The effects of oxygen variability during early development on the physiology of Atlantic salmon (*Salmo salar*)

by

Andrew T. Wood

Bachelor of Science (Hons)

Institute for Marine and Antarctic Studies

in collaboration with

Commonwealth Scientific and Industrial Research Organisation

Submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

University of Tasmania

August, 2017

Statements and Declarations

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Statement of Publication

Original research papers in peer-reviewed journals

Wood, A. T., Clark, T. D., Andrewartha, S. J., Elliott, N. G. and Frappell, P. B**.** (2017). Developmental hypoxia has negligible effects on long-term hypoxia tolerance and aerobic metabolism of Atlantic salmon (*Salmo salar*). *Physiol. Biochem. Zool.* **90**, 494-501.

Oral presentations

Wood, A.T., Clark, T.D., Andrewartha, S.J., Elliott, N.G. & Frappell, P.B. (2016). Are there long term effects of developmental hypoxia on the metabolic physiology of Atlantic salmon? Presentation at the Society of Experimental Biology Annual Meeting, Brighton, UK.

Wood, A., Andrewartha, S., Morash, A., Elliott, N., Frappell, P. (2014, December). The effect of oxygen concentration on the developmental physiology of Atlantic salmon (*Salmo salar*). Presentation at the Australian and New Zealand Society for Comparative Physiology and Biochemistry Meeting, Armidale, NSW.

Poster presentations

Wood, A., Clark, T.D., Andrewartha, S.J., Elliott, N.G. & Frappell, P.B. (2016). Does developmental hypoxia have enduring effects on the metabolism and morphology of Atlantic salmon (*Salmo salar*)? Poster presented at the Species on the Move Conference, Hobart, TAS.

Wood, A. (2014). The effect of oxygen concentration on the developmental physiology of Atlantic salmon, *Salmo salar*. Poster presented at The University of Tasmania Graduate Research Conference, Hobart, TAS.

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The following people and institutions contributed to the publication of work undertaken as part of this thesis:

Candidate: Andrew T. Wood, University of Tasmania and CSIRO, Hobart, Australia

Author 1: Timothy D. Clark, University of Tasmania and CSIRO, Hobart, Australia, Primary supervisor

Author 2: Sarah. J. Andrewartha, CSIRO, Hobart, Australia, Supervisor

Author 3: Nicholas G. Elliott, CSIRO, Hobart, Australia, Supervisor

Author 4: Peter B. Frappell, University of Tasmania, Hobart, Australia, Supervisor

Author details and their roles:

Paper 1 (Located in Chapter 4): Developmental hypoxia has negligible effects on long-term hypoxia tolerance and aerobic metabolism of Atlantic salmon (*Salmo salar*)

Candidate developed the research idea, conducted the experiment, collected and analysed the data and wrote the manuscript (80% proportion). Authors 1,2,3 and 4 assisted developing the research idea, analysing and interpreting the data, and revising the manuscript.

Signed: _____

Andrew T. Wood, Candidate

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Supervisor and Head of School Declaration

We the undersigned agree with the above stated "proportion of work undertaken" for each of the above published (or submitted) peer-reviewed manuscripts contributing to this thesis:

Signed:		
	Dr. Timothy D. Clark	**Prof. Chris G. Carter (PhD)**
	Supervisor	**Head of School**
	IMAS	**IMAS (Fisheries and Aquaculture Centre)**
	University of Tasmania	**University of Tasmania**
Date:	22 Aug 2017	22.08.2017

Acknowledgements

I am extremely grateful for the financial project and scholarship support of SALTAS. I also thank the University of Tasmania, the Institute for Marine and Antarctic Studies, the Society for Experimental Biology and ANZSCBP for conference travel support. I was also supported by an Australian Research Training Program scholarship.

This thesis would not have been possible without assistance from the SALTAS hatchery staff at Wayatinah, Tasmania. I am thankful for the help and guidance of Harley, Kristen, Brendan, Damien, Atefeh, Marnie and Aaron (sorry to anyone I have missed!). Special thanks to Brad Evans, Mark Hilder and Jody Wilson for supplying fish, sharing resources, and for advice about the practicalities of working at a salmon hatchery!

To my colleagues at CSIRO: Kathi Alter, Liss Bowden, Ben Maynard, Peter Kube, John McCulloch, Dion Frampton, Dave Cordell, Curtis Lind, Mat Cook, thank you for technical support, discussions, coffee and the interest you have displayed in my research. Special thanks to Harry King for assistance and advice about all things salmon, and for smoothing out CSIRO's bureaucracy. Richard Taylor, thank you for your assistance, advice and enthusiasm. Thanks to Matt Hamilton for help analysing data using R, and Tina Stephens for helping to navigate the procedures at UTAS.

To my supervisors Tim Clark, Sarah Andrewartha, Nick Elliott, Peter Frappell and Andrea Morash. Thank you for your guidance, support, constructive feedback and useful discussions, which have helped to shape me as an independent researcher. Nick, thank you for supporting the move to Tassie from the outset and making it become a reality.

To friends near and far, old and new, thank you for your support, for visiting me in Tassie, or sharing adventures with me in my new home state.

To my family, thank you for your love, and supporting me in pursuing this opportunity.

Georgie, thank you for your unconditional love, support, patience and understanding. You're a good one.

General abstract

Variable oxygen availability can challenge the performance and survival of anadromous salmon species from early development in freshwater redds to adult life in the open ocean. Salmon are also a highly valued aquaculture species, and may face similar hypoxic or hyperoxic challenges in hatcheries and sea cage rearing. This thesis investigates the physiological responses of Atlantic salmon (*Salmo salar*) early life stages to oxygen variability (hypoxia and hyperoxia) that is typically experienced in natural and aquaculture systems. In particular, the thesis focuses on how oxygen variability during incubation affects developmental trajectories that may cause long-term impacts.

Salmon incubating in natural under-gravel redds and aquaculture incubation systems can experience oxygen levels from < 20% to 180% dissolved oxygen (DO; % air saturation). While hypoxia is known to compromise the growth and development of salmon and stimulate physiological processes to improve oxygen uptake rate ($\dot{M}O_2$) or reduce metabolism, it is unclear whether hyperoxia alleviates respiratory stress and leads to improved performance. Across two experiments, we investigated how various hypoxia and hyperoxia levels affected growth, aerobic metabolism and hypoxia tolerance of salmon from fertilisation until yolk-sac absorption. Rearing in hyperoxia had no effect on $\dot{M}O_2$ or O_{2crit}, and a negligible effect on growth. On the other hand, salmon incubated in moderate (50% DO) or cyclical hypoxia (100-25% DO daily) grew and developed slower associated with reduced $\dot{M}O_2$ and critical oxygen level (O_{2crit}) prior to the eyed-egg stage. Severe hypoxia (~27% DO) caused near-complete mortality and deformities. Thus, during development, salmon appear most sensitive to hypoxia prior to hatching and respond to oxygen limitation by reducing their oxygen demand. There was no evidence to suggest that embryos or alevins compensated for hypoxia via mechanisms to increase oxygen supply.

Hypoxia exposure during incubation can permanently affect physiology by altering the developmental trajectory, thereby impacting later life performance. Hypoxia imposes limitations on the aerobic metabolic scope that may be compensated for by physiological modifications that increase the maximum attainable $\dot{M}O_2$. We tested how moderate or cyclical hypoxia from fertilisation until the fry stage affected subsequent aerobic performance, hypoxia tolerance and haematology of juveniles reared in normoxia. In

addition, aerobic performance was measured following a re-acclimation period of up to 44 days in hypoxia. Hypoxia exposure during incubation had no effect on aerobic performance, hypoxia tolerance or haematology, even following re-acclimation to hypoxia. Overall, acute hypoxia (<13 h) reduced aerobic scope, however acclimation to hypoxia (up to 44 days) increased blood-oxygen carrying capacity and reduced the limitation that acute hypoxia had on aerobic scope.

The results of this thesis demonstrate that the effects of oxygen limitation on salmon during hypoxia incubation are most severe between fertilisation and hatching. However, hypoxia incubation did not appear to impact the developmental trajectory of salmon, as there was negligible impact on later life aerobic performance and hypoxia acclimation capacity. I conclude that there may be negligible evolutionary advantages to anadromous salmon modifying their long-term physiological phenotype based on the oxygen levels encountered during incubation in natural redds. However, the considerable hypoxia acclimation capacity of Atlantic salmon can alleviate the limitation that hypoxia has on aerobic performance later in life.

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Abbreviations

ATP	Adenosine triphosphate
CSIRO	Commonwealth Scientific and Industrial Research Organisation
DD	Degree days
DO	Dissolved oxygen (% air saturation)
DO_{def}	Cumulative DO deficiency prior to LOE
DPF	Days post-fertilisation
[Hb]	Haemoglobin concentration
LOE	Loss of equilibrium
$\dot{M}O_2$	Oxygen uptake rate
O_{2crit}	Critical oxygen level
SALTAS	Salmon Enterprises of Tasmania Pty Ltd
SD	Standard deviation
SEM	Standard error of the mean
τ_s	Developmental stage
U_{crit}	Maximum sustainable swimming speed

Chapter 1: General introduction

The life cycle of salmonids

Natural life history

Salmonids are a family of fishes that naturally inhabit the temperate waters of the northern hemisphere. In general, salmonids are classified as anadromous species, breeding in freshwater streams and lakes followed by a period of growth and maturation in freshwater, coastal or oceanic waters. All salmonids are spawned in cool freshwater streams or lakes where they undergo 2-6 months of incubation depending on temperature (Pennell and McLean, 1996). The duration spent within each habitat varies between species and populations of salmon. For example, the freshwater growth period varies from 1 to 8 years in Atlantic salmon (*Salmo salar*) and the time spent in marine waters for maturation can vary from months to 7 years among all salmonids (Gibson, 1993; Laird, 1996). However, most salmonid species have the capability to complete their lifecycle wholly within freshwater (Gibson, 1993; Webb et al., 2007). Indeed, Atlantic salmon, brown trout (*Salmo trutta*), rainbow trout (*Oncorhynchus mykiss*) and sockeye salmon (*Oncorhynchus nerka*) have naturally occurring landlocked strains that never reach marine waters (Laird, 1996). Salmonids may be exposed to a wide range of environmental conditions throughout their life and across the variety of habitats that they occupy. Consequently, salmon can use a variety of physiological strategies to cope with environmental variation associated with factors like temperature, salinity and oxygen.

Aquaculture

The development of a global salmonid aquaculture industry has been aided by their flexible lifecycle, and sporting and culinary value. Salmonids are the most valuable commercial finfish species globally across all fisheries and aquaculture sectors, with most production coming from aquaculture (FAO, 2016). Salmonid aquaculture has also been successfully established in areas well outside their natural range. For example, Atlantic salmon originally sourced from the east coast of Canada now form part of a salmonid aquaculture industry in Australia which accounts for 76% (by value) of total Australian finfish aquaculture production (Elliott and Kube, 2009; Savage, 2015). Improved rearing technology has resulted in a dramatic expansion of salmonid aquaculture over the past 35 years (Laird, 1996).

Aquaculture rearing typically mimics the anadromous nature of many salmonids. Early salmon life stages are incubated and reared in freshwater hatcheries that are typically supplied by cool freshwater streams or rivers. Eggs and alevins are incubated in trays or troughs, before they are transferred into tanks or ponds prior to external feeding (Billard and Jensen, 1996; Pennell and McLean, 1996). Once the fish reach a stage where they are physiologically capable of seawater transfer (i.e. smoltification) they may be grown in closed-containment systems or in sea cages. Sea cage aquaculture typically takes place in coastal marine ecosystems for 12-18 months until harvest at 2-5 kg (Novotny and Pennell, 1996). However, in some cases salmon are grown to market size or maturation completely in freshwater. Despite efforts to provide favourable conditions for salmon in aquaculture by selecting suitable environments (e.g. in sea cages) or modifying water quality parameters (in the hatchery), salmonids can experience a variety of challenging environments similar to those encountered by wild fish (Appendix A; Burt et al., 2012; Johansson et al., 2007; McLean and Lim, 1985).

Aquatic oxygen variability

In comparison to air, water has a relatively low oxygen capacitance and diffusion coefficient (Dejours, 1981). As such, aquatic environments are susceptible to dramatic variation in oxygen level due to organisms consuming or producing oxygen faster than the oxygen diffusion rate. In fish, oxygen levels lower or higher than air saturation (hypoxia and hyperoxia, respectively) may alter physiological function. In a broad sense, the term hypoxia means a shortage of oxygen at the level that first affects physiological function (Farrell and Richards, 2009). However, the oxygen level that constitutes hypoxia varies between species, individuals and even the physiological process of interest. Therefore, throughout this thesis the terms hypoxia and hyperoxia will generally refer to oxygen levels below or above normoxia, respectively, where normoxia is defined as 100% dissolved oxygen (% air saturation; DO).

Oxygen availability during incubation and early rearing

Factors affecting the oxygen available to incubating salmon

Salmonid eggs range from 3-8 mm in size and are spawned as unattached eggs (i.e. not contained within an egg mass) (Billard and Jensen, 1996). Similar to many other aquatic

species, the quantity of oxygen available to salmonid embryos is largely determined by oxygen diffusion through the aquatic microenvironment surrounding the egg. An oxygen gradient (boundary layer) forms in the water immediately surrounding the egg as well as inside the egg capsule due to oxygen consumption of the embryo (Daykin, 1965). Similar oxygen boundary layers also form around the skin of alevins after hatching (Dhiyebi et al., 2013). Ultimately, the oxygen level in the aquatic environment may not accurately represent the amount of oxygen that reaches the embryo due to interactions between the egg and its physical surroundings. For example, decreased water flow reduces the oxygen that reaches the embryo by increasing the egg boundary layer thickness and the oxygen gradient within the boundary layer (Ciuhandu et al., 2007; Miller et al., 2008).

Crowding of embryos similarly decreases oxygen availability by increasing the oxygen gradient within the boundary layer, presumably due to water flow interruptions and overlapping boundary layers (Dhiyebi et al., 2013). Such disruptions to oxygen levels in the boundary layer can have implications for the early development of salmon embryos. For example, the growth of Chinook salmon (*Oncorhynchus tshawytscha)* was reduced when incubated at low flow rates and high density in Heath trays (McLean and Lim, 1985). Similarly, lower flow reduced the developmental rate and hatching success of Atlantic salmon (Hamor and Garside, 1976). In addition, the oxygen gradient within the boundary layer increases independent of water flow, possibly due to increasing metabolic oxygen demand with embryo growth without an associated increase in oxygen supply due to oxygen diffusion limitations (Dhiyebi et al., 2013; Miller et al., 2008). Overall, the nature of the oxygen gradient within the boundary layer may have a significant effect on oxygen availability to the embryo, especially in natural systems where aquatic conditions such as water flow, embryo density and oxygen availability can suffer vary considerably (Greig et al., 2007).

Oxygen levels in natural salmon redds

The natural incubation environment of salmonids is unique compared with other fishes. Salmon spawn their eggs into excavated depressions (redds) within the stream bed gravel before covering them with gravel at a depth of up to 80 cm (DeVries, 1997). Water oxygen levels within a redd are largely determined by hydrological processes. For example, oxygen levels within artificially constructed redds dropped below 10% DO depending on flow rate,

sediment infiltration and the interaction between groundwater and stream water within the hyporheic zone (Malcolm et al., 2006; Schindler Wildhaber et al., 2014; Sear et al., 2014). Oxygen levels in redds can negatively impact incubating salmon, for example, Atlantic salmon eggs grew slower when incubated in artificial redds at oxygen levels as low as 19% DO (Youngson et al., 2004). Similarly, mean dissolved oxygen lower than 6.7 mg L^{-1} (~53% DO) caused complete mortality in natural redds of sea trout (*Salmo trutta*) (Ingendahl, 2001). The dynamic oxygen conditions within salmon redds may have significant effects on the growth, survival and physiology of salmon pre- and post-hatching, and could potentially influence performance into adulthood.

Oxygen levels in aquaculture incubation systems

Artificial incubation of salmonids for aquaculture takes place in a variety of systems, usually with eggs placed on a mesh screen or open substrate to allow water flow through the egg mass to maintain a sufficient oxygen supply (Billard and Jensen, 1996). Despite attempts by aquaculturists to maintain optimal oxygen conditions in incubation systems, temporal and spatial variability in oxygen levels can occur. In Heath tray incubators, oxygen can decrease as water cascades vertically from one tray to another, especially at advanced developmental stages when oxygen consumption rates of the embryos are high (McLean and Lim, 1985). Egg densities are often high, with up to 500,000 eggs reared in a single incubator, potentially limiting oxygen available to the embryos due to interruptions of water flow and impacts on the boundary layers outlined above (Billard and Jensen, 1996). In light of the limited data on oxygen levels in hatchery incubation systems, we measured oxygen levels in a Heath tray incubation system at an Atlantic salmon hatchery in Tasmania (Appendix A). From the commencement of hatching, alevins experienced bouts of hypoxia to below 20% DO, several times per day which were typically confined to the centre or rear of the Heath tray (Figs. A.2 & A.3, Appendix A). In addition, hyperoxia was measured within the Heath tray system throughout the incubation period, increasing from 120% DO at the commencement of measurements to 190% DO near the completion of incubation. While supplemental oxygenation can routinely lead to such hyperoxic levels in an attempt to maintain optimal oxygen levels within incubation systems, hypoxia bouts still occur (Appendix A, Britton et al., 1983; McLean and Lim, 1985).

Variable oxygen availability as juveniles and adults

Oxygen levels in natural habitats

Following emergence from the redd, salmon may inhabit environments ranging from cool freshwater streams to lakes, rivers, estuaries and open oceans. Oxygen levels within global freshwater and marine systems are declining and hypoxic zones are increasing in prevalence due to anthropogenic influences (Diaz and Rosenberg, 2008; Vaquer-Sunyer and Duarte, 2008). For example, the number of hypoxic freshwater lakes has increased significantly in the past 100 years (Jenny et al., 2016). Owing to their migratory nature, salmonids may pass through, or reside in, hypoxic waters at multiple life stages as they traverse various aquatic environments. The capacity for salmon to efficiently and effectively alleviate the negative impacts of hypoxia via physiological and behavioural responses is likely to impact their long-term survival and fitness.

