 kPa (PO_2), resembling normoxic (21-19 kPa), intermediate hypoxic (14-12 kPa) and severely hypoxic (7-5 kPa) conditions. $\dot{M}O_2/g$ in 2nTx

and 3nNt alevins was higher (~8%) than in 2nNt alevins while 3nTx alevins, in turn, had elevated metabolic rates (~8%) in comparison to 2nTx and 3nNt alevins (Fig. 6.1 inset). Within intermediate and severe hypoxia, 2nNt alevins showed the lowest values for $\dot{M}O_2/g$, while 2nTx, 3nNt as well as 3nTx alevins were not statistically different (Fig. 6.1 inset).

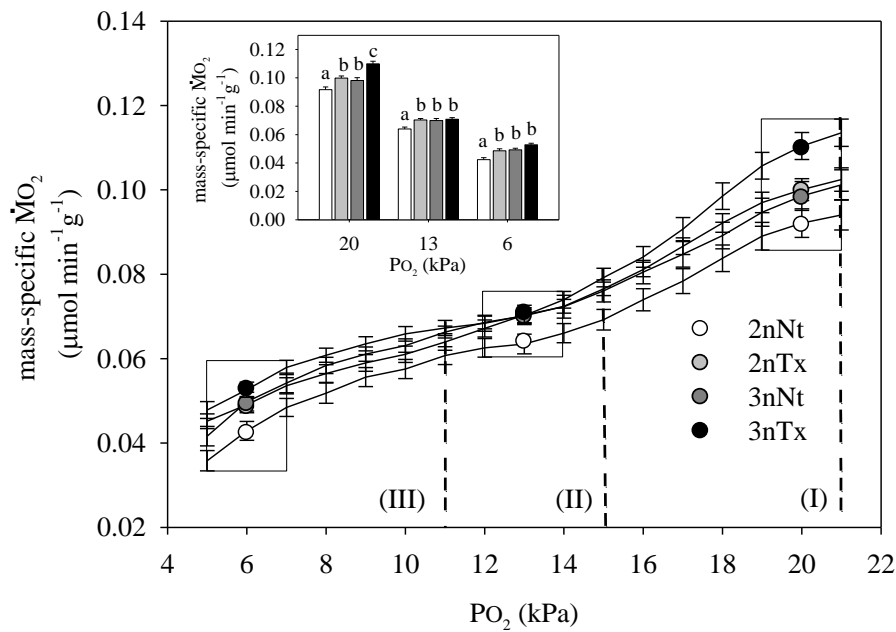


Figure 6.1. Changes in $\dot{M}O_2$ in response to progressive, acute hypoxia (21 \rightarrow 5 kPa) of 2nNt, 2nTx, 3nNt and 3nTx *S. salar* yolk-sac alevins measured at 8°C. Dashed lines separate sections where $\dot{M}O_2$ decreases (I, III) or remains constant (II) as determined by two-way repeated measures ANOVA. Inset panel shows $\dot{M}O_2$ data for normoxia, intermediate and severe hypoxia (data enclosed in boxes) and statistical differences ($P < 0.05$) are indicated using different letters. Data shown are means \pm S.E.

6.4.3. Series II: Simultaneous measurement of mass-specific metabolic rate, heart- 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. 6.1), here, $\dot{M}O_2/g$ in both groups decreased with declining levels of PO_2 compared to normoxic values (Fig 6.2a, 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 to 16% in Series I and as in Series I remained elevated at all levels of PO_2 in comparison to 2nNt alevins.

3nTx as well as 2nNt alevins had a similar f_H in normoxia (Fig 6.2b, e). Despite a small reduction in f_H in 2nNt alevins in intermediate hypoxia, it was not statistically different compared to normoxic values, unlike in severe hypoxia, where f_H in 2nNt alevins was reduced (~10%), while in 3nTx it remained similar to measurements in normoxia. In contrast to these findings, both 3nTx and 2nNt yolk-sac alevins reduced their f_V at severely hypoxic levels (Fig. 6.2c, f). In normoxic and intermediate hypoxic conditions, no difference in f_V was detected between genotypes.

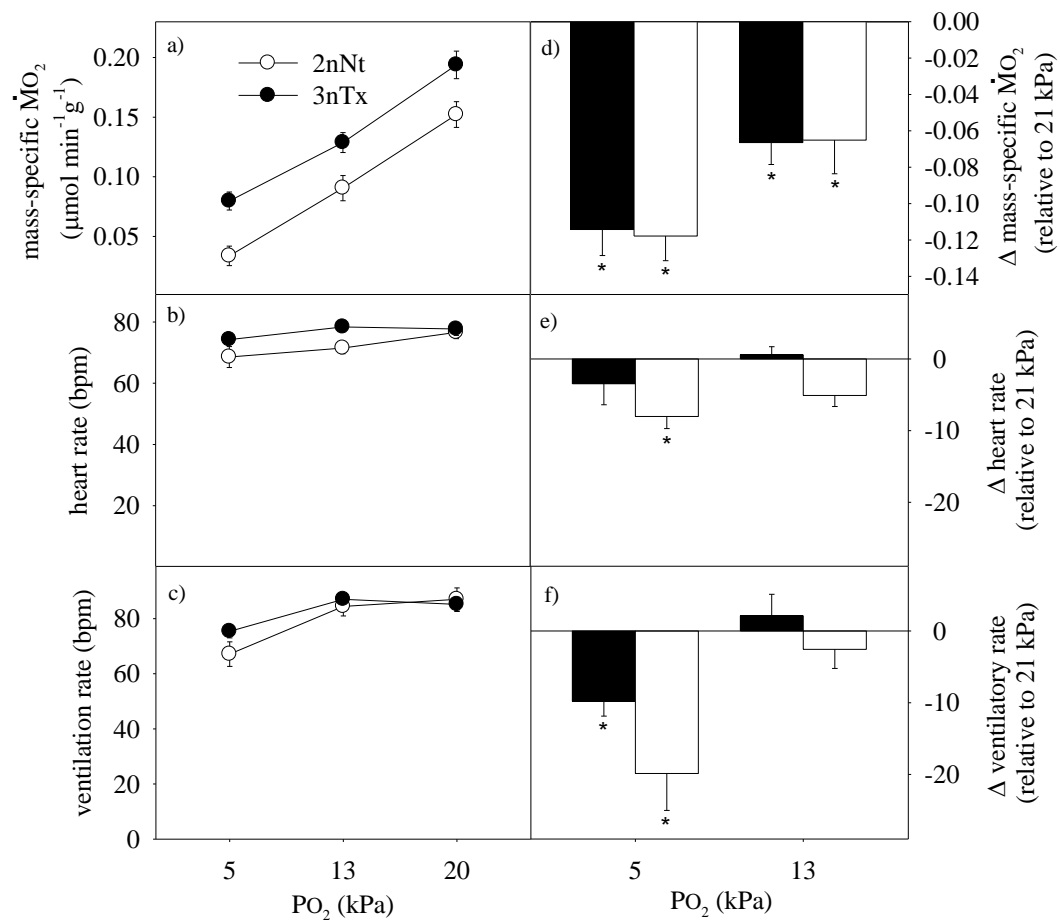


Figure 6.2. Changes in $\dot{M}O_2$ (a), heart rate f_H (b) and ventilation rate f_V (c) in 2nNt and 3nTx *S. salar* yolk-sac alevins in response to a stepwise (PO_2 of 21, 13 and 5 kPa) challenge of hypoxia. Right panels show relative changes in $\dot{M}O_2$ (d), heart rate f_H (e) and ventilation rate f_V (c) at 13 and 5 kPa with respect to values measured in normoxia (21 kPa). The asterisks indicate a significant difference ($P < 0.05$) in respective parameters compared to normoxic (control) conditions. All values are expressed as mean \pm SE.