Oxygen levels in aquaculture rearing systems

In aquaculture, salmon are transferred from incubation systems to tanks or ponds when the yolk-sac absorption is nearly complete and the fish are ready to begin feeding externally as fry (Pennell and McLean, 1996). Supplemental oxygenation is common during tank rearing to increase the carrying capacity of the rearing systems and prevent hypoxia (Pennell and McLean, 1996). Upon transfer to sea cages, typically within in-shore or near-shore marine ecosystems, salmon can be exposed to highly variable environments in which oxygen levels and other environmental variables like temperature and salinity can fluctuate widely (Novotny and Pennell, 1996). The oxygen concentration within sea cages varies spatially and temporally, and is influenced by temperature, water flow, fish density and water stratification (Burt et al., 2012; Johansson et al., 2007). For example, oxygen levels within and adjacent to sea cages containing Atlantic salmon within Macquarie Harbour, Tasmania, can drop below 20% DO and display high spatial and temporal variability (Dempster et al., 2016; Stehfest et al., 2017). However, when the opportunity exists, salmon are known to behaviourally regulate the DO to which they are exposed, with one study showing Atlantic salmon actively avoided areas where oxygen levels were below 35% DO within a sea cage (Stehfest et al., 2017). The dynamic oxygen environments within sea cages are likely to favour salmon with higher capacity for compensatory mechanisms to withstand or avoid hypoxia. Fish that can better cope with hypoxic conditions are likely to have higher growth and survival, attributes that are clearly favourable to maximise aquaculture production efficiency.

Effect of oxygen availability on fish physiology

Oxygen uptake and metabolism

Oxygen uptake

The total metabolic energy demands of fish are the sum of all energy requiring processes, such as swimming, digestion and maturation. Metabolic energy production (adenosine triphosphate) occurring within mitochondria via aerobic pathways (oxidative phosphorylation) is much more efficient than energy production in the absence of oxygen (anaerobic glycolysis). However, aerobic energy production can be limited by the amount of oxygen that can be transported to the tissues. Oxygen uptake relies on the cascade of oxygen from the environment to the mitochondria via the cardio-respiratory system (Fig. 1.1) (Dejours, 1981; Malte, 2011). Oxygen diffuses into the blood from water ventilated across the gills and binds to haemoglobin within the red blood cells. Blood is circulated by the heart throughout the tissues via the cardiovascular system, where oxygen offloads from the haemoglobin and diffuses through the interstitial fluid and tissue cells to the mitochondria.

During early development salmonids rely heavily on cutaneous diffusion for oxygen uptake, however, from approximately two weeks after hatching oxygen uptake is predominately via branchial diffusion and the cardio-respiratory system (Rombough and Ure, 1991; Wells and Pinder, 1996). Modifications to the cardio-respiratory system in response to hypoxia may increase the maximal oxygen uptake rate. A common response to acute hypoxia or exhaustive exercise in fish is the release of red blood cells from the spleen, which increases the total blood-oxygen carrying capacity (Lai et al., 2006; Pearson and Stevens, 1991; Wells and Weber, 1990). Acute hypoxia also reduces heart rate, increases cardiac stroke volume and increases gill ventilation rate, while prolonged exposure increases erythropoietin-induced red blood cell production (Gamperl and Driedzic, 2009; Lai et al., 2006; Perry et al., 2009; Wells, 2009). Ultimately, the ability to withstand prolonged periods of hypoxia depends on the capacity to match metabolic demands with oxygen supply from the environment, therefore minimising any reliance on anaerobic metabolism.

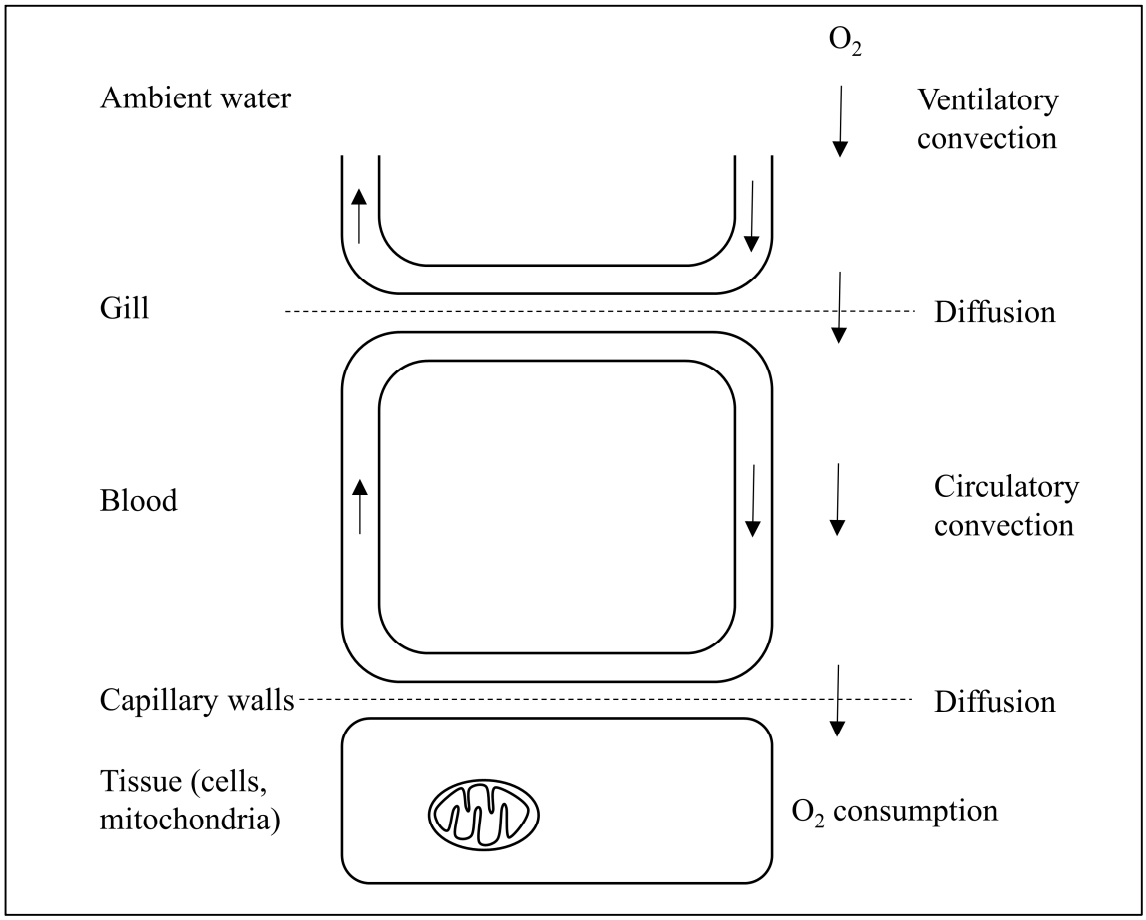

Figure 1.1: The oxygen cascade from ambient water to the mitochondria via diffusion and convection in fish.

Limitation of aerobic metabolism in hypoxia

The aerobic metabolic rate (oxygen uptake rate, $\dot{M}O_2$) of fish is bound by the minimum ($\dot{M}O_{2min}$) and maximum ($\dot{M}O_{2max}$) limits of oxygen uptake rate (Clark et al., 2013). The difference between the $\dot{M}O_{2min}$ and $\dot{M}O_{2max}$ (aerobic scope) represents the capacity for increasing oxygen uptake rates above resting levels to support aerobic activities such as swimming and digestion. Often, in a declining oxygen environment $\dot{M}O_{2max}$ and aerobic scope are progressively limited, therefore limiting the capacity for simultaneous oxygen-demanding processes (Fig. 1.2) (Claireaux and Chabot, 2016; Fry, 1947; Fry, 1971; Norin and Clark, 2016).

The limiting effect of hypoxia on $\dot{M}O_{2max}$ and aerobic scope can reduce the performance of fish. Indeed, non-lethal levels of hypoxia decrease aerobic scope, growth and maximum sustainable swimming speed (U_{crit}) of fish (Brett and Blackburn, 1981; Bushnell et al., 1984;

Chabot and Dutil, 1999; Fu et al., 2011; Petersen and Gamperl, 2010). If oxygen levels continue to decline, aerobic scope will eventually reach zero. This is the point where oxygen supply from the environment can no longer meet $\dot{M}O_{2min}$ (termed critical oxygen tension, O_{2crit}, see Fig. 1.2), such that $\dot{M}O_{2min}$ becomes oxy-conforming (i.e. dependent on environmental oxygen). Measurement of O_{2crit} estimates the level of hypoxia that can be endured without anaerobic metabolism, and has often been used as a measure of hypoxia tolerance (Rogers et al., 2016). If oxygen levels decline below O_{2crit}, fish rely on anaerobic metabolism to fill the gap between the oxygen required to meet baseline metabolism ($\dot{M}O_{2min}$) and oxygen supplied (oxygen uptake). Prolonged periods below O_{2crit} will result in loss of equilibrium (LOE, see Fig. 1.2) and eventually death. It is important to note that while both O_{2crit} and either the DO at LOE or time until LOE are widely used as indicators of hypoxia tolerance, they are measuring different physiological parameters compared with O_{2crit} (that are not yet clearly understood). Measurements of O_{2crit} represent the capacity to maintain aerobic metabolism in a hypoxic environment and prevent anaerobic metabolism occurring, whereas DO at LOE or time until LOE represent a combination of both O_{2crit} and anaerobic capacity once oxygen levels drop below O_{2crit}. Overall, the combined aerobic and anaerobic capacity throughout a range of oxygen levels can impact fish performance in a hypoxic environment. An ability to maintain aerobic capacity in hypoxic environments may minimise the need for anaerobic metabolism and reduce the impacts of hypoxia on ecologically important traits such as growth, survival and swimming speed.

Somewhat surprisingly, it has been reported that the population of Atlantic salmon used for Tasmanian aquaculture contains both oxy-conforming and oxy-regulating individuals (Barnes et al., 2011a; Barnes et al., 2011b). Such a finding indicates that the oxy-conforming proportion of fish within the population have a poor ability to withstand periods of hypoxia. However, the respirometry techniques used in those studies did not meet the recommended standards for aquatic organisms, and so the results should be viewed with caution pending independent validation (Chabot et al., 2016; Clark et al., 2013; McKenzie et al., 2007). There do not appear to be any other studies that have measured O_{2crit} in Tasmanian Atlantic salmon. This thesis provides an opportunity to investigate the hypoxia tolerance of Tasmanian Atlantic salmon and assess the validity of reports suggesting the presence of oxy-conforming individuals within the population.

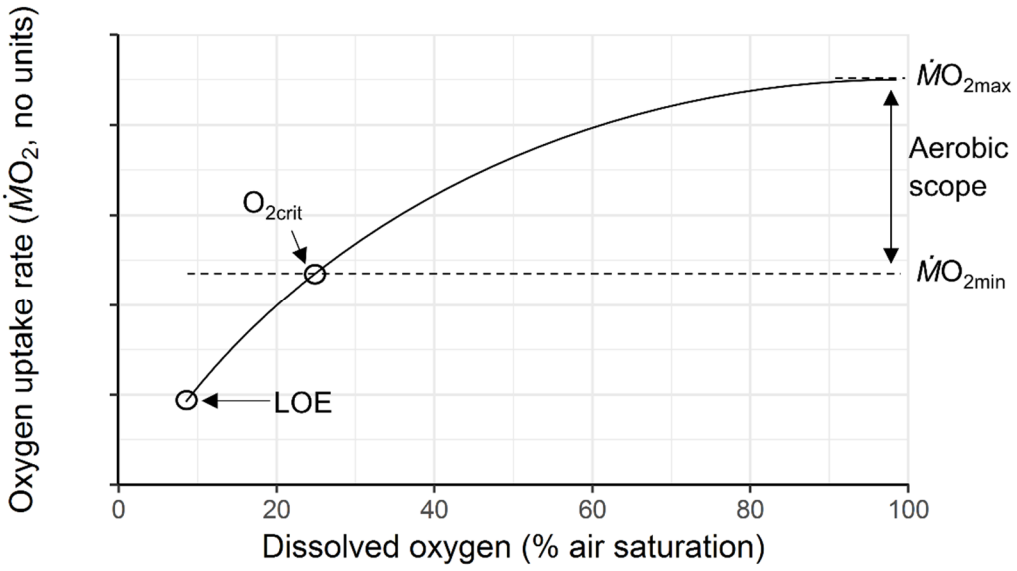

Figure 1.2: The limiting effect of declining dissolved oxygen on $\dot{M}O_{2max}$. The distance between $\dot{M}O_{2min}$ and $\dot{M}O_{2max}$ is the aerobic scope and the critical oxygen level (O_{2crit}) is the point where oxygen uptake rate no longer meets minimum metabolic energy requirements ($\dot{M}O_{2min}$). When oxygen levels fall below O_{2crit} loss of equilibrium (LOE) and death will eventually ensue. Modified from Fry (1971) and Claireaux and Chabot (2016).

Effects of varying oxygen levels during early development

Growth, development and hatching

The major proportion of energy expended during early fish development is devoted to growth (Rombough, 2011). Salmonid alevins have a low anaerobic capacity when compared to juveniles and adults, meaning that embryos have a limited capacity to maintain metabolism when oxygen levels drop below their O_{2crit} (Ninness et al., 2006). As a result, hypoxia often reduces the growth and development of salmonid embryos and alevins, but the reduction is dependent on the level of hypoxia and the stage of development in which they are exposed (Ciuhandu et al., 2005; Hamor and Garside, 1976; Hamor and Garside, 1977; Hamor and Garside, 1979; Shumway et al., 1964). On the other hand, hyperoxia (150% DO) has been reported to slightly increase growth and development in Atlantic salmon, although it had no effect on rainbow trout (Ciuhandu et al., 2005; Matschak et al., 1997; Polymeropoulos, 2013). Given the inconsistent reports of the effects of hyperoxia on growth and development in salmonids, further work is required to elucidate the impacts of hyperoxia at varying stages of development.

Oxygen level influences the time of hatching of salmonids, possibly due to respiratory stress caused by oxygen limitation (DiMichele and Powers, 1984; Dimichele and Taylor, 1981). The O_{2crit} generally increases with embryonic growth because the consequent increase in oxygen demand is not met by an increase in oxygen supply, owing to limitations in oxygen diffusion through the egg capsule and perivitelline (Rombough, 1988a; Rombough, 1988b). Hatching removes the oxygen diffusion barrier, resulting in an immediate ~25% DO decrease in O_{2crit} (Rombough, 1988a). Indeed, hypoxia exposure can cause premature hatching if the embryos are close to hatching. Atlantic salmon began hatching up to 3 weeks early when exposed to < 3% DO, however, hatching was faster and more synchronous closer to the normal hatching time (Oppen-Berntsen et al., 1990). Decreased growth and developmental rate during chronic hypoxia exposure may also delay hatching, because oxygen supply limitations will be delayed in smaller embryos with a lower total metabolic oxygen demand (Alderdice et al., 1958; Bloomer et al., 2016). In some cases hyperoxia has been reported to delay hatching, possibly by increasing the oxygen availability to the embryo and delaying the onset of respiratory stress that is thought to occur just prior to hatching. Rainbow trout transferred to hyperoxia of 275 mmHg (~163% DO) and 690 mmHg (440% DO) 3 days prior to hatching were delayed by 1 and 3 days until hatching, respectively (Latham and Just, 1989). In an extreme case, hyperoxia delayed hatching of killifish (*Fundulus heteroclitus*) by up to 1 month (Dimichele and Taylor, 1980). Removal of an oxygen diffusion barrier via hatching is an important developmental milestone that helps to enable oxygen supply to meet oxygen demand under variable oxygen conditions.

Physiological responses to variable oxygen levels

When oxygen declines to a level where $\dot{M}O_2$ cannot be maintained, developing salmonids must either decrease their metabolic oxygen demand or increase oxygen supply to match metabolic demands due to their limited capacity for anaerobic metabolism (Ninness et al., 2006). The physiological responses to hypoxia during salmonid incubation can vary throughout development due to asynchronous development of cardio-respiratory organs (Burggren and Reyna, 2011; Gorodilov, 1996). Arctic charr (*Salvelinus alpinus*) are capable of depressing their metabolism during periods of anoxia (Gnaiger et al., 1987). Similarly, the $\dot{M}O_2$ of rainbow trout embyros was reduced by approximately 40% when measured in 50% DO, however anaerobic metabolism was not measured (Miller et al., 2008). Reducing

metabolic oxygen demand via metabolic depression during prolonged periods of hypoxia exposure may result in slowed growth and development of hypoxia-exposed embryos.

Progressively increasing reliance on the cardio-respiratory system for oxygen uptake following hatching may result in physiological responses to hypoxia that differ from those prior to hatching (Rombough and Ure, 1991; Wells and Pinder, 1996). Zebrafish exposed to hypoxia increased vascularisation within the muscle tissue, increased blood perfusion, raised concentration of red blood cells and increased dorsal aorta diameter (Schwerte et al., 2003; Yaqoob et al., 2009; Yaqoob and Schwerte, 2010). Arctic charr exposed to hypoxia following hatching increased opercular movements and heart rate, which may aid in oxygen uptake via the gills and skin, but would also increase the energetic costs of respiration (McDonald and McMahon, 1977). Such hypoxia-induced cardio-respiratory modifications may increase oxygen uptake rate, thereby limiting the impacts of hypoxia. For example, Atlantic salmon exposed to hypoxia (50% DO) for 2 weeks following hatching had a higher $\dot{M}O_2$ than normoxia-incubated alevins (Polymeropoulos, 2013). Hypoxia induced cardio-respiratory modifications would be advantageous in order to maximise $\dot{M}O_2$ during periods of low oxygen experienced during incubation in redds and artificial incubation systems. Therefore, it is important to consider the timing and severity of oxygen variability in relation to developmental events, and it cannot be assumed that oxygen levels that effect development during a particular period will have similar effects throughout development.

Long-term effects of oxygen variability during development

Physiological responses of incubating organisms to oxygen variability may carry over to juveniles and adults by altering the course of development, resulting in a modified adult phenotype (Beaman et al., 2016; Burggren and Reyna, 2011). Indeed, incubation hypoxia exposure of an air breathing vertebrate (chicken, *Gallus gallus*) lowers metabolic rate ($\dot{V}O_2$) after hatching (Dzialowski et al., 2002). Physiological responses to incubation environments may be beneficial for later life performance, however this is not always the case (Beaman et al., 2016). As mentioned above, hypoxia incubation can induce physiological responses that may be beneficial to match oxygen supply with demand. The few studies that have investigated the long-term effects of hypoxia incubation on cardio-respiratory physiology in fish have found no effect on $\dot{M}O_2$ or hypoxia tolerance when fish were tested later in life (Robertson et al., 2014; Vanderplancke et al., 2015). However, evidence for long-term

impacts of temperature on physiology of fish suggests that incubation environment may be important for later life performance. Indeed, the thermal incubation regime of zebrafish can impact long-term swimming performance by affecting muscle cellularity, although the responses are not always beneficial (Scott and Johnston, 2012). Changes in muscle cellularity have also been reported in Atlantic salmon incubated in hypoxia (Matschak et al., 1997). Hypoxia may cause permanent detrimental effects on cardio-respiratory development that negatively impact performance. For example, chronic hypoxia (34% DO) exposure of rainbow trout from fertilisation to yolk-sac absorption reduces the swimming speed of trout fry even once returned to a normoxic environment (Johnston et al., 2013). As such, the potential detrimental impacts of oxygen variation during early development are equally important as beneficial physiological responses when considering the long-term impacts of the incubation environment.