6.4.4. Series III: Hsp expression

Western blot analysis of whole animal samples revealed that Hsp70 (inducible form) was undetectable in the control or hypoxia treated alevins (Fig. 6.3a). Unlike Hsp70, Hsc70 (constitutive form) and Hsp90 were detectable by western blot as shown by representative fluorograms (Fig. 6.3b, c). No differences in relative expression of Hsc70 between genotypes in the control samples kept in normoxic conditions were observed (Fig. 6.4a). However, Hsc70 levels in acute hypoxia treated GH-transgenic (2nTx and 3nTx) alevins were significantly lower than in non-transgenics (2nNT and 3nNT, $P = 0.048$). Equally, relative Hsp90 expression in control groups were similar but significantly lower in all hypoxia treated alevins (Fig. 6.4b, $P < 0.001$).

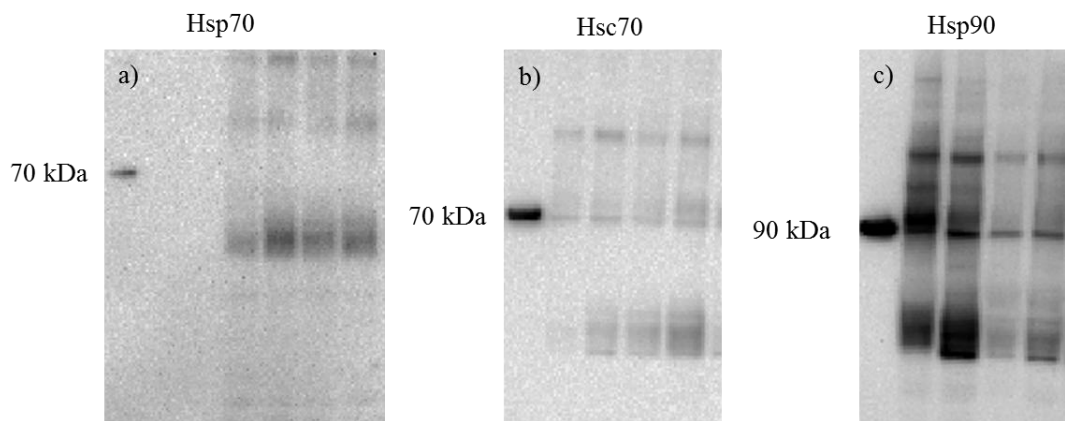


Figure 6.3. Representative fluorograms of a) Hsp70 and b) Hsc70 c) Hsp90 synthesis in *S. salar* yolk-sac alevin whole body homogenates. Molecular masses of proteins are indicated in kDa.

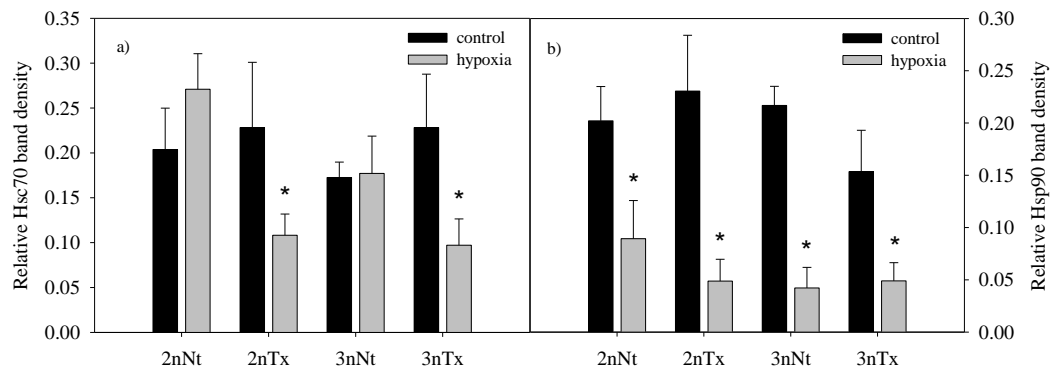


Figure 6. 4. Quantification of a) Hsp70 and b) Hsp90 synthesis in 2nNt, 2nTx, 3nNt and 3nTx *S. salar* yolk-sac alevin whole body homogenates in normoxia (control) and after acute, progressive hypoxia exposure (hypoxia). The bar graphs depict protein band density obtained from densitometric scans of fluorograms. The asterisks indicate a significant difference ($P < 0.05$) from respective control conditions within each genotype. All values are expressed as mean \pm SE.

6.5. Discussion

In the present study it was demonstrated that acute hypoxia exposure generally leads to a decrease in metabolic rate in *S.salar* yolk-sac alevins when measured at 8°C (as previously described in Chapter 3-5). In addition, evidence that GH-transgenesis as well as triploidy result in elevated mass-specific metabolic rates in normoxia as well as acute hypoxia and in combination (3nTx) appear to have additive effects, was presented. Furthermore the results of higher metabolic rates in triploid transgenic alevins compared to diploid non-transgenic alevins in a separate methodological approach while simultaneously measuring heart rate and ventilation rate were confirmed. Neither of these measures universally correlated with metabolic rate (as seen previously, Chapter 4 and 5) at this ontogenic stage. Hence, the increase in metabolic rate through GH-transgenesis and triploidy is not reflected in improved O₂ uptake through heart- or ventilation rate and therefore must relate to other physiological mechanisms to ensure O₂ delivery. The increase in metabolic rate was also not reflected in an altered expression of Hsps (Hsp70, Hsc70 and Hsp90) and hypoxia did not elicit a cellular stress response through increased Hsp expression. However, hypoxia induced differential Hsp expression with transgenics showing sensitivity in the expression of Hsc70 while Hsp90 expression was sensitive to hypoxia irrespective of the genotype.

6.5.1. Series I and Series II

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; Høileton and Randall 1967; Randall 1982)

to counteract the reduced availability of O₂ 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 O₂ across the skin is the primary avenue for O₂ uptake and the reliance on gills as the main respiratory organ occurs at the end of the larval stage (Wells and Pinder 1996a, b). 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, as well as ventilation from metabolic rate (as described in Chapter 4 and 5) as it 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 rate as well as ventilation rate from metabolic rate questions the relevance of convective O₂ transport in the O₂ 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 as well as cholinergic control of the heart is present shortly after hatching and altered by hypoxic exposure (Miller et al. 2011).

In this study, it was observed that normal diploid Atlantic salmon alevins acclimated to normoxia showed a bradycardic response and a decrease in ventilation rate to severe levels of hypoxia. In comparison to the previous findings (chapter 4 and 5) where a bradycardic response to acute hypoxia was only observed after hypoxic acclimation, here, the experimental procedure was slightly altered. In this procedure there were only 2 stepwise changes in acute hypoxia. Whereas there was less steps compared to the previous study (3 hypoxic steps), the initial decrease to a lower PO_2 (13 kPa compared to 15 kPa) could have resulted in an earlier onset of hypoxic bradycardia and lower ventilation rate at more severe hypoxia (5 kPa). In addition, *S. salar* alevins in this study were from a different population with a potentially different breeding and incubation history. In contrast, 3nTx alevins did not display this bradycardic response but a decrease in f_V at a low PO_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 preconditions for a cardiorespiratory response to hypoxia are met, but an increase in metabolism did not lead to increased O_2 uptake through heart- or ventilation rate.

Even though the reduction in $\dot{M}O_2$ with hypoxia is similar between genotypes in Series II (unlike in Series I), a more severe hypoxic cardiorespiratory response in 2nNt through a decrease in heart rate and a trend towards lower ventilation rate was observed. 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 smaller relative reduction in $\dot{M}O_2$ in severe hypoxia compared to normoxic values (60% in 3nTx vs. 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, genuine physiological modifications must be in place. 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.

It is generally accepted that GH-transgenesis, leading to accelerated growth rates, is compliant with an increase in metabolism in juvenile and adult salmon (Deitch 2006). In this study on alevins, it is demonstrated that in normoxia, GH-transgenesis as well as triploidy leads to an increase in metabolism, that is additive when the effects are combined. This raises the 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. In the past, it has been hypothesised that the increase in cell size and different shape observed (Benfey 1999), mainly a consequence of the additional genetic material within the nucleus, would have an impact on O_2 transport to the cell and therefore on overall metabolism (Maxime 2008). If in

agreement with Fick's law of diffusion, triploidy leads to an increase in cell volume and thus increases the distance for O₂ diffusion to the centre of the cell, a greater O₂ gradient has to be established to meet the O₂ demand, which in turn, increases O₂ 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 is very inconclusive and does not follow a coherent pattern (Benfey 1999; Maxime 2008). If triploidy affects the hematology of fish, it stands to reason to further investigate O₂ binding properties of embryonic/ larval hemoglobin in triploid fish.

In an exercise challenge, Hyndman et al. (2003) found no indication for additional aerobic metabolism in diploid and triploid trout. At higher temperatures however, the use of additional anaerobic metabolism was impaired in triploids. This is indicative of different thermal tolerances between diploids and triploids, as suggested by different growth and survival rates (Benfey et al. 1997; Galbreath et al. 2006). The effect of thermal limits on cardiorespiratory and metabolic physiology in transgenic fish remains to be examined and in combination with ploidal level, is likely to reveal more detailed physiological characteristics of the different genotypes.

6.5.2. Series III

On a cellular level, Hsps are differentially expressed after exposure to acute, progressive hypoxia and this expression is genotype dependent (transgenic, polyploid). Firstly, the reduction in the expression of Hsc70 and Hsp90 indicates

that this level of acute hypoxia, does not induce a cellular stress response as commonly seen for other environmental stressors (e.g. heat shock) where Hsp expression is increased (Basu et al. 2002). This result is in accordance with studies by Zarate and Bradley (2003) who show that Hsp30, Hsp70 and Hsp90 mRNA was not increased with hypoxia and therefore suggested that Hsps are not sensitive indicators of hatchery stress in Atlantic salmon. In a different study by Araisinen et al. (1998) cell specific expression of Hsps (36, 39, 51) is demonstrated and is increased in gill epithelial cells of rainbow trout in hypoxia. Red blood cells of rainbow trout did not display elevated levels of Hsp70 during anoxia, despite sustained non-stress protein synthesis (Currie and Tufts 1997). In the anoxia tolerant western painted turtle, short-term anoxia does not lead to an induction of Hsps (constitutive Hsp73, inducible Hsp72 and Hsp90) but are tissue specifically upregulated after long-term exposure (Ramaglia and Buck 2004). A similar pattern that may indicate an involvement of Hsp in long-term hypoxia tolerance was shown in the heart of the common frog, where hypoxic submergence increased Hsp70 expression (Currie and Boutilier 2001). These results illustrate the complexity of the Hsp response to hypoxia/anoxia and highlight the necessity to improve our understanding of its temporal, spatial and species specific regulation.

The hypoxic challenge experienced by the commonly regarded hypoxia intolerant *S.salar* in this study is relatively severe. The respiratory system of the salmon alevin is grossly different to the adult stages and therefore the Hsp response to hypoxia might equally be different. Susceptibility to hypoxia is thought to be greater during early life stages as energy resources are dedicated to growth. The

level of hypoxia experienced in this study however, does not elicit an Hsp response and might therefore not be an indicator for hypoxic stress. On the contrary, the differential downregulation of Hsps with hypoxia might be indicative for a reduced ability for protein synthesis due to the lack of aerobic energy production. Equally protein might be used as an energy source for gluconeogenesis. In this case potential protective mechanisms cannot be established and the cells ability to ensure homeostasis might be impaired.

While this could readily explain the Hsp90 expression pattern observed here, the reduction in Hsc70 is only restricted to GH-transgenic alevins. As 3nNt larvae display a similar increase in metabolic rate as 2nTx, the increased metabolism does not provide a plausible explanation for this finding. Other mechanisms must be taking place that explain the reduced expression in transgenics. Our knowledge of the cellular and molecular consequences resulting from GH-transgenesis to date are very limited. The increased growth entails a higher rate of gene translation as well as transcription. It is speculated that the increased genetic activity could therefore be diverted to processes involved in increased growth of certain tissues or cells, that in turn could reduce the available resources to maintain cellular homeostasis in others. As whole animal samples were used, tissue or cell specific protein expression can not be discerned. If growth was diverted to an increase in muscular growth for example, other tissues usually displaying high levels of Hsc70 may have undergone reduced transcriptional activity. An important role is seen in examining the tissue and cell specific Hsp expression patterns and comparing these results to adult GH-transgenic specimens, to understand whether the effects observed during early life remain

present in adulthood. It is advocated to study the effects of long-term hypoxia exposure on the cellular stress response throughout development to improve our understanding of the involvement of Hsps 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 animal, under consideration of the effects of polyploidy. It is concluded that GH transgenesis or artificially induced triploidy, increases metabolism and together alters cardiorespiratory function in hypoxia in *S. salar* during early development. It is further concluded that hypoxia does not elicit a cellular stress response but has significant impact on the animal's potential to maintain cellular homeostasis through the reduced expression of Hsps. There is a pivotal role in further investigating respiratory and cellular stress parameters in triploid larvae and comparing these to juvenile and adult life stages to conclusively elucidate physiological effects associated with polyploidy and its interaction with GH transgenesis. The differential Hsp expression could be a potential indicator/marker for hypoxic stress that could find application in the aquaculture industry.