Carry-over effects of oxygen variability may also extend beyond physiological modifications during early development (Jonsson and Jonsson, 2014). As mentioned above, hypoxia reduces growth and developmental rate in salmonids, while hyperoxia may increase growth. In natural systems salmon emerge from the redd into a competitive environment where smaller or later-emerging salmon are more likely to be predated upon, are at a competitive disadvantage and are likely to grow slower, which may ultimately delay smoltification (Einum and Fleming, 2000; Metcalfe et al., 1990; Roussel, 2007; Skoglund and Barlaup, 2006). Variable growth and development in response to oxygen variability may also have consequences for salmonid aquaculture rearing. The timing of transfer from the incubation system to tanks for external feeding is critical to maintain growth. Salmon that are transferred when too small or underdeveloped may crowd on the bottom of tanks as they are not ready to swim or feed, while larger fish may starve if they receive food too late (Pennell and McLean, 1996). The long-term consequences of oxygen variability may not only be constrained to physiological impacts, oxygen variability that alters growth and development may also have carry over effects in natural conditions and complicate the optimisation of aquaculture rearing protocols.

Thesis aims, objectives and structure

Aims and objectives

In light of the limited knowledge about the impacts of oxygen variability during incubation on aerobic physiology and the potential for hypoxia to influence aerobic performance later in life, this thesis investigates the impacts of hypoxia and hyperoxia during incubation on the physiology, growth and development of Atlantic salmon. In particular, this thesis aims to determine periods of development that are sensitive to oxygen variability during incubation. The thesis also assesses the potential for hypoxia to impact the developmental trajectory of salmon by measuring the aerobic performance of juveniles under various hypoxia regimes. While achieving these objectives, it was also possible to investigate the previous claims of the existence of oxy-conformers and oxy-regulators within the Tasmanian Atlantic salmon population. Notably, a theme of 'whole-animal oxygen supply and demand' runs through the thesis, whereby consistent techniques have been used to tease apart important windows of development and threshold oxygen levels that may lead to changes in performance.

Thesis structure

This thesis is compiled as four experimental chapters (Chapters 2-5) that are intended for peer-review publication as independent journal articles. Chapter 4 was published in 2016 in Physiological and Biochemical Zoology and is included within the thesis in its published form. In Chapter 2, 3 and 5 repetition has been removed where possible, however, there may be some overlap between chapters as each has been written with independent publication in mind. I was solely responsible for the majority of the work to produce this thesis, however, I use the terms 'we' and 'our' throughout to acknowledge the contributions of my supervisors and co-authors.

Chapter 2 investigates the effect of hypoxia (50% DO) and hyperoxia (150% DO) on incubating Atlantic salmon during multiple developmental windows from fertilisation until yolk-sac absorption. The impact of hypoxia and hyperoxia incubation on routine $\dot{M}O_2$, O_{2crit} and embryo mass is determined during each window.

Chapter 3 expands on the findings from Chapter 2 by investigating how severe and variable hypoxia incubation impacts developing salmonids from fertilisation until yolk-sac absorption. Salmon are incubated in moderate hypoxia (~63% DO), cyclical hypoxia (100-25% DO

daily) and severe hypoxia (25% DO). We evaluate the impact of hypoxia incubation on aerobic metabolism via measurements of routine $\dot{M}O_2$ and O_{2crit}, and monitor growth, development, survival and hatching.

Chapter 4 investigates the potential for hypoxia incubation to impact the developmental trajectory of salmon that were exposed to constant hypoxia (50% DO) from fertilisation until yolk-sac absorption in Chapter 2. We test for the influence of developmental hypoxia on aerobic performance by measuring $\dot{M}O_{2min}$, $\dot{M}O_{2max}$, aerobic scope and hypoxia tolerance (loss of equilibrium) in both normoxic and hypoxic conditions.

Chapter 5 investigates whether variable hypoxia incubation affects the developmental trajectory of salmon (from Chapter 3). Chapter 5 builds on previous knowledge by testing for long-term effects of moderate and cyclical hypoxia incubation (~63% DO and 100-25% DO daily, respectively) on later life hypoxia-acclimation capacity. Specifically, aerobic performance and hypoxia tolerance of juvenile salmon acclimated to hypoxia are quantified using measures of $\dot{M}O_{2min}$, $\dot{M}O_{2max}$, aerobic scope, O_{2crit} and hypoxia tolerance (loss of equilibrium) in both hypoxic and normoxic conditions.

Chapter 2: Developmental windows of hypoxia and hyperoxia exposure during incubation of Atlantic salmon (*Salmo salar*)

Abstract

Oxygen availability is highly variable during salmonid incubation in natural redds and aquaculture incubation systems. While hypoxia generally decreases growth and aerobic metabolism prior to hatching, and can also cause physiological modifications that increase oxygen delivery, less is known of the consequences of developmental hyperoxia. The impacts of hypoxia and hyperoxia during incubation are likely to be dependent on the window of exposure, as sensitivity to oxygen level can vary throughout development. Here, we test for the effects of hypoxia (50% dissolved oxygen: DO, % air saturation) and hyperoxia (150% DO) on the growth, routine aerobic metabolism ($\dot{M}O_{2rout}$) and hypoxia tolerance (O_{2crit}) of Atlantic salmon (*Salmo salar*) at various developmental windows during incubation. We exposed Atlantic salmon to either hypoxia or hyperoxia for seven developmental windows at various stages from fertilisation until 100 days post-fertilisation. Embryos exposed to hyperoxia (150% DO) did not differ from the normoxic group in growth, $\dot{M}O_{2rout}$ or O_{2crit} at any developmental window. In contrast, embryos exposed to hypoxia between fertilisation and hatching grew slower and had a lower $\dot{M}O_{2rout}$, but had higher hypoxia tolerance (lower O_{2crit}) than normoxic and hyperoxic counterparts. Interestingly, these differences were only apparent when the embryos were measured before hatching. Larvae (alevins) incubated in hypoxia following hatching grew similarly to normoxia-incubated alevins. Our results provide evidence that Atlantic salmon embryos are most sensitive to hypoxia before hatching, probably due to restricted oxygen diffusion through the egg. We suggest that prior to hatching, hypoxia-incubated embryos suffer oxygen-limited metabolic depression, thereby reducing growth and $\dot{M}O_2$.

Introduction

Oxygen availability during incubation

Embryonic and larval fish are susceptible to oxygen variability during incubation because they are mostly immobile and therefore cannot flee sub-optimal environmental conditions. Salmon in natural habitats deposit eggs into under-gravel redds in freshwater streams where they develop for several months (Groot, 1996; Webb et al., 2007). The oxygen available to incubating embryos and larvae (alevins) is dependent on the physical characteristics of the redd and the microenvironment surrounding the egg (Ciuhandu et al., 2007; Dhiyebi et al., 2013; Greig et al., 2007; Miller et al., 2008). Indeed, oxygen levels as low as 19% dissolved oxygen (DO; % air saturation) have been recorded in Atlantic salmon (*Salmo salar*) redds within natural streams, with significant variation between redd sites (Youngson et al., 2004). Similarly, salmonids incubating in aquaculture rearing systems can experience hypoxic conditions below 20% DO depending on the stage of development and location within the rearing system (Appendix A). Supplemental oxygenation of incubation water in an attempt to prevent hypoxic conditions in aquaculture can cause hyperoxic conditions up to 180% DO (Appendix A). These large variations in oxygen availability in both natural and artificial incubation systems have the potential to affect early salmonid development.

Effects of oxygen variability during incubation

Fish generally respond to periods of hypoxia by either increasing oxygen uptake capacity by modifying the structures used for oxygen delivery, or reducing their metabolic oxygen demand by a controlled down-regulation of cellular mechanisms involved in energy-dependent processes (Richards, 2009). Hypoxia delays the growth and development of embryonic and larval salmonids, possibly due to metabolic depression in response to the reduced oxygen content (Ciuhandu et al., 2005; Hamor and Garside, 1976; Miller et al., 2008; Polymeropoulos, 2013; Richards, 2009). Indeed, the oxygen uptake rate ($\dot{M}O_2$) of Atlantic salmon embryos incubated in hypoxia was lower from fertilisation until pre-hatch compared with normoxia-incubated embryos (Hamor and Garside, 1979). Similarly, rainbow trout (*Oncorhynchus mykiss*) embryos exposed to acute or chronic hypoxia before hatching reduced routine oxygen uptake rates ($\dot{M}O_{2rout}$) compared with embryos in normoxia, suggesting that embryos reduce their metabolic oxygen demand and that oxygen uptake may not be compensated in response to hypoxia in eggs (Miller et al., 2008). Hatching removes the oxygen diffusion limitations imposed by the egg capsule, and salmon larvae (alevins)

subsequently rely on both cutaneous diffusion and the cardio-respiratory system for oxygen uptake (Rombough and Ure, 1991; Wells and Pinder, 1996). Physiological modifications in response to hypoxia that improve cutaneous oxygen diffusion or cardio-respiratory function may improve oxygen uptake. For instance, zebrafish (*Danio rerio*) increased vascularisation and bullfrog (*Rana catesbeiana*) larvae reduced skin thickness and increased capillary mesh density during hypoxia exposure (Burggren and Mwalukoma, 1983; Yaqoob and Schwerte, 2010). The $\dot{M}O_2$ of Atlantic salmon alevins incubated in hypoxia for 2 weeks from hatching was higher than normoxia-incubated fish, when measured in normoxia (Polymeropoulos, 2013). Zebrafish embryos incubated in hypoxia (5% DO) for 4 h had a higher $\dot{M}O_{2rout}$ when oxygen levels dropped below O_{2crit} (Robertson et al., 2014). In combination, these responses to hypoxia incubation suggest that modifications to the cardio-respiratory system or structures involved in cutaneous diffusion may be associated with increases in oxygen uptake capacity.

While it is clear that hypoxia can affect the early growth, development and physiology of incubating fish, there have been considerably fewer reports investigating the effects of hyperoxia during early development. Oxygen diffusion to the embryo is limited by the egg capsule prior to hatching, therefore, increasing metabolic oxygen requirements with embryo growth are not met with an equivalent increase in oxygen uptake capacity (Miller et al., 2008; Rombough, 1988a). If oxygen uptake becomes limited in normoxic conditions then hyperoxia may delay or decrease the impacts of oxygen limitation by increasing the partial pressure gradient and therefore increasing the oxygen availability to the embryo. Indeed, rainbow trout hatching can be delayed in hyperoxia, possibly due to the alleviation of cardio-respiratory stress related to oxygen (Latham and Just, 1989). Hyperoxia-incubated (150% DO) Atlantic salmon alevins grew faster during 2 weeks of incubation, but did not have any associated changes in $\dot{M}O_2$ (Polymeropoulos, 2013). However, increased growth during hyperoxia incubation does not appear to be consistent, as the growth of hyperoxia-incubated rainbow was not affected prior to hatching (Ciuhandu et al., 2005). Evidence from bullfrog tadpoles suggests that hyperoxia may not affect oxygen uptake, as gas exchange organ morphology and haematology remained unchanged (Burggren and Mwalukoma, 1983; Pinder and Burggren, 1983). While there is some evidence that hyperoxia may alleviate respiratory stress during early development of fish, there has not yet been a comprehensive study of impacts throughout the entire developmental period, where oxygen sensitivity is variable (Rombough, 1988a).

Critical developmental windows for oxygen availability

The physiological response of developing embryos and larvae to environmental fluctuations are likely to depend on the developmental window in which they are exposed (Burggren and Reyna, 2011; Eme et al., 2015). During early development of salmon, the cardio-respiratory organs develop at different rates and during different developmental windows. For instance, heart contractions begin before gill and vasculature development is complete, and the changeover to adult erythrocytes does not occur until after hatching (Bianchini and Wright, 2013; Gorodilov, 1996). Thus, the characteristics and magnitude of responses to incubation in hypoxia or hyperoxia are likely to vary depending on the stage of development. In chum salmon (*Oncorhynchus keta*) the time delay until hatching was largest in salmon incubated in hypoxia during a critical developmental window between 100 and 200 degree days (prior to the eyed-egg stage) (Alderdice et al., 1958). However, there are few studies that investigate critical developmental windows of physiology in fish in response to oxygen variability, although, evidence suggests that responses to incubation temperature vary during different developmental windows. Indeed, the incubation temperature of lake whitefish (*Coregonus clupeaformis)* affected $\dot{M}O_2$ and heart rate during the first third of embryonic development, whereas growth was most sensitive to temperature during the final third of development (Eme et al., 2015; Mueller et al., 2015b). Salmon $\dot{M}O_2$ became limited (i.e. oxygen dropped below the critical level (O_{2crit})) at progressively higher oxygen levels from fertilisation until hatching, suggesting that hypoxia sensitivity of salmon is not constant throughout development (Rombough, 1988a). Modifications to the cardio-respiratory system that increase oxygen uptake may not be apparent until late developmental stages because developing salmonids rely primarily on cutaneous diffusion for oxygen delivery until several weeks after hatching (Gorodilov, 1996; Rombough and Ure, 1991). Variable oxygen levels may also result in physiological modifications or alterations in the timing of key developmental milestones in an attempt to maintain oxygen delivery (Spicer and Burggren, 2003). For example, the changeover from embryonic to adult erythrocytes and onset of cholinergic heart rate control was delayed in rainbow trout incubated in hypoxia (Bianchini and Wright, 2013; Miller et al., 2011). While evidence suggests that the physiological responses of salmon will vary depending on the developmental window of oxygen variability, our understanding is hampered by a lack of studies exploring the impacts of oxygen availability at defined periods during incubation.

Objectives

The objective of this chapter was to investigate how hypoxia and hyperoxia affect the routine oxygen uptake rate ($\dot{M}O_{2rout}$), hypoxia tolerance (O_{2crit}) and growth of Atlantic salmon embryos and alevins when exposed during seven windows of early development. We hypothesised that hypoxia exposure will decrease growth due to a decrease in $\dot{M}O_{2rout}$, with the largest effects of oxygen limitation during the window between fertilisation and hatching when oxygen availability to the embryo is limited by diffusion through the egg capsule. The inclusion of the hyperoxia treatment group enabled us to elucidate whether oxygen can be a limiting factor during normoxia incubation at any developmental window, whereby hyperoxia would alleviate negative impacts associated with oxygen limitation in the normoxia treatment.

Methods

Fertilisation and brood-stock source

All experiments were conducted at the Salmon Enterprises of Tasmania (SALTAS) hatchery at Wayatinah, Tasmania, Australia. Atlantic salmon were sourced from a population selectively bred for aquaculture in Tasmania, Australia that originated from the east-coast of Canada in the 1960s (Elliott and Kube, 2009). The eggs of four female Atlantic salmon were mixed and fertilised with the milt from one male to create four half-sibling families on 22 May 2014. We used half-sibling families to limit the impact of genetic variation on any potential effects of oxygen rearing environment (Anttila et al., 2013).

Incubation treatments

Within 2 hours of fertilisation the eggs were randomly allocated to two replicate mesh isolation baskets (18 x 14.5 x 5.5 cm) per incubation treatment within Heath trays (L x W x H = 39 x 32 x 5.5 cm; Marisource, USA). Each Heath tray held four isolation baskets with approximately 1750 eggs per basket (7000 eggs per Heath tray). Normoxia (~100% DO), hypoxia (~50% DO) and hyperoxia (~150% DO) incubation treatments commenced immediately upon stocking the isolation baskets and continued until 100 days post-fertilisation (DPF, ~800 degree days (DD)). Hypoxia exposure of 50% DO was chosen as a treatment that induced physiological changes based on previous studies of salmonids (Rombough 2007; Polymeropoulos 2013). Hyperoxia exposure of 150% DO was chosen

based on DO measurements made within Heath trays in an aquaculture hatchery (Appendix A). Eggs and alevins were exposed to either hypoxia or hyperoxia incubation treatments for one of the seven following developmental windows, where 50 represents hypoxia, 150 represents hyperoxia, F is fertilisation, E is eyed-egg, H is hatch, 2 is two weeks post-hatch and 4 is four weeks post-hatch. The developmental windows were from 0-41 DPF (50F-E, 150F-E), 41-69 DPF (50E-H, 150E-H), 0-69 DPF (50F-H, 150F-H), 69-84 DPF (50H-2, 150H-2), 0-84 DPF (50F-2, 150F-2), 84-100 DPF (502-4, 1502-4) and 0-100 DPF (50F-4, 150F-4; Fig. 2.1 & Table 2.1). Developmental windows and measurement time-points were selected to avoid measurements during the period of development prior to the 'eyed-egg' stage, when eggs are sensitive to mechanical shock (Billard and Jensen, 1996). Additionally, developmental windows were chosen based on evidence for physiological changes in hypoxia-incubated salmonids at 2 weeks post-hatch (Polymeropoulos, 2013). During the developmental windows where the eggs and alevins were not exposed to hypoxia or hyperoxia they were incubated in normoxia. Additionally, a group of control eggs and alevins were incubated in normoxia for the entire experimental period from 0-100 DPF. Care was taken to ensure that the eggs or alevins were not exposed to air for more than 15 s when the isolation baskets were transferred between the treatments. Replicate isolation baskets for each treatment group were always incubated in separate Heath trays.