7. General discussion

The development, survival and performance of fish depend on a variety of extrinsic factors and their interactions. The effects of these variables further depend on intrinsic factors such as the genetic “make-up” and maternal effects that shape the individual. In addition, the acute vs. chronic effects of the extrinsic factors might result in different responses at the inter- and intraspecific level.

This thesis has mainly focussed on the acute and chronic physiological responses of early developmental stages of Atlantic salmon to changes in oxygen and temperature, and their interaction with genetic modifications such as growth hormone transgenesis. In this section, the major findings of this study and future research directions are outlined.

The measurement of metabolic rate ($\cong \dot{M}O_2$) and cardiorespiratory variables such as heart rate and ventilation rate in very small animals such as salmon eggs or yolk-sac alevins (alevins) poses a challenge to researchers interested in studying developmental physiology. Despite major technological advances in the field of respirometry over the past decades, inter-study comparisons to obtain a general overview of these variables show that the data obtained varies significantly. This is partly explained by differences in methodological approaches, their accuracy and also biological factors such as the climatic history of an individual or a population. In Appendix 3, the literature data on $\dot{M}O_2$, heart rate and ventilation rate in salmonids at the hatching stage are summarised, and clearly demonstrate how varied the results have been. While experimental conditions vary slightly, values for $\dot{M}O_2$ differ in orders of magnitude when converted to similar units. The

literature data highlights the importance of accurate and careful measurements even of variables that appear to be easily obtained and are widely measured in the field of animal physiology. It also highlights the importance of accurately verifying the data obtained in every study or species investigated. It was found that the data in the present study falls well within the range of the data presented in the literature for all variables (Appendix 3).

In this study, closed system respirometry to measure $\dot{M}O_2$ in Atlantic salmon embryos and alevins was used. Closed system respirometry is based upon the measurement of the oxygen consumption (an indirect measure of $\dot{M}O_2$) in a sealed system. However, oxygen exchange across the walls of respirometry chambers can lead to a significant error in estimating the rate of oxygen consumption that requires accurate validation of the data obtained. In Chapter 2, the need for correction in the most accurate use of conventional polystyrene multiwell plates with integrated optodes for the use in aquatic respirometry is demonstrated. In doing so it was shown how to account for the gas exchange across the wells, a generalised formula for this purpose was provided how to calibrate small respirometry chambers using micro-osmotic pumps in combination with oxygen scavengers was demonstrated. The adoption of this method allows the measurement of $\dot{M}O_2$ in large sample sizes of small aquatic animals and will likely popularise and improve the accuracy of data obtained using this measurement system in future research.

Applying this method in the following chapters, the hypoxic metabolic response of *S. salar* embryos and yolk-sac alevins was characterised. Generally, the acute

hypoxic response is hypometabolism at both developmental stages. The present data indicate that the metabolic depression is likely to be related to oxygen transport limitation, rather than an active down-regulation. In embryos, this is supported by the fact that metabolic rate and perivitelline PO_2 ($PO_{2(in)}$) both decrease in an exponential fashion, suggesting that O_2 uptake is dependent on the physical presence of the egg capsule. Additionally, in both embryos and alevins small changes in environmental oxygen cause an almost instant decrease in $\dot{M}O_2$. Therefore, the data here supports previous suggestions that at these early life stages, aquatic vertebrates are oxygen conformers rather than regulators (Pelster 1999), and $\dot{M}O_2$ in a diffusive system is governed by the supply, not the demand for oxygen.

Oxygen regulation implies the ability to maintain $\dot{M}O_2$ to some extent in hypoxia, even in hypoxia intolerant species such as salmonids, whereas oxygen conformation implies a reduction in $\dot{M}O_2$ accompanying the reduction in PO_2 . In the case of Atlantic salmon embryos and alevins, the concept of P_{crit} as an indicator for hypoxia tolerance is probably not the ideal. It is suggested that other indicators for hypoxia tolerance during embryonic and larval development might be required that will aid in defining indicators for hypoxic stress. These could include differences in swimming performance or thermal tolerance or the sensitivity of the cardiac performance to hypoxia (P_{crit} for heart rate) for example, as well as differences in hypoxia inducible gene or protein expression (potentially Hsps).

The $\dot{M}O_2$ curves in alevins that were measured in Chapter 3, 4, 5, 6 using the closed system approach generally show a triphasic pattern, which is more clearly described in Chapter 6 and exemplified in Appendix 3. Here, an initial decline in $\dot{M}O_2$ with decreasing PO_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 again 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 $\dot{M}O_2$.

It is noteworthy that the $\dot{M}O_2$ measured in embryos (Appendix 1), which does not show a triphasic pattern but an exponential decrease in $\dot{M}O_2$ with hypoxia, indicates that the triphasic pattern in alevins contains a biological signal.

It is possible that the initial, steep decrease in $\dot{M}O_2$ however, 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. 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 were established at a minimum, 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 greater ability to maintain

$\dot{M}O_2$, which could be indicative of a switch to a more “O₂ efficient” metabolic pathway, that is triggered at a specific PO₂. During the exposure to a gradually decreasing PO₂, the alevins might have adjusted to hypoxia through other pathways of ATP production that support the maintenance of $\dot{M}O_2$. A measure of activity 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, creatin phosphokinase) might add further insight into changes in metabolic pathways.

Another, simply explanation could be that if section I corresponds to an elevated $\dot{M}O_2$ due to an unsettled state of the animal, section 2 might then correspond to the true settled state. The decrease in $\dot{M}O_2$ in section III might then be analogous to the P_{crit} where cellular changes are made to further reduce metabolic rate as part of “metabolic depression”.

The oxygen transport limitation hypothesis in embryos and alevins in hypoxia is further supported by the observation that independent of previous acclimation conditions (hypoxia or temperature) or measurement temperature, $\dot{M}O_2$ s at severe levels of hypoxia (~5 kPa) are similar. Hence, the Q₁₀-driven increase in oxygen demand observed in normoxia, can no longer be sustained at these severe levels. In addition, the finding that relative to $\dot{M}O_2$ in normoxia, the extent of metabolic depression independent of acclimation or measurement conditions is similar (~60% when comparing $\dot{M}O_2$ at 21 kPa with 5 kPa), tends to suggest a physical limitation to the ability to obtain oxygen from the environment rather than a regulative process.

The findings on the cardiorespiratory response to acute hypoxia confirm previous suggestions on vertebrate embryos and larvae (Barrionuevo and Burggren 1999; Mortola et al. 2009; Burggren 2004; Pelster 1999). Here, heart rate and ventilation rate are largely insensitive to hypoxia, hence, uncoupled from $\dot{M}O_2$ and most likely not involved in oxygen convection at this developmental stage.

The heart beat is more likely to serve other purposes such as angiogenesis (Burggren 2013) and the gills appear to be required for ionic regulation when cutaneous oxygen uptake occurs by diffusion. There is great potential in studying the purpose of the embryonic heart beat and ventilation as well as hemoglobin concentration or O_2 binding properties during vertebrate ontogeny, as this might uncover other important functions of these organs and tissues.

In Chapter 4, it was shown that chronic hypoxia results in physiological modifications leading to elevated $\dot{M}O_2$ s after re-exposure to normoxia and advances to the onset of the cardiorespiratory hypoxic response in alevins. The physiological adaptations underlying these changes are not known but are likely to involve alterations to respiratory structures (e.g. gills, cutaneous thickness) or changes in oxygen extraction through changes in cardiac output via cardiac stroke volume or for example hemoglobin concentration. Recent work on the embryonic hemoglobin isoform (Hb_E , Bianchini 2012) however, indicates an increased Hb_E concentration with higher $Hb-O_2$ affinity in chronic hypoxia raised rainbow trout embryos. This could then partly explain the increases in oxygen uptake observed here. More work on Hb_E characteristics and other potential mechanisms will be required to resolve the chronic effects of hypoxia. The increased sensitivity of

heart rate and ventilation rate to severe hypoxia after hypoxic acclimation are either due to changes to the onset of cardiorespiratory control mechanisms or simply a result of the lack of oxygen to supply cardiac metabolism.

Previous work suggests a reduction in \dot{M}_{O_2} in chronic hypoxia, as a developmental delay caused by hypoxia would reduce the demand for oxygen (Miller et al. 2008). This might be the case, as a developmental delay in the hypoxia acclimated alevins demonstrated by slower yolk absorption was observed in this study. The re-exposure to normoxic (relative hyperoxic) conditions however, suggests that the relative elevated oxygen supply allows increased oxygen uptake.

Further detail surrounding the relationship between oxygen uptake and its supply was obtained when looking at the response of \dot{M}_{O_2} to acute and chronic hyperoxic conditions in Chapter 5. While chronic hyperoxia did not alter \dot{M}_{O_2} or cardiorespiratory function in hyperoxia or hypoxia, the acute hyperoxic response was hypermetabolism, indicating that alevins in normoxia are not oxygen transport limited and that the oxygen demand matches the oxygen supply.

A hypoxia acclimated alevin in normoxia therefore experiences a higher than usual oxygen supply and can elevate its demand. This was well demonstrated when comparing the \dot{M}_{O_2} between acclimation groups at their respective acclimation conditions (Fig. 4.3). Here the \dot{M}_{O_2} was set to the same intrinsic level irrespective of acclimation PO_2 . This concept of “metabolic compensation” and

the effects of acute and chronic effects of changes in oxygen on metabolic rate is summarised in Fig. 7.1.

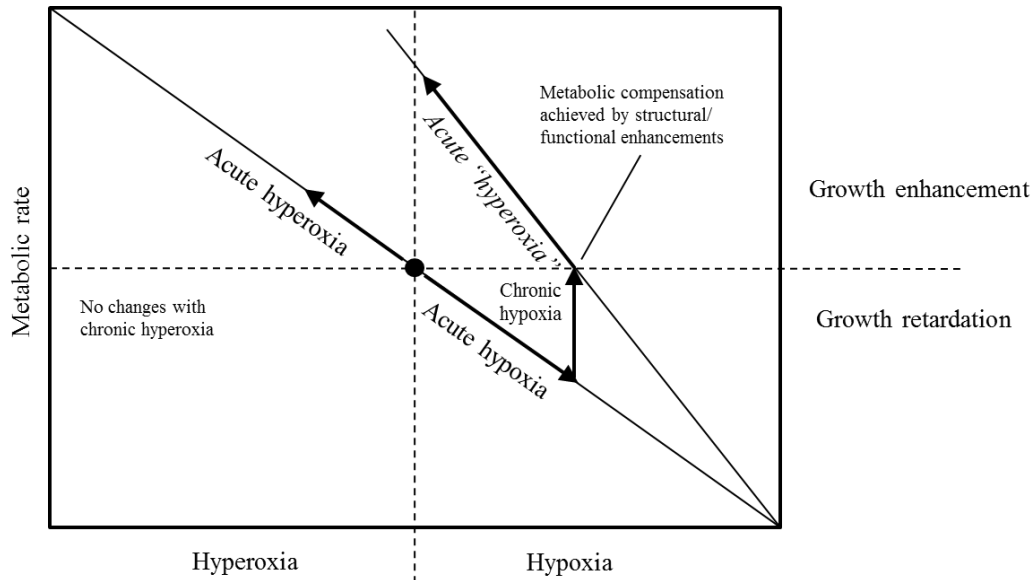


Figure 7.1. Conceptual diagram of the effects of acute and chronic changes in environmental O_2 levels on metabolic rate in Atlantic salmon yolk-sac alevins relative to normoxia (black circle). Acute hypoxia causes hypometabolism and acute hyperoxia causes hypermetabolism, indicating dependency of metabolic rate on O_2 availability. Chronic hypoxia acclimated alevins show signs of growth retardation. However, they elevate metabolic rate when re-exposed to normoxia. This is indicative of metabolic compensation where the metabolic rate of hypoxia acclimated alevins at their acclimation PO_2 is similar to normoxia acclimated alevins in normoxia. In this case, a PO_2 above their acclimation PO_2 is relative “hyperoxia”. Chronic hyperoxia causes growth enhancement but has no effect on metabolic rate.

In contrast, chronic hyperoxia did not result in changes to intrinsic levels of $\dot{M}O_2$. However, despite acute hyperoxia increasing $\dot{M}O_2$ and development being accelerated through greater oxygen availability, this increase could be of a transient nature and alevins might adapt to the increased oxygen supply without altering $\dot{M}O_2$. Also, hyperoxia might not trigger genetic pathways that result in chronic changes to oxygen uptake through functional or structural modifications.

On another note, the metabolic compensation observed in hypoxia acclimated alevins was seen at different measurement temperatures (Fig. 7.2). Comparing \dot{M}_{O_2} at different measurement temperatures, it was found that the set-point for metabolic rate in a hypoxia acclimated alevin at 4°C for example, is the same at its acclimation PO_2 as in a normoxia reared alevin at 8°C in normoxia. Theoretically they should display the same \dot{M}_{O_2} and therefore growth rate unless the elevated \dot{M}_{O_2} is not chronically maintained after returning to normoxic conditions. This would have profound implications on the relationship between growth, hypoxia and temperature. A study examining growth and metabolic rate at different temperatures in hypoxia reared alevins is required to understand the mechanisms underlying these compensatory mechanisms and adaptations. In reference to rearing practices of fish, there is value in analysing the cost/ value relationship of oxygenation vs. incubation temperature on growth.