Drum-screened river water was supplied to a Heath tray system modified to ensure an independent water supply to each tray at 10 L min^{-1}. The temperature was maintained at ~8°C and the DO held at ~100% (normoxia), ~50% (hypoxia) or 150% (hyperoxia) by an OxyGuard Pacific monitoring system (OxyGuard, Denmark) with a submersible heater and nitrogen or oxygen injection into 200 L treatment sumps (Table 2.1). The nitrogen injection system failed on three occasions throughout the 100 day incubation period, such that DO in the hypoxia treatment rose to normoxic levels for a maximum continuous period of 41 h prior to being rectified. Nonetheless, embryos held in hypoxia from fertilisation until 4 weeks post-hatch spent 97% of their incubation between 45 and 55% DO (grand mean 51.1 ± 7.6% DO including nitrogen system failures). Water quality was monitored on at least 3 days each week and maintained between 0 - 0.24 mg L^{-1} NH3-N (ammonia), 0 - 0.2 mg L^{-1} NH_2-N (nitrite) and 6.8 7.8 pH. According to industry best practices, the eggs and alevins were treated with formalin at 1.5-2 mL L^{-1} for 15 min three times weekly from 70 DD to 340 DD to prevent fungal growth, and dead eggs and alevins removed from 280 DD to 950 DD. Additionally, at the eyed stage (280 DD) eggs were physically agitated to assist the removal

of dead embryos. Eggs and alevins were held in relative darkness by covering the Heath tray incubation system with black plastic sheet when access to Heath trays was not required.

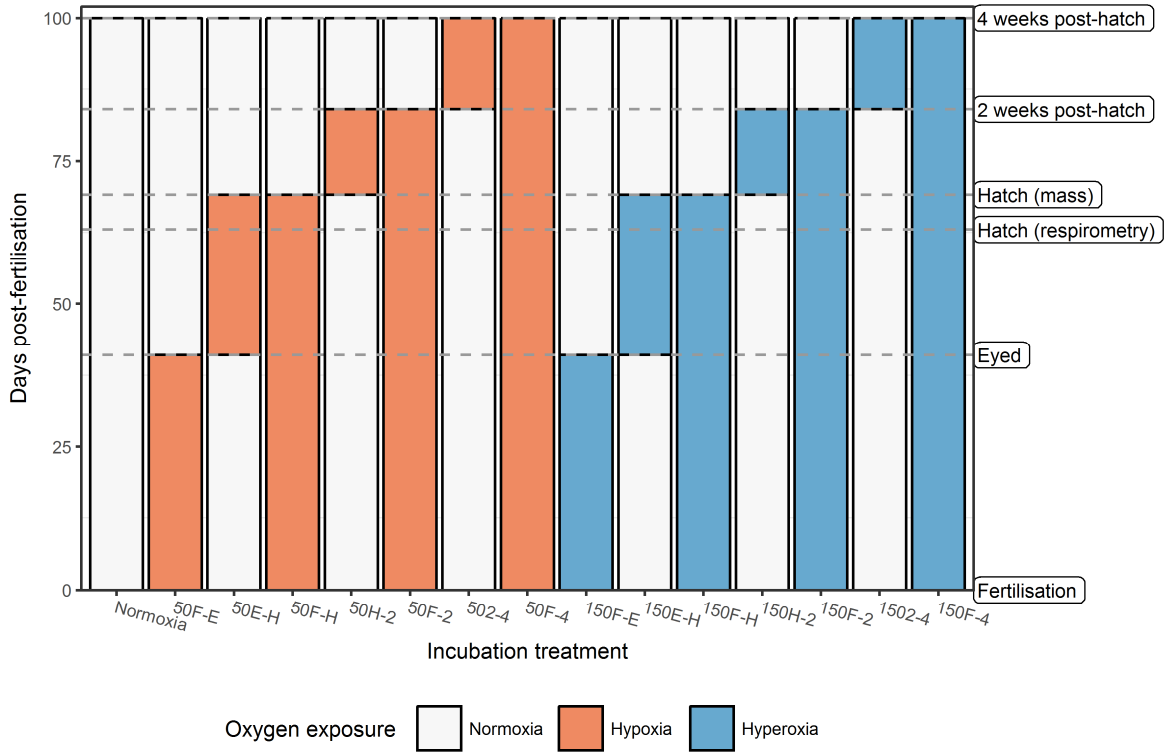

Figure 2.1: Developmental windows for hypoxia (50% DO) or hyperoxia (150% DO) incubation of Atlantic salmon (*Salmo salar*) embryos and alevins. F = fertilisation, E = eyed-egg, H = hatch, 2 = two weeks post-hatch, 4 = four weeks post-hatch. Measurement time-points are indicated with dashed lines.

Embryo mass

Embryo mass was measured at 40 DPF, 68 DPF, 83 DPF and 99 DPF. Each incubation treatment was measured at the completion of the developmental window and at each measurement time-point following a return to normoxia (Fig. 2.1 & Table 2.2). Eggs and alevins were weighed whole (with yolk) as well as following the removal of the egg and/or yolk-sac. Whole egg and alevin followed by yolk-free embryo and alevin mass were measured to ± 0.0001 g with a Sartorius LC3200D balance (Sartorius, Germany) after being blotted with tissue paper to remove excess water.

Table 2.1: Mean dissolved oxygen (DO; % air saturation) and temperature (± SD) for the normoxia, hypoxia (50% DO) and hyperoxia (150% DO) incubation treatments within each developmental window (F = fertilisation, E = eyed-egg, H = hatch, 2 = two weeks post-hatch, 4 = four weeks post-hatch). DPF = days post-fertilisation, DD = degree days

	Stage of development			
	0-41 DPF	**41-69 DPF**	**69-81 DPF**	**81-100 DPF**
	0-329 DD	**329-552 DD**	**552-647 DD**	**647-798 DD**
Treatment	**F-E**	**E-H**	**H-2**	**2-4**
	Dissolved oxygen (% air saturation)			
Normoxia	100.9 ± 0.7	99.8 ± 2.7	98.4 ± 1.1	99.6 ± 0.7
Hypoxia	50.5 ± 3.6	50.9 ± 5.8	56.1 ± 16.4	50.0 ± 0.2
Hyperoxia	150.7 ± 1.5	150.0 ± 0.7	150.0 ± 0.4	150.0 ± 0.4
	Temperature (°C)			
Normoxia	8.06 ± 0.22	7.96 ± 0.03	7.95 ± 0.01	7.94 ± 0.01
Hypoxia	8.02 ± 0.14	7.96 ± 0.03	7.95 ± 0.01	7.95 ± 0.01
Hyperoxia	8.00 ± 0.12	7.96 ± 0.03	7.95 ± 0.01	7.95 ± 0.02

Respirometry

Oxygen uptake rate ($\dot{M}O_2$) measurements were conducted at 40 DPF (eyed), 63 DPF (hatch) and 83 DPF (2 weeks post-hatch; Fig. 2.1, Table 2.2). Each incubation treatment group was measured immediately prior to the completion of the developmental window and again at each measurement time-point once returned to normoxia. However, due to delayed hatching the 50F-E and 50F-H incubation treatment groups were excluded from respirometry at the hatching stage. Additionally, the normoxia group was measured at every measurement time-point. On the measurement day an approximately equal number of individuals were randomly selected from each replicate isolation basket per treatment and sealed in respirometers in water at 150-170% DO (elevated oxygen levels ensured that fish could be loaded into respirometers and allowed to settled before DO dropped below 100%). Oxygen uptake rates were measured for individual embryos or alevins within closed respirometers and at least one respirometer remained empty to measure background respiration. Respirometers were constructed from aluminium plates (approx. 12.8 cm x 8.6 cm x 2.1 cm) containing 24 (15.7 mm diameter x 19.1 mm deep; 2.7 mL) individual respirometry chambers with a glass base. Each respirometer was sealed by an acrylic lid with a rubber o-ring. The inside of each lid was concave and had a central vent constructed from a modified 18 gauge needle and tygon tubing extending at least 2 cm above the lid. The vent allowed air bubbles and excess water to

be removed during sealing whilst also preventing pressurisation, O_2 diffusion, and back-draw of air into the chamber during $\dot{M}O_2$ measurements. Dissolved oxygen was measured using a 24 channel SDR SensorDish reader and oxygen sensor spots adhered to the glass base of each respirometer (PreSens Precision Sensing GmbH, Regensburg, Germany).

Table 2.2: Sample sizes for mass and respirometry measurements of Atlantic salmon (*Salmo salar*) exposed to normoxia, hypoxia (50% DO = 50) or hyperoxia (150% DO = 150) during different developmental windows (F = fertilisation, E = eyed-egg, H = hatch, 2 = two weeks post-hatch, 4 = four weeks post-hatch).

	Mass				**Respirometry**		
Incubation treatment	Eyed	Hatch	2 weeks post-hatch	4 weeks post-hatch	Eyed	Hatch	2 weeks post-hatch
Normoxia	22	23	24	24	11	21	9
50F-E	21	24	24	24	12	-	12
50E-H	-	27	26	24	-	14	13
50F-H	-	24	23	24	-	-	8
50H-2	-	-	24	24	-	-	9
50F-2	-	-	24	22	-	-	10
502-4	-	-	-	21	-	-	-
50F-4	-	-	-	22	-	-	-
150F-E	22	24	24	24	11	21	13
150E-H	-	21	21	24	-	17	12
150F-H	-	24	24	24	-	19	14
150H-2	-	-	22	23	-	-	12
150F-2	-	-	24	24	-	-	12
1502-4	-	-	-	22	-	-	-
150F-4	-	-	-	22	-	-	-

The respirometers contained one 3.9 mm stainless steel ball bearing to mix water and a stainless steel mesh stage to prevent the embryo or alevin touching the oxygen sensor spot or ball bearings. The respirometer plates and SensorDish readers were placed on a Ratek orbital shaker plate (Ratek, Australia) that was set at the lowest speed that ensured the ball bearing was continuously moving within the respirometer and therefore mixing the water. Temperature in the respirometers was maintained at 7.9 ± 0.81°C (mean ± SD) by placing the shaker plate and up to four respirometer plates and SensorDish readers in an upright refrigerator modified with a custom built temperature controller. The oxygen sensor spots were calibrated before and after each $\dot{M}O_2$ measurement at ~8°C at 100 % DO with aerated water and 0 % DO by adding 30 g L^{-1} sodium sulfite to create oxygen-free water.

Dissolved oxygen concentration was measured until the DO for all respirometers being simultaneously measured fell below 5% DO. Upon completion of $\dot{M}O_2$ measurements the embryos and larvae were removed from the chambers and weighed as described above.

Data analysis and statistics

The rate of declining DO (% DO min $^{-1}$) was dependent on fish size and determined using least-squares regression for consecutive 1200 s (eyed), 600 s (hatch) and 450 s (2 weeks post-hatch) slopes. Only oxygen uptake measurements below 100% DO were included to ensure that the temperature had stabilised and the embryos had acclimated to the respirometer. The rate of declining DO for background respiration measurements were determined for the entire measurement period once DO stabilised. Oxygen uptake rate ($\dot{M}O_2$, μg O_2 min^{-1}) was calculated using equation (2.1).

$$\dot{M}O_2(\mu g\ O_2\ min^{-1}) = \frac{\Delta\ DO}{\Delta\ t} \times \left(P_B - (P_S \times RH)\right) \times \beta_{O_2} \times vol \times 0.2094 \quad (2.1)$$

where DO is the fractional dissolved oxygen saturation, t is time in minutes, P_B is barometric pressure (kpa), P_S is the calculated saturation vapour pressure of water (kpa; Antoine equation), h is the fractional relative humidity, βO_2 is the oxygen capacitance of freshwater adjusted for temperature (~563.3 μg L^{-1} kpa^{-1} at 8°C; see (Dejours, 1981)) and vol is the volume of the respirometry chamber minus fish tissue volume in L (assuming 1 g wet tissue mass = 1 mL volume). The mean background respiration for all respirometry measurements was subtracted from each individual $\dot{M}O_2$ measurement (background respiration was always less than 8% of embryo $\dot{M}O_2$).

The O_{2crit} for each individual was determined by fitting a piecewise linear regression (broken-stick) with the slope above O_{2crit} set at 0, using the 'segmented' package within R (Muggeo, 2008). The O_{2crit} was determined as the breakpoint of the piecewise linear regression, and routine $\dot{M}O_2$ ($\dot{M}O_{2rout}$) was calculated as the intercept of the slope at DO concentrations above O_{2crit}.

All statistical analyses were performed using R along with the packages lsmeans and car (Fox and Weisberg, 2011; Lenth, 2016; R Core Team, 2016). Differences in O_{2crit}, $\dot{M}O_{2rout}$ and

embryo mass were compared between incubation treatment groups within each measurement time-point. Yolk-free embryo mass and O_{2crit} were compared using ANOVA (Type 3 SS). Routine $\dot{M}O_2$ was compared using ANCOVA (Type 3 SS) with yolk-free embryo mass as a covariate. Pairwise comparisons were conducted via least-square means and Tukey adjusted P-values. All values are presented as mean ± SEM (standard error of the mean) unless stated otherwise.

Results

Embryo and alevin mass

At the eyed-egg developmental stage the embryos that had been exposed to hypoxia from fertilisation (50F-E) were ~30% smaller than embryos that were incubated in normoxia and hyperoxia (150F-E; both $P < 0.0001$, Fig. 2.2A). At hatching, embryos that had been incubated in hyperoxia were a similar mass to those incubated in normoxia (0.0454 ± 0.0013 g, all $P > 0.80$, Fig. 2.2B). However, embryos incubated in hypoxia from fertilisation to eyed-egg or from eyed-egg to hatching stage (50F-E or 50E-H, respectively) were ~8% smaller than those incubated in normoxia (both $P < 0.0001$, Fig. 2.2B). Embryos incubated in hypoxia from fertilisation until hatching stage (50F-H) were ~40% smaller than normoxia-incubated embryos and ~25% smaller than those partially incubated in hypoxia (all $P < 0.0001$, Fig. 2.2B).

At 2 weeks post-hatch all hyperoxia-incubated embryos had similar masses to those incubated in normoxia (0.0765 ± 0.0017 g, all $P > 0.0930$, Fig. 2.2C). Out of the embryos that were incubated in hypoxia, only the embryos incubated from hatching stage until two-weeks post-hatch (50H-2 treatment group) were a similar mass to normoxia-incubated embryos ($P = 0.0657$, Fig. 2.2C). Embryos incubated in hypoxia from fertilisation until eyed-egg (50F-E) and from eyed-egg to hatching stage (50E-H) were ~11% smaller than embryos incubated in normoxia ($P = 0.0034$ and $P = 0.0062$, respectively, Fig. 2.2C). However, embryos exposed to hypoxia for a longer period, i.e. fertilisation until hatching stage and fertilisation until 2 weeks post-hatch (50F-H and 50F-2 treatment groups), were ~26% smaller than embryos incubated in normoxia (both $P < 0.0001$). These embryos incubated for longer in hypoxia (50F-H and 50F-2) were also between 17% and 20% smaller than embryos incubated for a shorter developmental window in hypoxia (50H-2, 50E-H and 50F-E, all $P < 0.0008$, Fig. 2.2C).

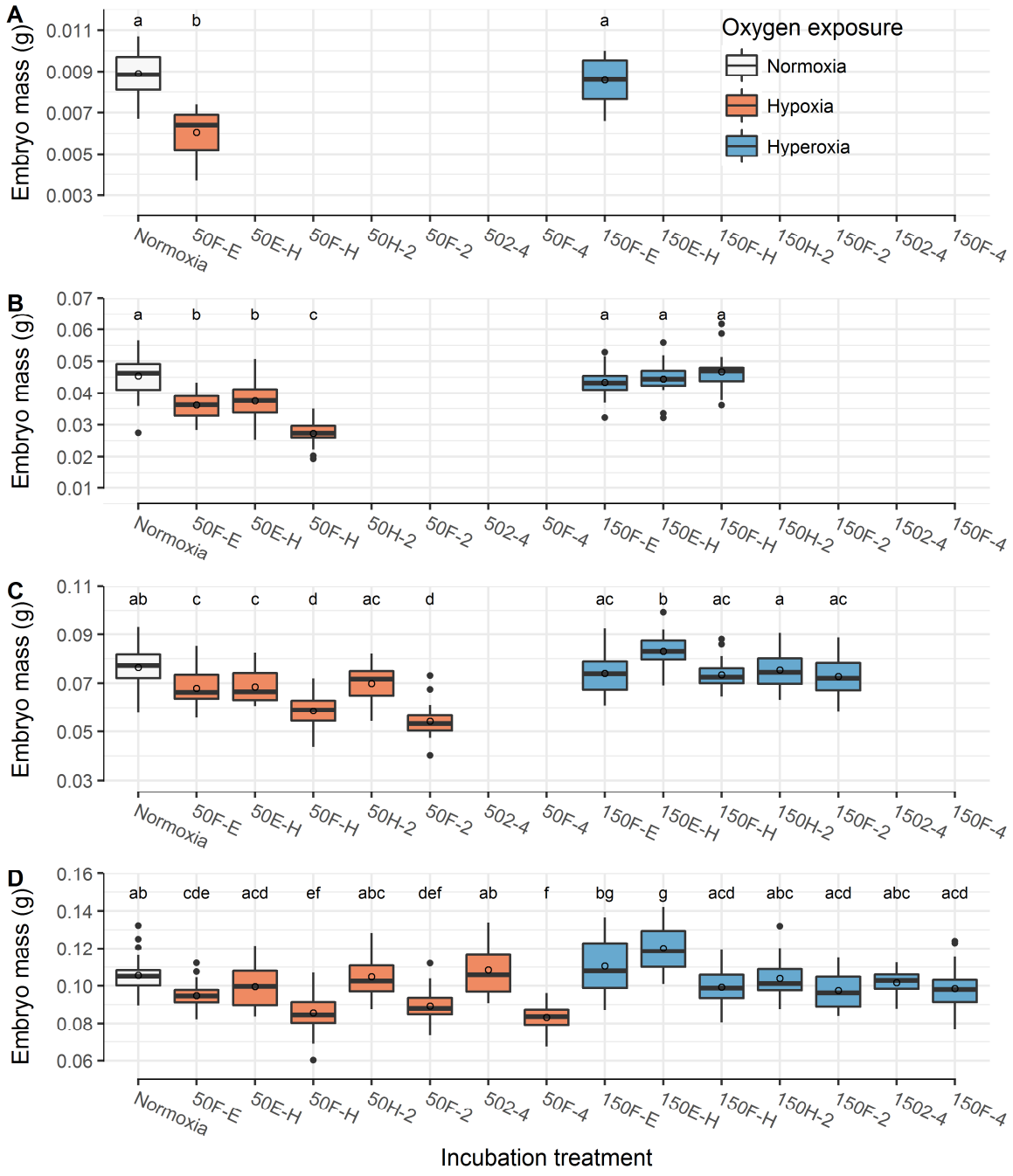

Figure 2.2: Boxplots of embryo mass measured at the eyed stage (A), hatch stage (B), 2 weeks post-hatch (C) and 4 weeks post-hatch (D) following normoxia, hypoxia (50% DO) or hyperoxia (150% DO) incubation during different developmental windows (see Fig. 2.1). Different letters represent significant differences between incubation treatment groups within each measurement time-point (Tukey, $P < 0.05$). The solid central line is the median, the box denotes 25th and 75th percentile, whiskers extend to the highest or lowest value within 1.5*inter-quartile range and filled circles are outliers beyond that range. Mean values are represented by open circles.