Furthermore, the metabolic compensation observed, indicates that the physiological changes during hypoxic acclimation, allowing an increased \dot{M}_{O_2} , might cause the developmental delay and smaller body mass. Hence, structural improvements for increased O_2 uptake change the developmental trajectory and delay normal development.

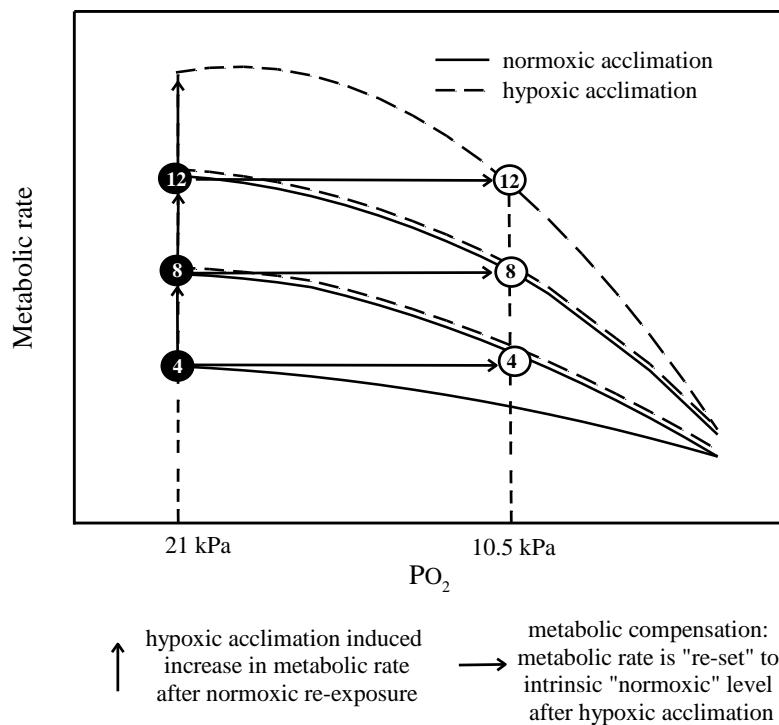


Figure 7.2. Simplified diagram of the concept of metabolic compensation at different measurement temperatures in Atlantic salmon yolk-sac alevins. Irrespective of the measurement temperature (4, 8, 12°C, black circles with white font), metabolic rate in normoxia, after 15 days of chronic hypoxia (10.5 kPa, dashed lines) is increased by a set amount (upwards arrows) compared with normoxia reared alevins (solid lines). This is likely to be achieved by means of structural or functional adaptations. At each temperature, the metabolic rate at the level of O_2 the groups (normoxia vs. hypoxia) were acclimated to, was similar (sideways arrows, white circles with black font). This metabolic compensation further suggests, that alevins raised at 4°C in hypoxia, will display the same metabolic rate as an animal raised at 8°C in normoxia and should hypothetically have a similar growth rate.

Further on this topic, the developmental plasticity seen after hypoxic acclimation allows speculations about developmental trajectories and critical windows during development. If for example, hypoxia delays development and alters mechanisms of oxygen uptake as well as cardiorespiratory sensitivity, it is of great importance in terms of survival if these animals have the ability to compensate for the developmental perturbations experienced. The concept of developmental trajectories (Burggren and Reyna 2011) indicates that some animals reared in adverse environmental conditions that delay development for example, have the ability of “self-repair” and catch up on development with normally reared

conspecifics when returning to normal conditions. Understanding the temporal and qualitative patterns of developmental plasticity is pivotal information for conservation purposes (for example to assess environmental impacts in fish spawning grounds) as well as aquaculture practices (to improve rearing conditions).

Despite finding indications for differences in relation to incubation temperature in Chapter 4, these results are difficult to interpret. There was a general trend towards an increase in metabolic rate, heart rate as well as ventilation rate with an increase in incubation temperature from 4°C to 8°C. However, the animals from these two acclimation groups were not at similar developmental stages and therefore causalities can not clearly be defined. While an increase in incubation temperature might lead to elevated heart rates for example, there is evidence that heart rate also increases with development. Hence, no obvious conclusions can be drawn.

Nevertheless, differences in the pattern of the response relating to incubation temperature were seen in alevins incubated at 4°C in the flow-through system. Alevins here showed a greater increase in metabolic rate after normoxic re-exposure with concomitant increases in ventilation rate than were observed at 8°C incubation.

A possible explanation could be that alevins incubated at 4°C are more developed (despite younger age in terms of degree days) and display a more advanced response to hypoxia by increasing O₂ delivery through ventilation when metabolic rate is elevated above normal conditions. It is unlikely that

hyperventilation is a sign of delayed development as the hyperventilatory response to hypoxia is a characteristic of later life stages in salmonids (Perry et al. 2009). The lower acclimation temperature might have allowed a more advanced development through a more efficient use of energy stores. In this particular case, oxygen uptake across the gills could be more advanced and higher ventilation rates enable higher O₂ uptake. Whether 4°C is closer to the thermal optimum for development than 8°C at this stage needs to be further assessed.

So far, the environmental effects that may influence physiological and developmental aspects of fish have been discussed. The following section focusses on intrinsic aspects and their interactions with environmental stressors.

In Chapter 3, a study on *S. salar* embryos, evidence that maternal effects such as the differences in egg size that are due to maternal body size, influence $\dot{M}O_2$ and hypoxia induced hatching was found. This could be the result of an advantageous egg surface area relative to the metabolic demand in the embryos from larger eggs from repeat spawning females. This is indicative of an increased hypoxia tolerance in embryos from larger eggs from repeat spawners and likely to have an impact on their hatching success, and thus the survival and fitness of the species. These findings add to the currently increasing information available contradicting the “*bigger is worse during incubation*” hypothesis.

Increases in growth rates through growth hormone transgenesis that have great impact on developmental rates in fish and its interaction with polyploidy was investigated in Chapter 6. It was found that either growth hormone transgenesis or triploidy alone increase $\dot{M}O_2$ and together are additive effects. Differences in

cardiorespiratory function between diploids and triploid transgenics were observed when exposed to acute, severe hypoxia. Finally, differential expression of cellular stress proteins (Hsps) after acute, progressive hypoxia was shown, that in one case (Hsc70) was dependent on whether or not fish were transgenics. These novel findings are critical in understanding the effects of genetic modifications in animals. However, the causes leading to these changes need to be more intensively studied.

It is well documented in adult salmon, that the chronic expression of growth hormone (GH) increases $\dot{M}O_2$, and so the finding in the present study at this early developmental stage can be interpreted. The increase in $\dot{M}O_2$ with triploidy in contrast has found little evidence in the past. The increase in $\dot{M}O_2$ observed during early development here, is likely to be related to changes in cell size or the diversion of energy sources towards growth. Improved growth is not well documented in triploids and triploids are generally regarded as more susceptible to environmental stress. It appears that despite the additional genetic material and an elevated $\dot{M}O_2$, these effects do not translate into higher growth rates. If cell size is affected by triploidy through the additional genetic material (50%) and overall body size is maintained through hypoplasia (e.g. 50% less cells), oxygen uptake into the cell (being a diffusive process) might be effected that changes overall $\dot{M}O_2$ without altering growth or body size compared with diploids. Indeed, triploid Atlantic salmon display significantly less myocytes in juveniles (32%, Greenlee et al. 1995) and reduced numbers of erythrocytes have been reported for Coho salmon (32%, Small and Benfey 1987). Clearly, the effects of triploidy on respiratory function need to be more intensively studied in the future.

The acute hypoxic stress the alevins were exposed to in this study did not elicit a typical cellular stress response (i.e. increase in Hsp expression) but led to a decreased Hsp expression instead. In the case of Hsc70 the expression was dependent on the genotype. The decreased expression limited to GH-transgenics could indicate that they are more susceptible to hypoxia. Hsp90 expression generally decreased in hypoxia, independent of the genotype, which could be indicative for a general reduction in protein synthesis. A decrease in Hsp or overall protein expression could potentially serve as an indicator for hypoxic stress.

In conclusion, the acute and chronic physiological responses to environmental challenges during development in fish are different, and this needs to be taken into consideration when making assumptions on climatic effects on survival and fitness of a species, and on hatchery practices to improve production efficiency.

In terms of hatchery practices in particular, there is great potential to more clearly define the effects of environmental challenges on developmental trajectories. Oxygen and temperature are the critical and easily controllable variables in hatcheries and therefore, challenging developing fish during critical windows of development might lead to improvements in e.g. growth or in robustness (e.g. hypoxia tolerance) of certain strains. Incorporating studies of hypoxia tolerant strains into existing breeding programs could have a substantial impact on their effectiveness and sustainability in the long term. Equally understanding the physiological effects of environmental changes will assist in preventing adverse conditions of the fish and in all likelihood improve the health of the population.

The understanding of maternal effects, genetic manipulation and acclimation also needs to be improved in relation to the selection of hypoxia tolerant strains in aquaculture to reduce mortalities during events of environmental hypoxia.. Finally, this study demonstrated that changes to the oxygen environment has substantial effects on Atlantic salmon during early development and that these effects will likely affect the aerobic performance/ scope later in life and ultimately the survival of individuals.

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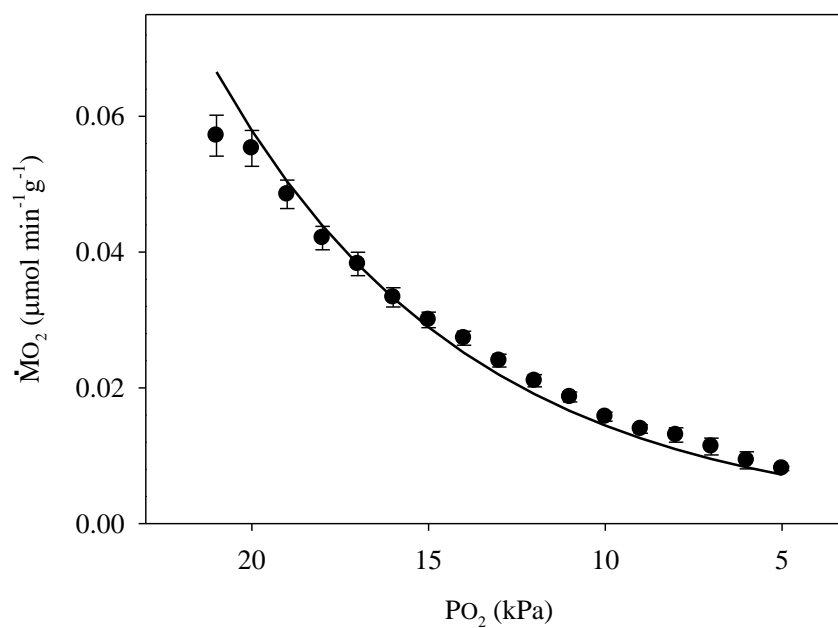
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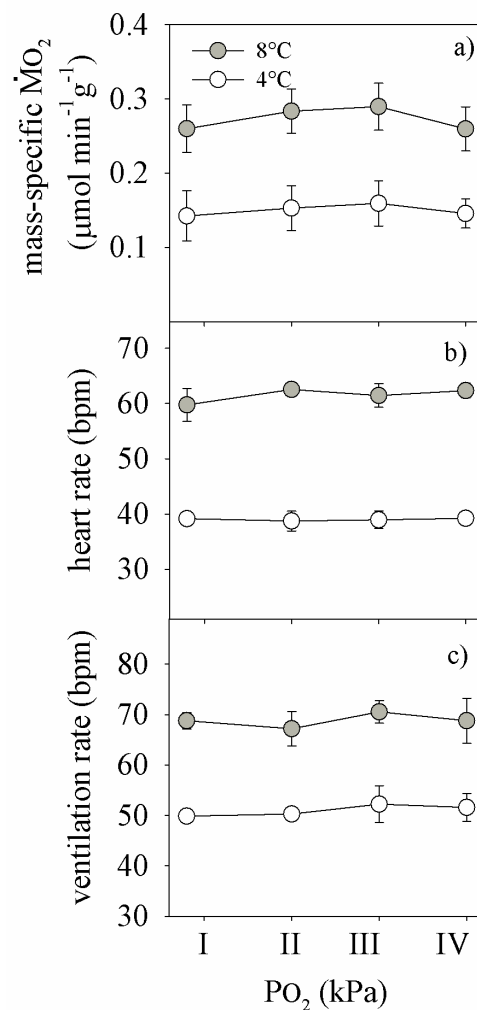
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Appendices