At 4 weeks post-hatch embryos incubated in hyperoxia from eyed-egg until hatching stage (150E-H) were ~12% larger than embryos incubated in normoxia (0.1201 ± 0.0025 vs. 0.1057 ± 0.0020 g, respectively; P = 0.0005, Fig. 2.2D). However, all other hyperoxia treatment groups had similar masses to the normoxia incubation group (all P > 0.36, Fig. 2.2D). Embryos that were incubated in hypoxia from the eyed-egg stage onwards (50E-H, 50H-2 and 502-4 treatment groups) were a similar mass to the normoxia incubation treatment (all P > 0.83, Fig. 2.2D). While those embryos that commenced incubation in hypoxia from fertilisation (50F-E, 50F-H, 50F-2 and 50F-4 treatment groups) were smaller than those incubated in normoxia (normoxia vs. 50F-E: P = 0.041, all others P < 0.0001, Fig. 2.2D).

Routine $\dot{M}O_2$ and hypoxia tolerance

At the eyed developmental stage $\dot{M}O_{2rout}$ of embryos incubated in hypoxia (50F-E) was ~23% and ~19% lower (comparison of least-square means) than embryos incubated in normoxia or hyperoxia (150F-E), respectively (P = 0.004 and P = 0.029, respectively, Fig. 2.4A, B). At hatching ($F_{(4,86)}$ = 0.6167, P = 0.652, Fig. 2.4C, D) and at 2 weeks post-hatch ($F_{(10,112)}$ = 1.305, P = 0.237, Fig. 2.4E, F) $\dot{M}O_{2rout}$ was similar between all incubation treatment groups.

At the eyed-egg stage the O_{2crit} of embryos incubated in hypoxia from fertilisation (50F-E, 38.3 ± 1.3% DO) was ~7% DO lower (i.e. more hypoxia tolerant) than those incubated in either normoxia (44.7 ± 1.6% DO; P = 0.012) or hyperoxia (44.8 ± 1.5% DO, 150F-E; P = 0.003, Fig. 2.3A). At hatching, the O_{2crit} of embryos incubated in hypoxia from eyed-egg to hatching stage (50E-H) or hyperoxia within any developmental window (150F-E, 150E-H and 150F-H) was similar to those incubated in normoxia (all P > 0.0755, Fig. 2.3B). However, the O_{2crit} of embryos incubated in hyperoxia from fertilisation to hatching (150F-H, 23.2 ± 0.93 % DO) was ~5.7% DO higher than embryos incubated in hypoxia from eyed-egg to hatching (50E-H, 17.5 ± 0.83% DO; P = 0.0012, Fig. 2.3B). At 2 weeks post-hatch all incubation treatment groups had a similar, and markedly lower, O_{2crit} of 16.4 ± 0.4% DO ($F_{(10, 113)}$ = 0.719, P = 0.705, Fig. 2.3C).

Discussion

Hypoxia incubation prior to hatching decreased growth, which supports our hypothesis that Atlantic salmon are most sensitive to hypoxia exposure between fertilisation and hatching. In contrast, hyperoxia had only negligible effects on growth and did not affect oxygen uptake

rate. Similarly, there was no evidence to support the hypothesis that hypoxia exposure induced physiological modifications to improve oxygen delivery (and therefore oxygen uptake under hypoxic conditions). We discuss these findings below in the context of oxygen variability in natural and artificial rearing environments.

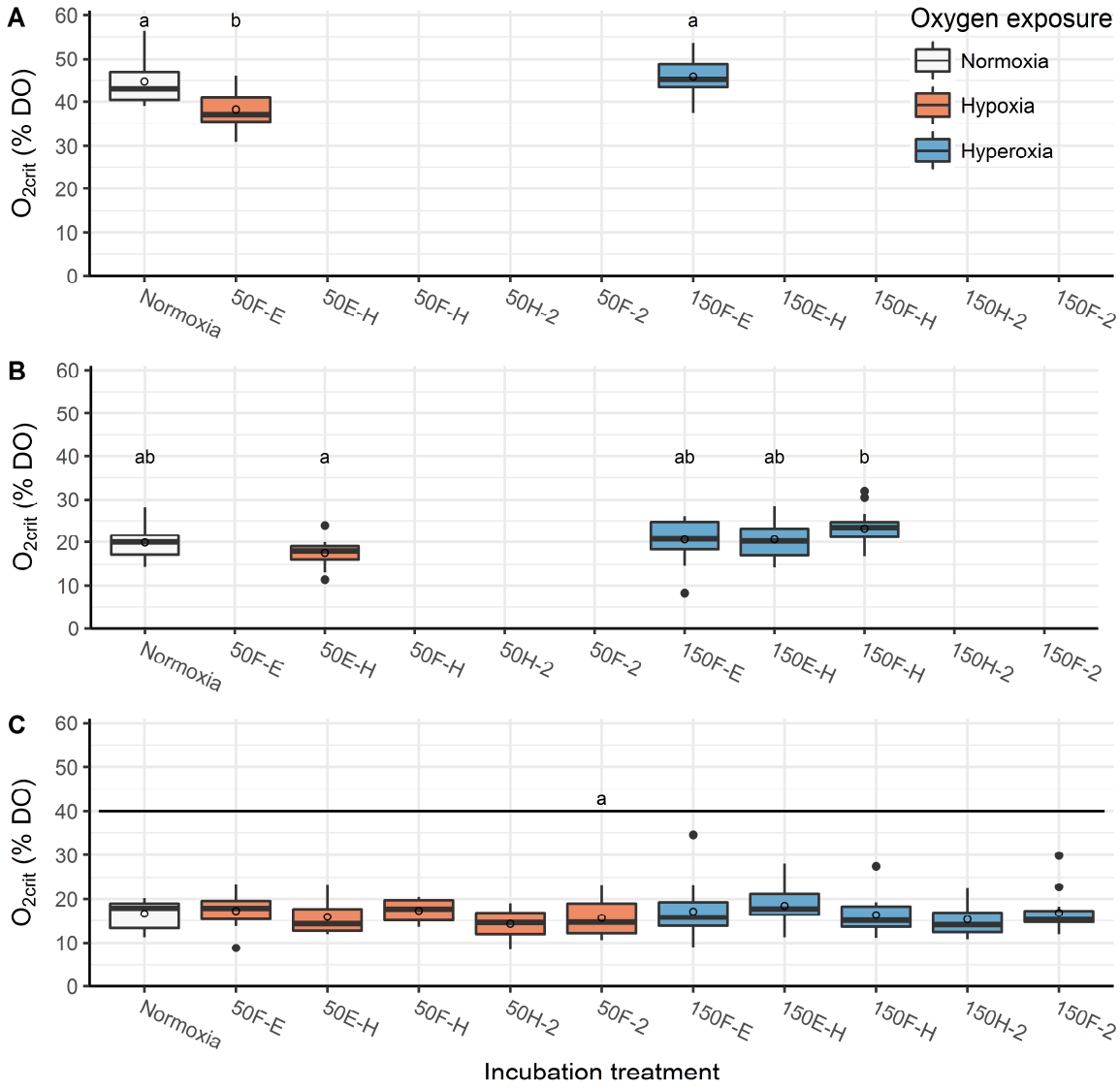

Figure 2.3: Boxplots of critical DO level (O_{2crit}) at the eyed-egg (A), hatch (B) and 2 week post-hatch (C) developmental stages for Atlantic salmon (*Salmo salar*) incubated in hypoxia (50% DO) or hyperoxia (150% DO) during different developmental windows (see Fig. 2.1). Different letters represent significant differences between incubation treatment groups within each measurement time-point (Tukey, $P < 0.05$). The solid central line is the median, the box denotes 25th and 75th percentile, whiskers extend to the highest or lowest value within 1.5*inter-quartile range and filled circles are outliers beyond that range. Mean values are represented by open circles.

Growth responses to hypoxia and hyperoxia

Growth was slower in embryos incubated in hypoxia from fertilisation until hatching, similar to other studies that have found slower growth for salmonid embryos incubated in hypoxia pre-hatch (Fig. 2.2) (Ciuhandu et al., 2005; Hamor and Garside, 1977). Hypoxia exposure after hatching did not affect growth (Fig. 2.2C, D), which contrasts with a previous study where Atlantic salmon alevins incubated at a similar DO (50%) for 2 weeks were ~10% smaller than normoxia-incubated alevins (Polymeropoulos, 2013). The mean O_{2crit} of post-hatch alevins across all incubation treatment groups in the present study was between 14.2 – 20.6% DO, which was well below the hypoxia treatment level of 50% DO. Therefore, we would not necessarily expect the hypoxia level in the present study to be severe enough to restrict the growth of alevins following hatching because aerobic metabolism would not be limited.

Enhanced growth (compared with normoxia) was measured in embryos exposed to hyperoxia from eyed-egg to hatching stage (150E-H) when measured at 4 weeks post-hatch (i.e. after ~4 weeks return to normoxia) (Fig. 2.2D). This is a surprising result, because increased growth would have had to occur during a developmental window when alevins were incubating in normoxia. In addition, hyperoxia incubation at any other developmental window did not improve growth compared to normoxia-incubated salmon. The developmental period from eyed-egg to hatching stage is likely to be the most sensitive to hyperoxia exposure, because it is when embryonic O_{2crit} is highest and therefore oxygen uptake is most likely to be limited (Rombough, 1988a). Hyperoxia-incubated salmonid embryos have been reported to hatch later than those in normoxia, indicating that hyperoxia may alleviate respiratory stress caused by limited oxygen supply in normoxic conditions (Dimichele and Taylor, 1980; Latham and Just, 1989). As such, oxygen availability in normoxic conditions may be limited close to hatching, and therefore a relative increase in oxygen availability caused by hyperoxia exposure may cause an immediate increase in growth rate. However, there were no immediate growth enhancements (i.e. when measured at the completion of hyperoxia exposure) in hyperoxia-incubated salmon between eyed-egg and hatching stage (150E-H, 150F-H, 150F-2, and 150F-4) compared to normoxia-incubated embryos. The lack of hyperoxia growth response during the developmental window immediately prior to hatching is similar to a previous study where hyperoxia incubation prior to hatching did not improve the growth of pre-hatch rainbow trout (Ciuhandu et al., 2005). These results suggest suggests

that oxygen uptake is not limited when salmonid embryos are incubated in normoxia, even immediately prior to hatching when the capacity for oxygen uptake rate to meet metabolic oxygen demand is most severely affected by oxygen diffusion limitations through the egg capsule (Rombough and Ure, 1991; Wells and Pinder, 1996).

Aerobic metabolism and hypoxia tolerance

Reduced $\dot{M}O_2$ and O_{2crit} in hypoxia-incubated embryos at the eyed-egg stage, in combination with decreased growth, indicates that embryonic metabolism may have been depressed when they were exposed to 50% DO prior to hatching (Figs. 2.3A & 2.4A, B). Metabolic depression reduces embryonic energy requirements by down-regulating energy intensive processes such as protein synthesis to match available oxygen levels, thereby reducing the need for anaerobic respiration (Richards, 2010). Metabolic depression has been previously measured via calorimetry in Arctic charr (*Salvelinus alpinus)* embryos, where 8 h of anoxia caused 80% metabolic depression which was not recovered until they were returned to normoxia for 24 – 36 h (Gnaiger et al., 1987). In the present study, $\dot{M}O_2$ was reduced in hypoxia, however, it would be necessary to quantify the extent of anaerobic metabolism to confirm the magnitude of metabolic depression in Atlantic salmon embryos. The critical oxygen level to maintain routine $\dot{M}O_2$ (O_{2crit}) of some embryos at the eyed-egg stage was above 50% DO. Therefore, it is possible that embryos exposed to long-term hypoxia (50% DO) would need to depress their metabolic energy requirements (thereby reducing O_{2crit} below 50% DO) to meet the available oxygen supply and avoid long-term anaerobic metabolism. Following hatching, $\dot{M}O_2$ and O_{2crit} were no longer reduced in hypoxia-incubated embryos, indicating that any metabolic depression associated with hypoxia exposure had ceased. Hatching is associated with a relative increase in oxygen availability to the embryo, and this translated to a decrease in O_{2crit} of normoxia-incubated embryos from 44.7 ± 1.7% DO at the eyed-egg stage to 19.8 ± 0.8% DO at the hatching stage (Fig. 2.3) (Dhiyebi et al., 2013). As a result of the relative increase in oxygen availability associated with hatching, metabolic depression may have stopped in hypoxia-incubated alevins (Gnaiger et al., 1987; Rissanen et al., 2006). As such, the lack of physiological and growth response to hypoxia following hatching was possibly due to oxygen levels that were not severe enough to cause cardio-respiratory stress or limit the capacity for oxygen uptake to meet metabolic oxygen demand.

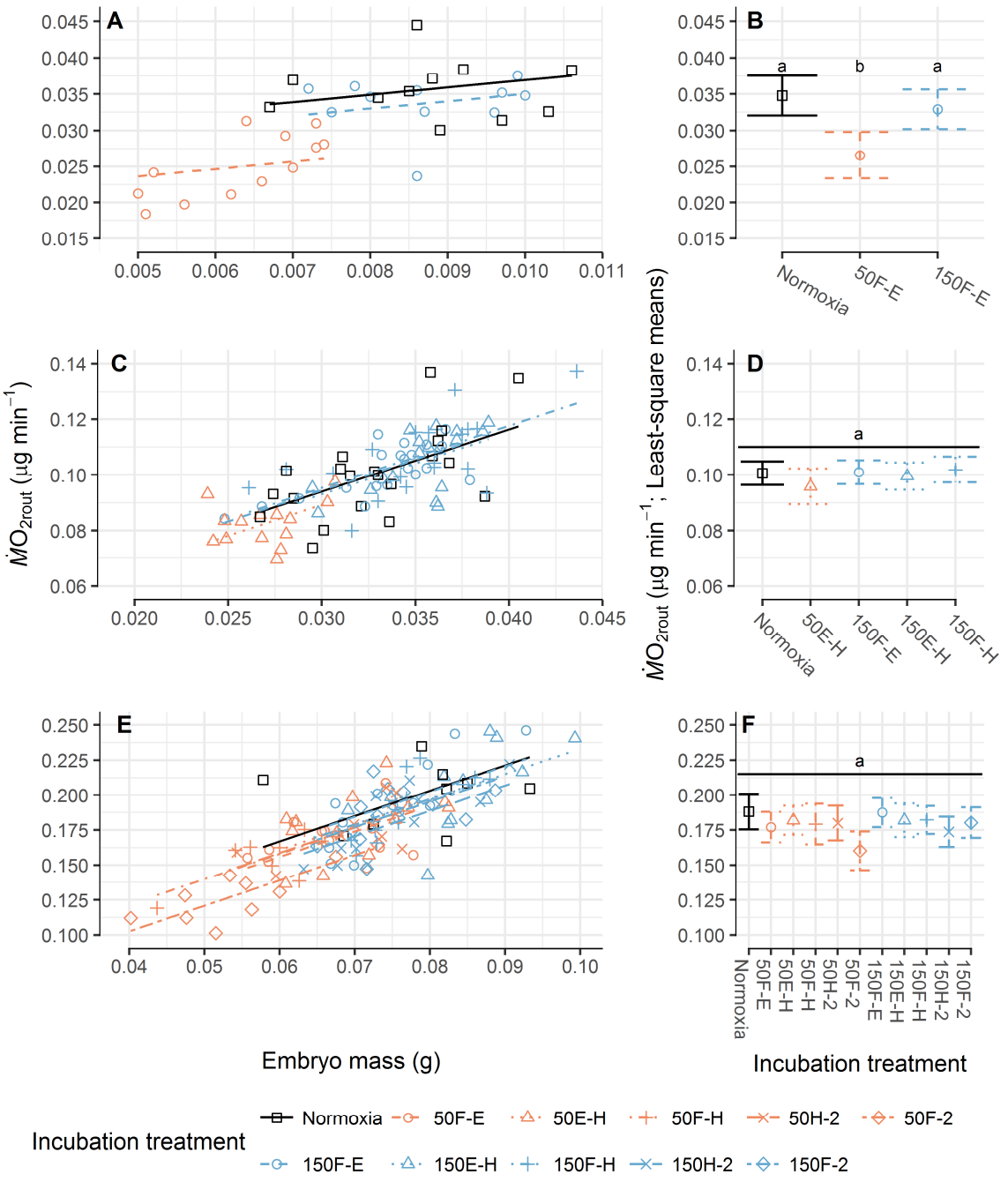

Figure 2.4: Linear relationships between routine metabolic rate ($\dot{M}O_{2rout}$) and embryo mass at the eyed-egg (A), hatch (C) and 2 weeks post-hatch (E) stages of Atlantic salmon (*Salmo salar*) exposed to hypoxia (50% DO) or hyperoxia (150% DO) during different developmental windows (see Fig. 2.1). Data points represent individuals and lines represent linear regressions for each developmental stage (common slopes were used since there was no covariate interaction between embryo mass and incubation treatment). The least-square means are shown for the eyed-egg (B), hatch (D) and 2 week post-hatch (E) developmental stages with error bars representing 95% confidence intervals. Different letters indicate significant differences between incubation treatment groups (Tukey, $P < 0.05$). Statistical values are reported on page 29.

Decreased $\dot{M}O_2$ and O_{2crit} of hypoxia-incubated embryos at the eyed-egg stage may also be caused by differences in the developmental stage of embryos (Figs. 2.3A & 2.4A). In the study we did not determine the developmental stage of embryos prior to measurement, however, hypoxia-incubated embryos were likely at an earlier developmental stage at each measurement time-point because the developmental rate of Atlantic salmon is delayed by up to 7 days prior to hatching when incubated in 50% DO (Hamor and Garside, 1976). The mass-specific $\dot{M}O_2$ of Atlantic salmon embryos is highly variable between developmental stages (Garside, 1959; Hamor and Garside, 1979). As such, the decrease in $\dot{M}O_2$ of embryos incubated in 50% DO in the present study may be caused by lower metabolic oxygen requirements at an earlier developmental stage. However, following hatching there was no effect of hypoxia on $\dot{M}O_2$, even though the developmental stage of hypoxia-incubated embryos is likely to remain delayed after hatching. Further increasing the complexity of future studies, incorporating the effects of both post-fertilisation age and developmental stage will help to further refine our understanding of the metabolic requirements of salmon embryos in the face of variable oxygen supply.

Hypoxia did not affect the $\dot{M}O_{2rout}$ of Atlantic salmon following hatching (Fig. 2.4C-F). This finding is in contrast with those reported for Atlantic salmon incubated in hypoxia (50% DO) for 2 weeks after hatching, where $\dot{M}O_2$ was higher than in normoxia-incubated embryos (Polymeropoulos, 2013). Increased $\dot{M}O_2$ has also been reported in hypoxia-incubated zebrafish, although only at oxygen levels below 25% DO (Robertson et al., 2014). We suggest that the lack of physiological response to hypoxia incubation of post-hatch Atlantic salmon in the present study may be because the hypoxia treatment level (50% DO) was not severe enough to induce physiological modifications aimed at increasing oxygen delivery. Indeed, the mean O_{2crit} of post-hatch alevins from any incubation treatment group did not exceed 20.6% DO, meaning that an oxygen level of 50% DO would not limit $\dot{M}O_{2rout}$ of alevins. However, oxygen levels in under-gravel redds and aquaculture incubation systems can fall well below 20% DO, possibly causing physiological modifications to alleviate respiratory stress. To better understand the effects of hypoxia from an ecological and physiological context, future studies of Atlantic salmon alevins should consider the varying hypoxia thresholds that induce physiological stress throughout development to ensure incubation in sufficiently stressful conditions.

Conclusions

Our results suggest that during early development Atlantic salmon are most sensitive to hypoxia exposure at the stages between fertilisation and hatching. Lower growth, $\dot{M}O_{2rout}$ and O_{2crit} in hypoxia-incubated embryos at the eyed-egg stage are likely consequences of metabolic depression, but future investigation of the anaerobic contribution to metabolism in hypoxia would help to support this suggestion. Hyperoxia had negligible effects during all developmental windows, while hypoxia ceased to have an effect on growth, $\dot{M}O_{2rout}$ and O_{2crit} following hatching. Overall, the hypoxia (50% DO) and hyperoxia (150% DO) levels used in the present study are unlikely to significantly impact the incubation of Atlantic salmon in natural or aquaculture incubation systems. However, more severe but transient levels of hypoxia and hyperoxia are common in natural habitats and aquaculture incubation systems, and may be capable of inducing physiological modifications during development.

Chapter 3: The effect of hypoxia exposure during development on the survival, growth and metabolic physiology of Atlantic salmon

Abstract

Salmonids incubating in natural redds and artificial systems are susceptible to periods of hypoxia caused by hydrological events, embryo crowding or management protocols. Hypoxia during early development can reduce salmon growth, development and survival, and delay hatching, potentially impacting future performance and survival upon leaving the redd or during subsequent aquaculture rearing. However, salmon embryos can also limit the effects of hypoxia by metabolic depression, premature hatching or physiological modifications to improve oxygen delivery. Notably, most studies have measured the effects of constant hypoxia during incubation, however, cyclical hypoxia exposures generally have greater relevance to natural and aquaculture systems. Here, we investigated the effects of constant and cyclical hypoxia on Atlantic salmon (*Salmo salar*) from fertilisation, through hatching, until 113 days post-fertilisation. We incubated salmon in either normoxia (100% DO), moderate hypoxia (~63% DO), severe hypoxia (~27% DO) or cyclical hypoxia (~100-25% DO daily). Severe hypoxia produced deformed embryos, severely decreased growth and development and resulted in 99.3% mortality by 113 days post-fertilisation. Cyclical hypoxia did not affect survival but slowed overall growth and development and delayed hatching in comparison with the normoxic group. At the eyed-egg stage $\dot{M}O_2$ was reduced in cyclical and moderate hypoxia incubating salmon, suggesting embryos compensate for hypoxia by depressing their metabolism. This study suggests that Atlantic salmon have a considerable capacity to survive daily bouts of extreme hypoxia, however, the consequent reductions in growth, development and hatching may still have implications for future survival.

Introduction

Prevalence of hypoxia during incubation

Incubating salmon are largely immobile and are therefore susceptible to varying oxygen levels in the incubation environment. In natural redds, the oxygen conditions within the egg pocket are dependent on the oxygen level in the water, stream flow characteristics (flow rate and groundwater mixing), egg density and developmental stage (Ciuhandu et al., 2007; Dhiyebi et al., 2013; Greig et al., 2007; Miller et al., 2008). Temporal variation in hydrological conditions can result in highly dynamic oxygen conditions within the redd where oxygen drops below 5% DO (Malcolm et al., 2006; Schindler Wildhaber et al., 2014; Sear et al., 2014).

Salmon reared for aquaculture are incubated in artificial systems designed to maintain normoxic conditions during early development, however, the oxygen level these systems can also depend on egg density, developmental stage, water flow, and management protocols (Billard and Jensen, 1996; Dhiyebi et al., 2013; McLean and Lim, 1985; Miller et al., 2008). For example, oxygen levels can fluctuate to below 20% DO multiple times in a day at certain locations within Heath tray incubators containing hatching stage Atlantic salmon (*Salmo salar*; Appendix A). In addition, the oxygen level in Heath tray systems can decline as water cascades vertically through consecutive trays (Appendix A; McLean and Lim, 1985). Similar periods of localised hypoxia occur in other intensive rearing systems such as keeper channels, where oxygen levels as low as 1 mg L^{-1} (~10% DO) were measured in pockets of crowded alevins (Britton et al., 1983). Thus, incubating salmon in both natural and aquaculture systems can experience periods of hypoxia that may cause a significant physiological challenge due to the limited supply of oxygen not meeting metabolic demands.

Effects of hypoxia during incubation

Hypoxia during early salmon development can slow growth, developmental rate and delay hatching (Ciuhandu et al., 2005; Hamor and Garside, 1976). In Chapter 2 shows that Atlantic salmon incubated in 50% DO were smaller than those incubated in normoxia, however, only when exposed to hypoxia between fertilisation and hatching. Sufficiently severe hypoxia levels can also reduce growth at later life stages, with a ~20% reduction in the mass of Arctic charr (*Salvelinus alpinus*) exposed to 33 mmHg (~25% DO) for 47 days following hatching (McDonald and McMahon, 1977). Delays in development have also been reported for

Atlantic salmon exposed to 50% DO for 2 weeks following hatching (Polymeropoulos, 2013). It is generally acknowledged that the severity of growth or development impairments is dependent on the magnitude and frequency of hypoxia during incubation (Hamor and Garside, 1976; Hamor and Garside, 1977).

Chapter 2 shows that Atlantic salmon incubated in hypoxia (50% DO) from fertilisation until eyed-egg stage had a lower routine oxygen uptake rate ($\dot{M}O_{2rout}$), possibly due to metabolic depression. Similar reductions in $\dot{M}O_2$ have been measured in rainbow trout (*Oncorhynchus mykiss*) incubated in hypoxia up until hatching (Miller et al., 2008). However, following hatching salmon become increasingly reliant on the cardio-respiratory system for oxygen uptake, which may increase the opportunity for physiological responses to mitigate the limiting effects of hypoxia (Rombough and Ure, 1991; Wells and Pinder, 1996). For example, after 47 days of hypoxia-incubation following hatching, Arctic charr increased heart rate, ventilation rate and gill lamellae surface area in comparison to normoxia-incubated fish (McDonald and McMahon, 1977). The limitations of hypoxia incubation on metabolism can negatively impact growth and development, however salmon may be able to compensate for oxygen limitation with a variety of physiological responses as the cardio-respiratory system develops.

Near hatching the oxygen level required to maintain the metabolic energy requirements of salmonid embryos increases to near normoxic levels (Rombough, 1988a). A decrease in oxygen availability close to hatching can cause respiratory stress that induces premature hatching. For example, rainbow trout hatching was initiated by exposure to oxygen levels below 94 mmHg (~60% DO) 1 day prior to hatching in normoxia-incubated embryos (Latham and Just, 1989). Hatching of Atlantic salmon was induced up to 3 weeks earlier than hatching in normoxia when embryos were transferred from normoxia to extreme hypoxia below 4 mmHg (~3% DO) (Oppen-Berntsen et al., 1990). However, the effect of hypoxia on hatching is dependent on the time of exposure. Indeed, chum salmon (*Oncorhynchus keta*) only hatched prematurely when exposed to hypoxia at ~0.8 mg L^{-1} (~ 7% DO) at 452 degree days, and hatching was instead delayed when they were exposed to constant hypoxia at earlier developmental stages (Alderdice et al., 1958). Reduced growth and development associated with hypoxia incubation at early developmental stages may delay hatching and reduce size and developmental stage at hatching. Bouts of hypoxia (30% DO) from fertilisation also reduce Atlantic salmon embryo size at hatching (Hamor and Garside, 1977).

Similarly, Atlantic salmon exposed to hypoxia as low as 10% DO for intermittent periods before 371 degree days hatched later and were developmentally delayed at hatching compared to embryos incubated in normoxia (Bloomer et al., 2016). Such sub-lethal impairments of hypoxia-incubated salmonids may have lasting impacts on their performance and fitness. Salmon that are smaller and emerge later are more likely to succumb to predation, are outcompeted for resources and may delay migration to marine environments (Einum and Fleming, 2000; Metcalfe et al., 1990; Skoglund and Barlaup, 2006).

The effects of intermittent or cyclical hypoxia

Notably, current knowledge regarding the effects of hypoxia on incubating salmon has stemmed primarily from experiments using constant levels of hypoxia. Little is known about the effects of cyclical or intermittent hypoxia incubation of fish despite the fact that it is more relevant to natural and aquaculture systems (Appendix A; Malcolm et al., 2006; Sear et al., 2014). However, evidence from other species and life stages suggests that responses may vary between cyclical hypoxia and constant hypoxia incubation. For example, spotted salamander (*Ambystoma maculatum*) embryos incubated in constant hypoxia (~14% DO) had greater reductions in growth and development when compared with cyclical hypoxia incubation below ~3 kpa (~14% DO) for 13 hours day^{-1} (Mills and Barnhart, 1999; Valls and Mills, 2007). Similarly, sub-adult European sea bass (*Dicentrarchus labrax*) exposed to constant hypoxia (40% DO) grew slower than fish exposed to a 12 hour daily hypoxia cycle (40% DO) (Thetmeyer et al., 1999). These findings may be dependent on the magnitude of hypoxia, as the growth rate of southern flounder (*Paralichthys lethostigma*) exposed to daily cyclical hypoxia (94 - 40% DO) was faster than those in constant hypoxia of ~40% DO, but similar to fish exposed to constant hypoxia of ~68% DO (Taylor and Miller, 2001).

Differential physiological responses to cyclical hypoxia compared to constant hypoxia have also been reported in fish. Adult killifish (*Fundulus heteroclitus*) reduced critical oxygen level (O_{2crit}) when exposed to cyclical (12 hours day^{-1}) or constant hypoxia of 5 kPa (~24% DO) or 2 kPa (~10% DO) compared to normoxia acclimated fish (Borowiec et al., 2015). However, only killifish exposed to constant hypoxia had increased haematocrit and haemoglobin concentrations, whereas those acclimated to cyclical hypoxia had similar haematocrit and haemoglobin concentrations but increased capillarity of glycolytic muscle (Borowiec et al., 2015). Given the dynamic oxygen levels in which salmonids develop, it is

important to understand whether the reduced magnitude of responses to cyclical hypoxia compared to constant hypoxia in other species and age classes are transferrable. Specifically, whether any differential responses may occur in developing salmon upon exposure to constant versus cyclical hypoxia.

Objectives

This study compared the growth, development, hatching, oxygen uptake rate ($\dot{M}O_2$) and hypoxia tolerance (O_{2crit}) of Atlantic salmon incubated in daily cyclical hypoxia with those incubated in constant hypoxia or normoxia. Based on the evidence for reduced growth and $\dot{M}O_2$ in hypoxia-incubated salmon embryos, we hypothesised that in general hypoxia will reduce growth and developmental rates with associated reductions in $\dot{M}O_2$ and increased time to hatch. In addition, we predicted that the magnitude of the response to cyclical hypoxia will be lower than severe hypoxia but larger than normoxia, because of the reduced duration spent at severe hypoxia levels.

Methods

Fertilisation and brood-stock source

The methods used here are similar to those in Chapter 2 and will be briefly described with reference to the previous chapter. All-female salmon embryos were collected from sixteen Tasmanian Atlantic salmon families (see Chapter 2) that were created by the fertilisation of eggs from female salmon with the milt from sex-reversed female (neo-male) salmon on 21 May 2015. An equal proportion of fertilised eggs from each family were thoroughly mixed together and transported in freshwater to the CSIRO Marine Laboratories, Hobart, Tasmania within 4 hours of fertilisation.

Incubation treatments

Fertilised eggs were randomly allocated between 8 Heath trays (L x W x H = 39 x 32 x 5.5 cm; Marisource, USA) at ~8°C at a density of ~2100 eggs per tray. Embryos and larvae (alevins) were exposed from within 4 hours of fertilisation until 113 days post-fertilisation (DPF) to one of four treatments (2 replicate Heath trays per treatment): 102.6 ± 1.1% DO at 8.13 ± 0.18°C (normoxia; mean ± SD, Fig. 3.1A), 63.0 ± 3.3% DO at 8.05 ± 0.16°C (moderate hypoxia, Fig. 3.1B), 27.0 ± 4.0% DO at 7.88 ± 0.19°C (severe hypoxia, Fig. 3.1C)

or 24 hour cyclical hypoxia between a mean daily maximum and minimum of 99.3 ± 2.2% DO and 27.3 ± 5.3% DO (overall mean: 65.6 ± 22.7% DO; 8.14 ± 0.19°C; Fig. 3.1D). The cyclical hypoxia treatment spent approximately 2 hours per day both below 30% DO and above 95% DO, with DO increasing or decreasing between maximum and minimum levels at approximately 6.5% DO h^{-1} (Fig. 3.1E).

Modified Heath trays received an individual water supply at 10 L min^{-1}. The moderate hypoxia, severe hypoxia and cyclical hypoxia incubation treatments received freshwater from separate 200 L treatment sumps that exchanged water with a 600 L semi-closed recirculating freshwater filtration system (Tropical Marine Centre, UK). The normoxia treatment received water directly from the 600 L semi-closed recirculating filtration system. Dissolved oxygen concentrations were maintained in the 200 L treatment sumps and 600 L filtration system by injecting either nitrogen (hypoxia treatments) or oxygen (normoxia treatment) gas controlled by an OxyGuard Pacific oxygen monitoring system (OxyGuard, Denmark) and PowerLab 4SP (ADInstruments, Australia). Water temperature and room temperature were maintained at ~8°C by a software-controlled custom heat exchanger system (Building Automation Controls, Tasmania, Australia). Water quality was monitored at least every second day and maintained between 0 – 1.36 mg L^{-1} NH_3-N (ammonia), 0 – 0.015 mg L^{-1} NH_2-N (nitrite), 7.15 – 7.8 pH and 20 – 70 mg L-1 $CaCO_3$ (alkalinity) through periodic exchange with dechlorinated tap water. The light:dark cycle was set to 14:10 h.

The eggs were treated with formalin to prevent fungal growth (as described in Chapter 2) on 5 occasions between 8 and 33 DPF and physically agitated once between 40 and 42 DPF for the normoxia, moderate hypoxia and cyclical hypoxia treatment and at 56 DPF for the severe hypoxia treatment. Dead eggs and alevins were counted and removed as necessary from 40 to 113 DPF.

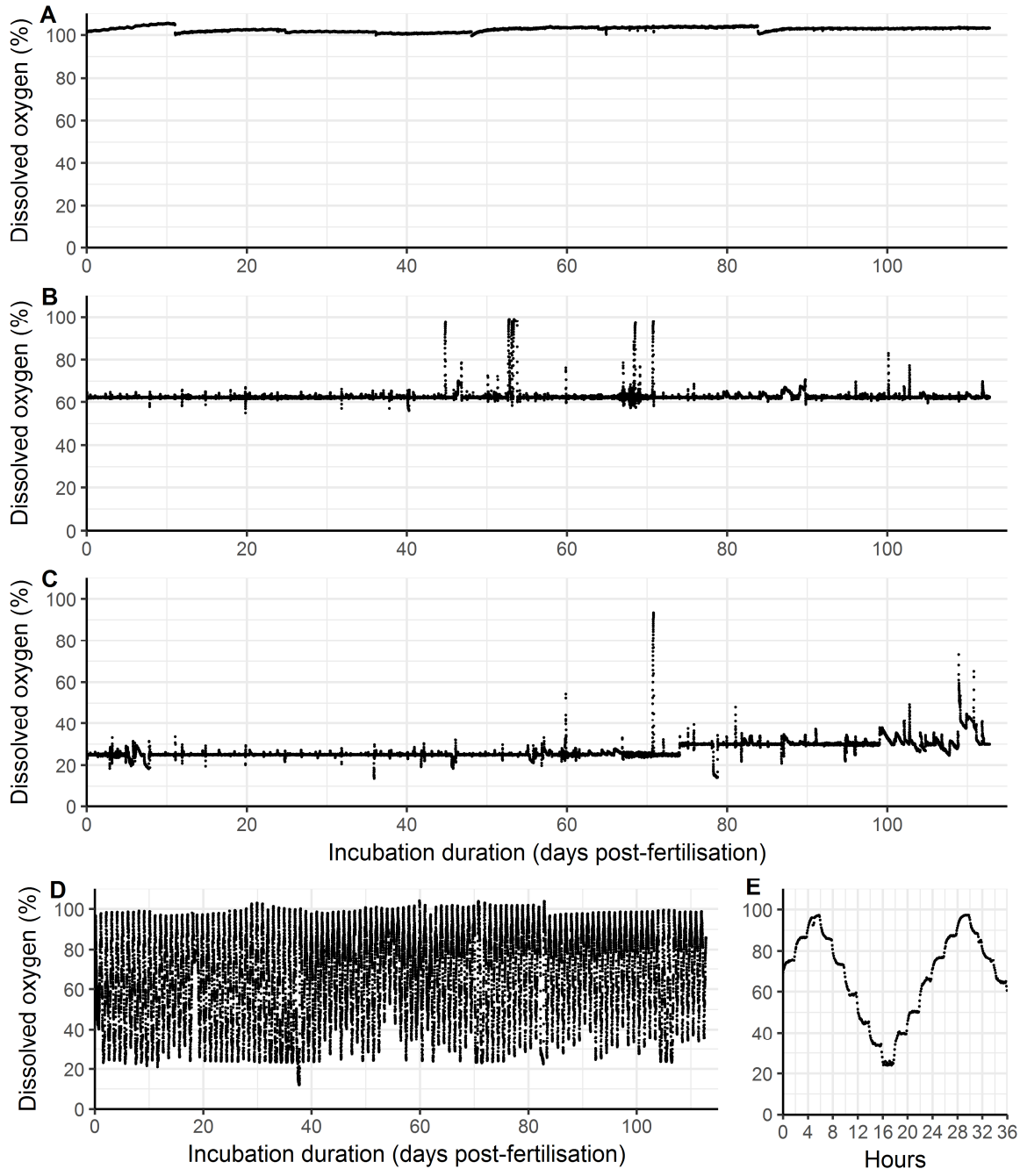

Figure 3.1: Dissolved oxygen level (% air saturation) measured within the normoxia (A), moderate hypoxia (~63% DO; B), severe hypoxia (~27% DO; C) or cyclical hypoxia (100-25% DO daily; D) treatment sumps throughout the incubation treatment exposure period. Panel E shows dissolved oxygen measurements from the cyclical hypoxia treatment sump for a 36 hour period showing a typical hypoxia cycle repeated throughout incubation.