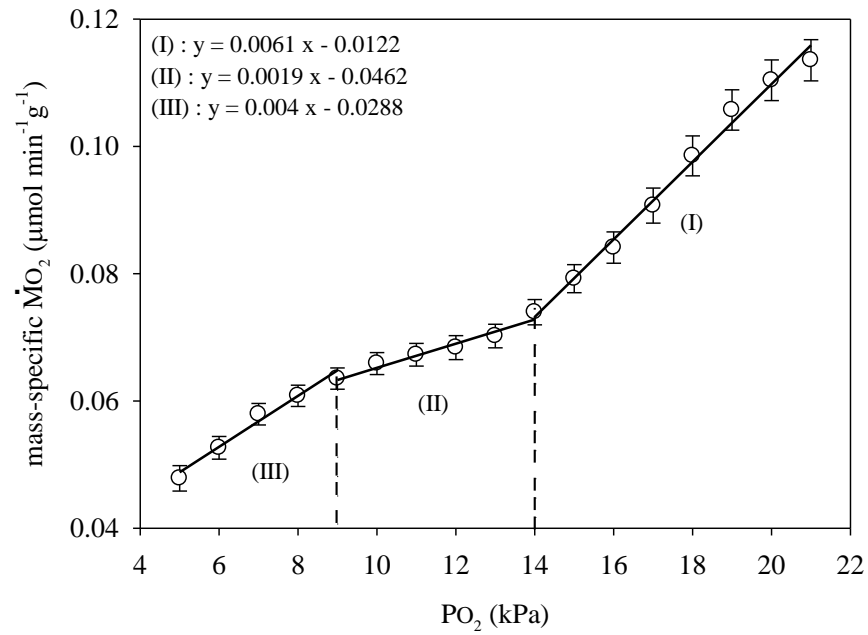
Appendix 1. Changes in mass-specific $\dot{M}O_2$ with decreasing levels of PO_2 in mature Atlantic salmon eggs. $\dot{M}O_2$ decreased with decreasing PO_2 (between 21 kPa and 5 kPa) in an exponential fashion ($y = 0.0036e^{0.1389x}$, $r^2 = 0.95$).



Appendix 2. Control experiments for simultaneous measurement of $\dot{M}O_2$, f_H and f_V (see chapter 2.4. for methodological approach and chapter 4.2 for experimental animals). Here, animals were not subjected to a stepwise hypoxic challenge but the water was kept at $PO_2 = 21$ kPa for the entire experimental period (numbers I to IV indicate time intervals that correspond to measurement periods of experiments where animals were exposed to stepwise hypoxia $21 \rightarrow 15 \rightarrow 10 \rightarrow 5$ kPa). Instead, the procedure of sealing and flushing of the chamber was repeated four times while f_H and f_V was measured after the same principle as described previously. Neither $\dot{M}O_2$, f_H and f_V altered during these experiments, indicating that the experimental duration had no effect on the variables measured ($P > 0.05$).



Appendix 3. Changes in mean \pm S.E. mass-specific $\dot{M}O_2$ in response to a progressive decline in PO_2 ($21 \rightarrow 5$ kPa) at 8°C of *S. salar* alevins incubated to 8°C in normoxia. Least squares linear regressions were fitted to triphasic response (I, II, III, separated by dashed lines) as determined in Chapter 6 (Fig. 6.1). Linear regression analysis revealed that the slopes of the linear regressions are all significantly different from each other ($P < 0.05$).



Appendix 4. Literature data on metabolic rate ($\dot{M}O_2$), heart rate (f_H), ventilation rate (f_V) in salmonid eggs and yolk-sac alevins at different measurement temperatures.

species	developmental stage [^]	incubation temperature (°C)	measurement temperature (°C)	$\dot{M}O_2$ ($\mu\text{mol min}^{-1}$)	$\dot{M}O_2$ ($\mu\text{mol min}^{-1} \text{g}^{-1}$)*	Q_{10} .adjusted $\dot{M}O_2$ ($\mu\text{mol min}^{-1} \text{g}^{-1}$)	f_H (bpm)	f_V (bpm)	study
<i>S. salar</i>	eggs	8	8	0.005*	0.057	-	-	-	present study
<i>S. salar</i>	alevins	8	8	0.006*	0.114	-	77	87	present study
				0.008 ⁺	0.152				
<i>S. salar</i>	alevins	4-10	10	0.0024	0.273	0.222	-	-	Wells and Pinder 1996b
<i>S. salar</i>	eggs	3	3	0.000013	0.00022	-	78	-	Klinkhardt et al. 1987
		8	8	0.000016	0.00027	-	93	125	
<i>S. salar</i>	eggs	5	5	0.0024	0.040	-	-	-	Hamor and Garside 1978 [#]
		10	10	0.0048	0.080	0.065	-	-	
<i>S. salar</i>	eggs	10	10	-	0.00089	0.00072	-	-	Hayes et al. 1951
<i>S. salar</i>	eggs	-	4.4	0.0000001	0.0018	0.0026	-	-	Einum et al. 2002
<i>S. salar</i>	alevins	10	8	-	-	-	20	-	Fisher 1942
<i>O. mykiss</i>	alevins	10	10	-	-	-	65	-	Miller et al. 2011
<i>O. mykiss</i>	eggs	10	10	0.0032	0.052	-	-	-	Miller et al. 2008
<i>O. mykiss</i>	eggs	10	10	0.018	0.306	0.250	-	-	Ninness et al. 2006a,b
	alevins	10	10	0.024	0.399	0.325	-	-	
<i>O. mykiss</i>	alevins	10	10	-	-	-	65	42	Holeton 1971
<i>O. mykiss</i>	alevins	-	-	-	0.243	-	-	-	Hamdorf 1961
			4	-	-	-	33	-	
<i>O. mykiss</i>	alevins	2-3	8	-	-	-	47	-	Mirkovic and Rombough 1998 [°]
			12	-	-	-	66	-	
<i>O. tshawytscha</i>	alevins	~10	12	-	0.100	0.066	-	-	Rombough and Ure 1991
			6	-	-	-	46	-	
<i>S. fontinalis</i>	alevins	6	9	-	-	-	56	-	Benfey and Bennett 2009
			12	-	-	-	66	-	

S. salar = *Salmo salar*; *O. Mykiss* = *Oncorhynchus mykiss*; *S. fontinalis* = *Salvelinus fontinalis*; *S. gairdneri* = *Salmo gairdneri*.

[^] if not otherwise stated, data for developmental stage was chosen as eggs shortly before hatching (eggs) or newly hatched alevins (alevins); *where accurate values were not explicitly provided by the author, it was estimated using the UN-SCAN-IT gel 6.1 software from the original graphs and converted to matching units; in order to calculate mass specific $\dot{M}O_2$ in cases where values for body mass were not provided by the author, mass as determined in the present study where used according to the developmental stage to allow comparison between studies; [°]Estimated using the formula ($\log f_H = 1.84 + 0.259 \log M + 0.03817 T$) according to Mirkovic and Rombough (1971); Q_{10} adjustments were performed on the basis of a Q_{10} of 2.8, the average of what was determined in chapter 4 at incubation temperatures of 4°C and 8°C. f_H = heart rate; M = yolk-free mass; T = temperature; [#] Hamor and Garside is the only study reviewed here that used open-flow respirometry to determine $\dot{M}O_2$. All other studies used closed system respirometry; ^{*}Values determined using the SensorDishReader system (see chapter 2.2) or ⁺flow through respirometer (see chapter 2.4).

Appendix 5. Microscopic photograph of newly hatched *Salmo salar* yolk-sac alevin.