Mass, developmental stage and hatching

Embryo and alevin developmental stage was determined periodically between 26 and 113 DPF. Embryos and alevins were dissected from their egg and/or their yolk sac removed in 2X

Holtfreter's solution (NaCl: 0.7 g, KCl: 0.1 g, $CaCl_2$: 0.2 g, $NaHCO_3$: 0.4 g, dissolved in 1 L distilled water) (Dunham, 2011; Gorodilov, 1996). Following dissection, the embryos and larvae were viewed with a dissecting microscope and assigned a numerical developmental stage (τ_s) determined using the morphological features described by Gorodilov (1996). Eggs and alevins were weighed to ± 0.001 g as described in Chapter 2 from 33-113 DPF with a Sartorius LC3200D balance (Sartorius, Germany).

Hatching was monitored daily between 54 to 66 DPF by counting all eggs and alevins in each replicate treatment tray for the normoxia, moderate hypoxia and cyclical hypoxia incubation treatment groups. The severe hypoxia treatment was excluded from hatch counts due to high mortality and low and sporadic hatching success. It was assumed that any unhatched eggs at 66 DPF would fail to hatch.

Respirometry

Oxygen uptake rate ($\dot{M}O_2$) measurements were conducted at similar developmental stages for each incubation treatment group. Measurements took place at four development stages: 'eyed stage' (161.5-163.5 τ_s), ~10 days pre-hatch (236.5-241 τ_s), ~3 days pre-hatch (256-265 τ_s) and ~10 days post-hatch (335-360 τ_s; Table 3.1). Oxygen uptake rates were measured for individual embryos (2.7 mL respirometers) or alevins (15.5 mL respirometers) within closed respirometers. On each measurement day, approximately equal numbers of individuals were randomly selected from each replicate Heath tray per incubation treatment group and sealed in respirometers in water from the corresponding incubation treatment system. Individuals were removed from the cyclical hypoxia incubation treatment when the DO was greater than 80% DO.

Respirometry measurements commenced at ~110 % DO (achieved by bubbling pure oxygen gas through the water) for the normoxia and cyclical hypoxia treatment and ~75 % DO for the moderate hypoxia treatment. Dissolved oxygen concentration was measured until the oxygen decline had ceased (representing mortality) or the oxygen level was lower than 5% DO in respirometers containing embryos or alevins. At least one respirometer per plate contained only water during each $\dot{M}O_2$ measurement period to measure background respiration for the 2.7 mL (embryo) respirometers. Background respiration for the 15.5 mL (alevin) respirometers was measured during two measurement periods where all

respirometers contained only water from the normoxia incubation system. Upon completion of $\dot{M}O_2$ measurements the embryos and larvae were removed from the chambers and weighed as whole eggs or alevins and yolk-free embryos and alevins as described previously.

The respirometer setup was similar to that in Chapter 2, with the addition of six well respirometers (33.6 mm diameter x 20.5 mm deep; 15.5 mL). Temperature in the respirometers was maintained at 8.04 ± 0.24°C (± SD) by placing the respirometer system in a chest freezer modified with a custom built temperature controller.

Table 3.1: Respirometry measurement timing and sample sizes of Atlantic salmon (*Salmo salar*) exposed to normoxia, moderate hypoxia (~63% DO) or cyclical hypoxia (100-25% DO daily).

Development group	Developmental stage (τ_s)	Incubation treatment	Days post-fertilisation	Accumulated thermal exposure (degree days)	Sample size
Eyed	161.5-163.5	Normoxia	35	300	22
		Moderate hypoxia	35	298	22
		Cyclical hypoxia	39	335	26
10 days pre-hatch	236.5-241	Normoxia	47	390	27
		Moderate hypoxia	49	403	16
		Cyclical hypoxia	52	433	18
3 days pre-hatch	256-265	Normoxia	54	448	10
		Moderate hypoxia	56	459	11
		Cyclical hypoxia	58	482	22
10 days post-hatch	335-360	Normoxia	67	555	15
		Moderate hypoxia	70	572	18
		Cyclical hypoxia	72	596	21

Data analysis and statistics

The rate of declining DO (% DO min $^{-1}$) was determined using least-squares regression for consecutive 1200 s (eyed, 10 days pre-hatch and 3 days pre-hatch) or 1800 s slopes (10 days post-hatch), and $\dot{M}O_2$ (μg O_2 min^{-1}) calculated using equation (2.1) in Chapter 2. The mean background respiration of 2.7 mL respirometers for each measurement period was subtracted from all fish $\dot{M}O_2$ measurements for that measurement period. The mean background

respiration for all 15.5 mL respirometers during the two background respiration measurement periods was subtracted from all fish $\dot{M}O_2$ measurements in 15.5 mL respirometers. The O_{2crit} and routine $\dot{M}O_2$ ($\dot{M}O_{2rout}$) was determined as described in Chapter 2.

The accumulated thermal exposure (degree days) was calculated for each incubation treatment using equation (3.1).

$$Degree\ days = \sum_{t=0}^{t=end} \left(\frac{Temperature_t}{Duration_T}\right) \quad (3.1)$$

where t=0 is fertilisation, t=end is the end of the period of interest and $Duration_T$ is the time spent at $Temperature_t$ in days. For example, 1 day spent at 8°C is 8 degree days.

All statistical analyses were performed using R along with the packages 'lsmeans', 'car' and 'drc' (Fox and Weisberg, 2011; Lenth, 2016; R Core Team, 2016; Ritz et al., 2016). Differences in O_{2crit} between incubation treatment groups and developmental stages were tested using ANOVA. Routine $\dot{M}O_2$ was compared between incubation treatments within each developmental stage group using ANCOVA with embryo mass as a covariate. Linear regressions were fitted for each of embryo mass or developmental stage and degree days to compare growth and developmental rate between incubation treatment groups using ANCOVA. Linear regressions between embryo mass and degree days were transformed using a boxcox transformation in order to meet the assumptions of linear regression. Degree days to hatch and hatching success were analysed using a time-to-event approach. A 5 parameter log-logistic regression was fitted to the proportion of eggs hatched against degree days (excluding eggs that died prior to the hatching period), where the lower asymptote was assumed to be 0 (Ritz et al., 2016; Ritz et al., 2013). The fitted model was then used for pairwise comparisons of degree days to hatch (percentage of eggs that ultimately hatched) and total percentage of eggs hatched (upper asymptote). Survival was compared between incubation treatments using a binomial generalised linear model (GLM). All pairwise comparisons were conducted using P-value adjustment using the Tukey HSD method.

Results

Growth and developmental rate

Growth per degree day was similar for fish incubated in moderate hypoxia and normoxia (P = 0.3466; Fig. 3.2A & Table 3.2). Fish incubated in cyclical hypoxia grew slower than those incubated in moderate hypoxia, but at a similar rate to those incubated in normoxia (P = 0.0027 and P = 0.2220, respectively, Fig. 3.2A). Growth was slowest in severe hypoxia compared with all other incubation treatment groups (P < 0.0001, Fig. 3.2A). At 113 DPF (~904 degree days) alevins incubated in normoxia (0.166 ± 0.005 g) and moderate hypoxia (0.166 ± 0.005 g) were larger than those incubated in cyclical hypoxia (0.146 ± 0.004 g; P = 0.03 and 0.027, respectively.

Embryos incubated in cyclical hypoxia were delayed by ~40 degree days (5 days at 8°C) to reach the same developmental stage as normoxia and moderate hypoxia incubated embryos, despite similar developmental rates (P < 0.0001, Fig. 3.2B & Table 3.2). However, between the eyed-egg stage and 113 DPF the developmental rate was similar for fish incubated in normoxia, moderate hypoxia and cyclical hypoxia (P > 0.48, Fig. 3.2B). Incubation in severe hypoxia reduced the developmental rate compared with all other treatment groups (P < 0.0001, Fig. 3.2B).

Oxygen uptake and hypoxia tolerance

At the eyed-egg developmental stage the $\dot{M}O_{2rout}$ of fish incubated in moderate hypoxia and cyclical hypoxia was ~20% lower for a given embryo mass (comparison of least-square means) than those incubated in normoxia (both P < 0.0001, Fig. 3.3A, B). However, at 10 days pre-hatch the $\dot{M}O_{2rout}$ of embryos incubated in normoxia and cyclical hypoxia was similar (P = 0.123), whereas the $\dot{M}O_{2rout}$ of fish incubated in moderate hypoxia was ~10% lower than the normoxia incubation treatment (P = 0.0005, Fig. 3.3C, D). At 3 days pre-hatch fish incubated in all three oxygen regimes had similar $\dot{M}O_{2rout}$ ($F_{(2,39)}$ = 2.45, P = 0.094, Fig. 3.3E, F). At 10 days post-hatch the $\dot{M}O_{2rout}$ of fish incubating in moderate hypoxia was ~7% higher than those incubating in normoxia (P = 0.0064, Fig. 3.3G, H), whereas $\dot{M}O_{2rout}$ was similar between fish incubated in cyclical hypoxia and those incubated in moderate hypoxia and normoxia (P = 0.759 and P = 0.06, respectively, Fig. 3.3G, H).

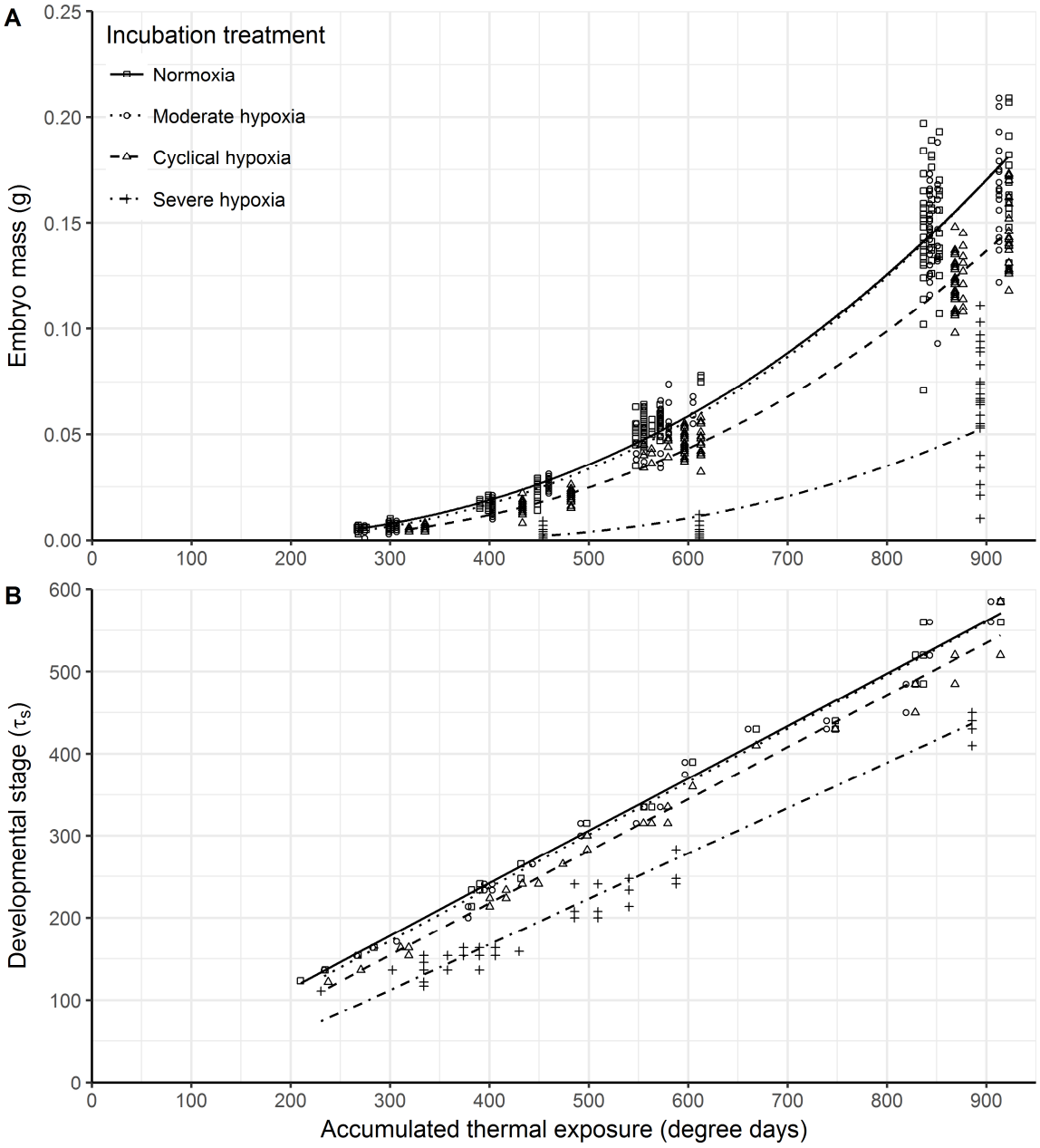

Figure 3.2: The relationship between embryo mass (A) or developmental stage (B) and accumulated thermal exposure in Atlantic salmon (*Salmo salar*) incubated in normoxia, moderate hypoxia (~63% DO), cyclical hypoxia (100-25% DO daily) or severe hypoxia (~27% DO). Points are individuals and lines are regressions (in (A) model predictions were calculated using embryo mass with exponent of 0.43). See Table 3.2 for regression equations.

At the eyed-egg and 10 days pre-hatch developmental stages the O_{2crit} of fish incubated in cyclical hypoxia was 4.2% DO and 4.8% DO lower, respectively, than fish incubated in normoxia ($P = 0.0019$ and $P = 0.0001$, respectively, Fig. 3.4A, B), however, there was no O_{2crit} difference between fish incubated in normoxia and moderate hypoxia ($P > 0.09$, Fig.

3.4A, B). Additionally, O_{2crit} was similar for fish incubated in normoxia, moderate hypoxia and cyclical hypoxia at either 3 days pre-hatch or 10 days post-hatch ($F_{(2,40)}$ = 1.53, P = 0.228 and $F_{(2,51)}$ = 2.43, P = 0.098, respectively; Fig. 3.4C, D). Across all treatments the O_{2crit} increased from between ~25-30% DO at the eyed stage to ~35-40% DO at 10 days pre-hatch and ~43-46% DO at 3 days pre-hatch, followed by a decrease to ~17-19% DO at 10 days post-hatch (all pairwise comparisons P < 0.004, Fig. 3.4A-D).

Table 3.2: Regression equations for relationships between embryo mass or developmental stage and accumulated thermal exposure (degree days) of Atlantic salmon (*Salmo salar*) embryos and alevins incubated in normoxia (~100% DO), moderate hypoxia (~62% DO), cyclical hypoxia (~100 – 25% DO daily) and severe hypoxia (~25% DO). EM = embryo mass (g, boxcox transformed), DS = developmental stage (τ_s, see Gorodilov (1996)), DD = degree days.

	Incubation treatment	**Regression equation**	**R^2**	**P-value**
Embryo mass (g)	Normoxia	$EM^{0.43}$ = -0.0473+0.000571*DD	0.975	<0.001
	Moderate hypoxia	$EM^{0.43}$ = -0.0613+0.000587*DD	0.980	<0.001
	Cyclical hypoxia	$EM^{0.43}$ = -0.0725+0.000553*DD	0.986	<0.001
	Severe hypoxia	$EM^{0.43}$ = -0.1482+0.000481*DD	0.734	<0.001
Developmental stage (τ_s)	Normoxia	DS = -13.49+0.639*DD	0.991	<0.001
	Moderate hypoxia	DS = -22.38+0.647*DD	0.987	<0.001
	Cyclical hypoxia	DS = -35.74+0.634*DD	0.991	<0.001
	Severe hypoxia	DS = -54.08+0.554*DD	0.980	<0.001

Hatching

A higher proportion of eggs incubated in normoxia hatched (96.49 ± 0.33% of eggs that survived until hatching) compared with fish incubated in moderate hypoxia (95.20 ± 0.38%) or cyclical hypoxia (94.31 ± 0.41%; P = 0.010 and P < 0.001, respectively; Table 3.4 & Fig. 3.5). However, the proportion of eggs hatched was similar between the moderate and cyclical hypoxia incubation treatment groups (P = 0.109, Table 3.4 & Fig. 3.5). Hatching of the cyclical hypoxia treatment was delayed by 18-21 degree days at 10%, 50% and 90% of eggs hatched compared to both the normoxia and moderate hypoxia incubation treatment groups (all P < 0.0001, Table 3.4 & Fig. 3.5). The accumulated degree days required for 10% of eggs to hatch was similar for normoxia and moderate hypoxia incubation (P = 0.363; Fig 3.5), whereas the moderate hypoxia treatment was delayed by 1 degree day and 3.1 degree days to reach 50% and 90% eggs hatched, respectively (both P < 0.0001, Table 3.4 & Fig. 3.5).

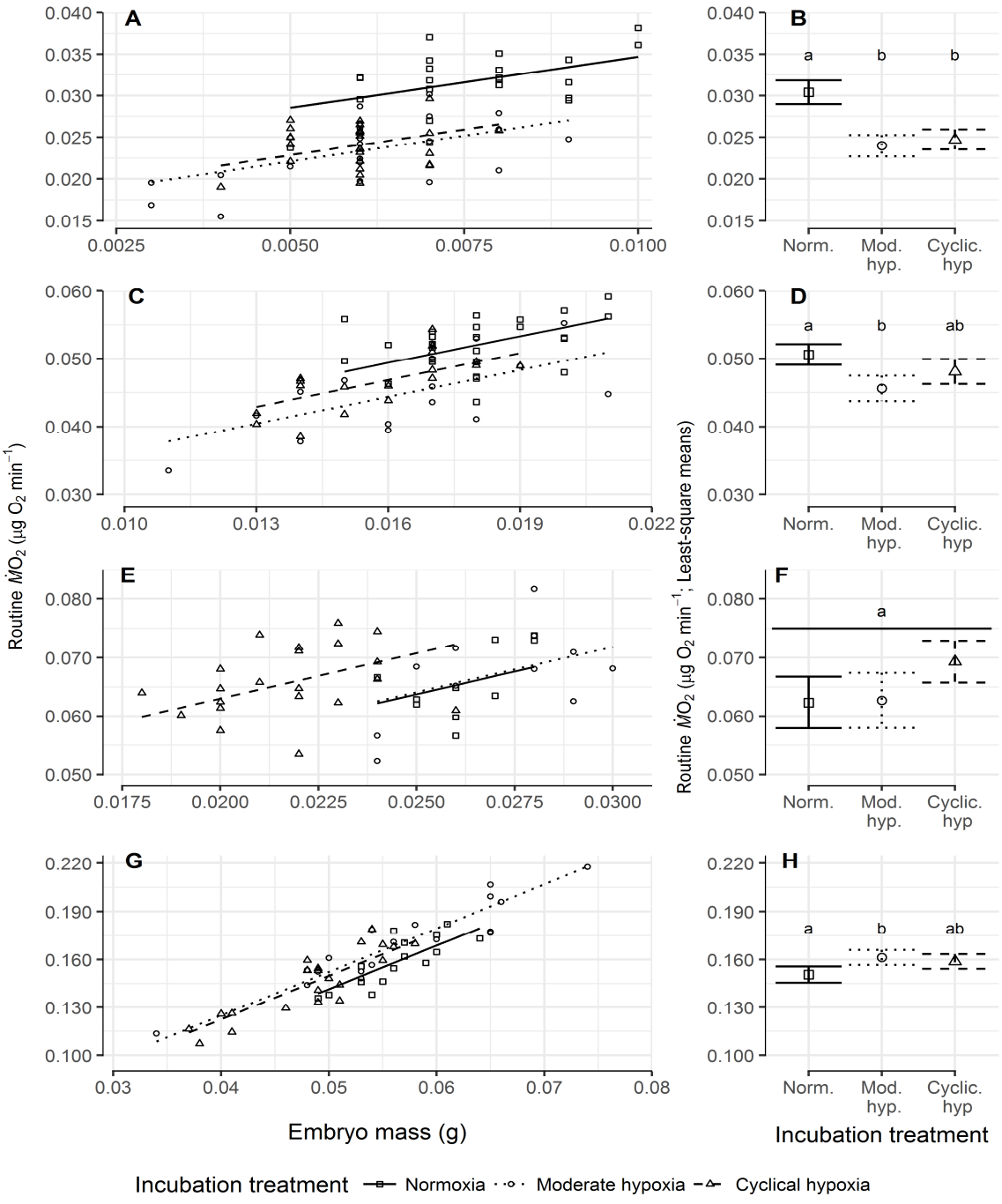

Figure 3.3: The relationship between embryo mass and routine $\dot{M}O_2$ of Atlantic salmon (*Salmo salar*) incubated in normoxia, moderate hypoxia (~63% DO) or cyclical hypoxia (100-25% DO) at eyed-egg (A), 10 days pre-hatch (C), 3 days pre-hatch (E) or 10 days post-hatch (G). Data points are individuals and lines are linear regressions for each developmental stage (common slopes were used since there was no covariate interaction between embryo mass and incubation treatment). The least-square means for routine $\dot{M}O_2$ are also shown for the eyed-egg (B), 10 days pre-hatch (D), 3 days pre-hatch (F) or 10 days post-hatch (H) developmental stages with error bars representing 95% confidence intervals. Different letters indicate significant differences between incubation treatment groups (Tukey, $P < 0.05$). Norm. = normoxia, mod. hyp. = moderate hypoxia, cyclic. hyp. = cyclical hypoxia.

Survival

Survival throughout the entire experimental period (from fertilisation until 113 DPF) was lower in the severe hypoxia incubation treatment (0.7 ± 0.1%) compared to all other treatments ($P < 0.0001$, Table 3.3). Survival of the other three treatments (normoxia, moderate hypoxia and cyclical hypoxia groups) was similar across this timeframe (74.5-76.2%, all $P > 0.30$, Table 3.3). Similarly, survival from fertilisation until egg-shocking and between egg-shocking and hatching was similar between the normoxia, moderate hypoxia and cyclical hypoxia treatment groups (80.9-81.2%, $X^2 = 0.274$, $P = 0.87$ and 99.3-99.6%, $X^2 = 2.75$, $P = 0.25$; Table 3.3). However, following hatching survival was higher in the normoxia incubation treatment (96.0 ± 0.4%) than both the moderate and cyclical hypoxia treatment groups (both 93.8 ± 0.4% and $P = 0.0002$, Table 3.3).

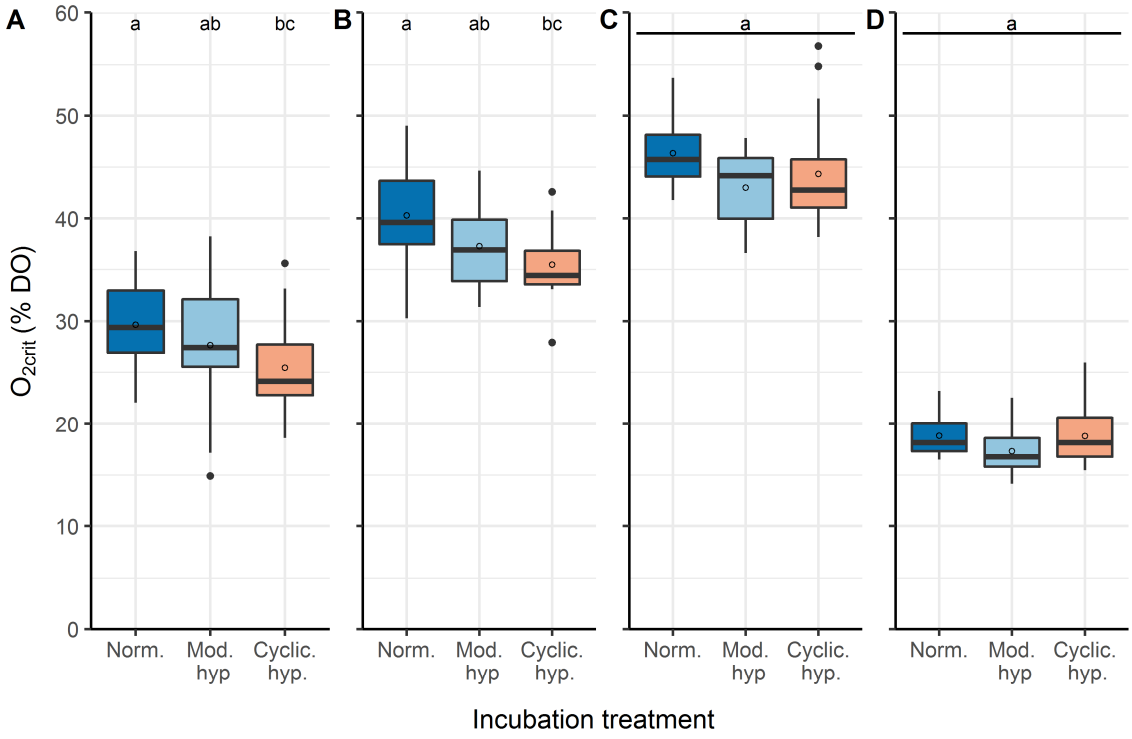

Figure 3.4: Boxplots of critical oxygen level (O_{2crit}) of Atlantic salmon (*Salmo salar*) incubated in normoxia, moderate hypoxia (~63% DO) or cyclical hypoxia (100-25% DO daily) at the eyed-egg stage (A), 10 days pre-hatch (B), 3 days pre-hatch (C) or 10 days post-hatch (D). Different letters represent significant differences between incubation treatment groups within each measurement time-point (Tukey, $P < 0.05$). The solid central line is the median, the box denotes 25th and 75th percentile, whiskers extend to the highest or lowest value within 1.5*inter-quartile range and filled circles are outliers beyond that range. Mean values are represented by open circles. Norm. = normoxia, mod. hyp. = moderate hypoxia, cyclic. hyp. = cyclical hypoxia.

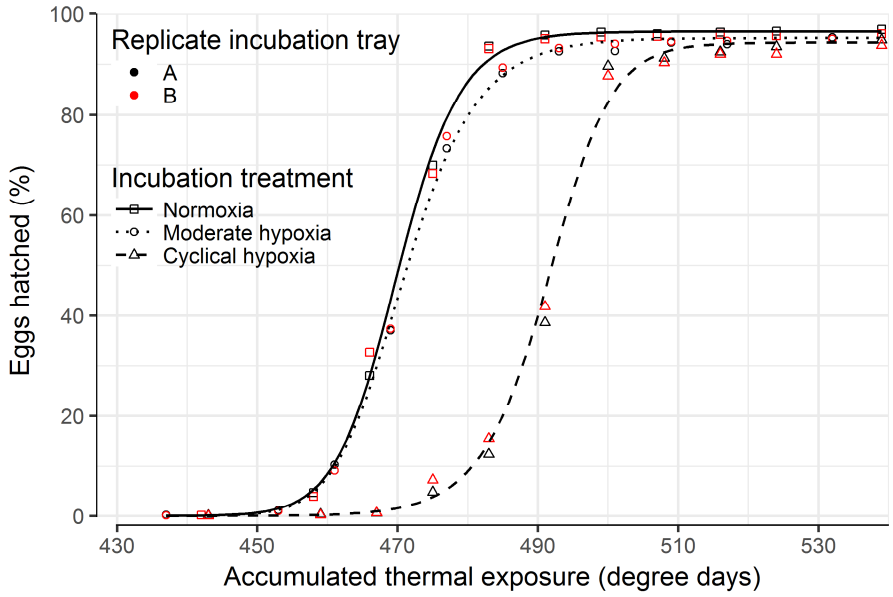

Figure 3.5: Relationship between hatching and degree days of Atlantic salmon (*Salmo salar*) incubated in normoxia, moderate hypoxia (~63% DO) or cyclical hypoxia (100-25% DO daily). Points are percentage hatched for each replication tray (i.e. 2 per treatment – A & B) and lines are fitted 5 parameter log-logistic curves (Control: $y =$ 0.965/〖(1+exp(-100(log(x)-log(468.84))))〗^1.187, Moderate hypoxia: $y =$ 0.952/〖(1+exp(-79.5(log(x)-log(466.5))))〗^1.780, Cyclical hypoxia: $y =$ 0.943/〖(1+exp(-122(log(x)-log(493.9))))〗^0.664).

Discussion

Overall, Atlantic salmon have a considerable capacity to survive severe cyclical hypoxia, but constant exposure to severe hypoxia (25% DO) causes dramatic reductions in growth, development and survival. Despite reductions in $\dot{M}O_{2rout}$, embryos incubated in moderate hypoxia grew, developed and hatched similarly to normoxia incubated embryos. In contrast, embryos incubated in cyclical hypoxia were smaller, developmentally delayed and hatched later compared to normoxia-incubated embryos. These findings help to highlight the ecological and aquaculture rearing implications of developmental oxygen availability during incubation and the potential impacts on future survival.

Cyclical hypoxia does not affect survival

Interestingly, survival from fertilisation until 113 DPF was not affected by cyclical hypoxia exposure (down to 25% DO), while nearly all fish incubated in constant severe hypoxia

(~27% DO) died by 113 DPF (Table 3.3). These differences in survival persist despite most embryos in the cyclical hypoxia treatment experiencing daily bouts of hypoxia below their O_{2crit} from approximately the eyed-egg stage until hatching (Fig. 3.4). To survive during hypoxic periods when the oxygen level is below O_{2crit} embryos must either depress their metabolism or meet metabolic energy demands via anaerobic metabolism. Indeed, salmonid embryos and alevins have capacity for anaerobic metabolism and the ability to depress their metabolism, although these mechanisms have not been fully elucidated (Gnaiger et al., 1987; Ninness et al., 2006). Cyclical hypoxia did not affect the survival of embryos, indicating that that the capacity for anaerobic respiration and metabolic depression combats intermittent periods of severe hypoxia below O_{2crit}.

Table 3.3: Survival of Atlantic salmon (*Salmo salar*) embryos and alevins incubating in normoxia, moderate hypoxia (~63% DO), cyclical hypoxia (100-25% DO daily) or severe hypoxia (~27% DO). Different superscript letters indicate significant differences (Tukey, $P < 0.05$) between incubation treatment groups within each incubation period. The severe hypoxia treatment was only included in the fertilisation to 113 DPF period due to low hatching success.

		Survival during incubation period		
Incubation treatment	**Fertilisation to egg-shocking**	**Egg shocking to 1 day pre-hatch**	**1 day pre-hatch to 113 DPF**	**Fertilisation to 113 DPF**
Normoxia	80.9 ± 0.6%[a]	99.4 ± 0.1%[a]	96.0 ± 0.4%[a]	76.2 ± 0.7%[a]
Moderate Hypoxia	81.0 ± 0.6%[a]	99.3 ± 0.1%[a]	93.8 ± 0.4%[b]	74.5 ± 0.7%[a]
Cyclical Hypoxia	81.3 ± 0.6%[a]	99.6 ± 0.1%[a]	93.8 ± 0.4%[b]	74.9 ± 0.6%[a]
Severe Hypoxia	-	-	-	0.7 ± 0.1%[b]

Impacts of hypoxia on growth and development

Embryos incubated in cyclical hypoxia were smaller at 113 DPF and developmentally delayed from eyed-egg to 113 DPF compared with those incubated in normoxia and constant moderate hypoxia. However, embryos incubated in cyclical and moderate hypoxia grew and developed at similar rates to embryos incubated in normoxia from the eyed-egg stage until 113 DPF (Fig. 3.2A, B). Our results suggest that cyclical hypoxia reduces growth and developmental rates between fertilisation and the eyed-egg stage, translating to smaller and developmentally delayed embryos throughout the rest of the developmental period. The results of this chapter are similar to reduced growth measured in Atlantic salmon incubated in moderate hypoxia (50% DO) between fertilisation and the eyed-egg stage in Chapter 2 (Fig. 2.2), and also to the finding that Pacific salmon incubated in hypoxia between 120 and 190

degree days (prior to eyed-egg stage) experience more substantially delayed hatching (Alderdice et al., 1958). Therefore, the developmental window between fertilisation and the eyed-egg stage appears to be particularly sensitive to hypoxia. It is notable that constant severe hypoxia (25% DO) considerably reduced growth and development and caused unquantified embryo deformities (Fig. 3.2A, B). However, the impact of daily bouts of hypoxia to the same level as the severe hypoxia treatment (25% DO) were relatively benign. Findings were similar for southern flounder reared in cyclical hypoxia (90-40% DO daily), which grew faster than fish reared in severe hypoxia (40% DO) (Taylor and Miller, 2001). Thus, the results here suggest that the daily normoxic respite associated with daily cyclical hypoxia incubation will limit the reductions of growth and development which occur in constant severe hypoxia.

Table 3.4: Degree days required to reach 10, 50 and 90% hatched (of eggs that hatched) and hatching success of Atlantic salmon (*Salmo salar*) eggs incubated in normoxia, moderate hypoxia (~63% DO) or cyclical hypoxia (100-25% DO daily). Different superscript letters indicate significant differences between incubation treatment groups (Tukey, $P < 0.05$). N.B. The severe hypoxia (~27% DO) treatment was not included due to low and sporadic hatching.

Incubation treatment	**Accumulated thermal exposure (degree days) to percent hatch**			**Hatching success**
	10%	**50%**	**90%**	
Normoxia	460.5 ± 0.2^{a}	469.9 ± 0.2^{a}	480.1 ± 0.3^{a}	$96.49 \pm 0.33\%^{a}$
Moderate hypoxia	460.8 ± 0.2^{a}	470.9 ± 0.2^{b}	483.2 ± 0.3^{b}	$95.20 \pm 0.38\%^{b}$
Cyclical hypoxia	480.3 ± 0.3^{b}	491.4 ± 0.2^{c}	501.0 ± 0.2^{c}	$94.31 \pm 0.41\%^{b}$

Cyclical hypoxia delays hatching

Egg hatching was delayed in the moderate hypoxia incubation treatment compared to the normoxia treatment, however the delay in hatching was negligible (~1 degree day; 3 hours at 8°C, Fig. 3.5 & Table 3.4). Embryos incubated in cyclical hypoxia were more substantially delayed and hatched ~21 degree days later (~2.6 days at 8°C) than normoxia-incubated eggs. However, calculating embryo mass or developmental stage using growth or developmental stage relationships predicted that when 50% of the eggs had hatched, the cyclical hypoxia incubated embryos were smaller and developmentally delayed compared to normoxia-incubated embryos (embryo mass: 0.0204 and 0.0299 g, respectively; developmental stage: 275.8 and 286.8 τ_s, respectively, Tables 3.2 & 3.4). Thus, hatching may have been developmentally premature in addition to being chronologically delayed. Delayed hatching of

hypoxia-incubated embryos associated with delayed development has been reported in other studies of salmonids. For example, Atlantic salmon exposed to cyclical hypoxia as low as 10% DO between 134-275 or 229-371 degree days hatched later than normoxia-incubated embryos and were developmentally delayed, but achieved a similar body length (Bloomer et al., 2016).

In the present study, hatching of the cyclical hypoxia incubation treatment at a smaller embryo mass may have been induced due to respiratory stress associated with hypoxia. Indeed, the O_{2crit} of embryos at 10 and 3 days pre-hatch was above 35% DO, which is higher than the daily bout of 25% DO to which the embryos were exposed within the cyclical hypoxia treatment (Fig. 3.4B, C). A daily hypoxia exposure below O_{2crit} may have caused respiratory stress that was alleviated by hatching, therefore reducing oxygen diffusion limitations associated with the egg capsule. Hypoxia exposure also induces premature hatching in rainbow trout and Atlantic salmon (Bloomer et al., 2016; Latham and Just, 1989; Oppen-Berntsen et al., 1990). We suggest that hatching was chronologically delayed in cyclical hypoxia due to slower growth and development and that cyclical hypoxia probably induced hatching at a smaller size and earlier developmental stage due to respiratory stress.

Oxygen uptake and hypoxia tolerance

At the eyed-egg stage, routine $\dot{M}O_2$ was similarly reduced (~20%) in eggs incubated in moderate and cyclical hypoxia compared to normoxia-incubated embryos. The reductions in $\dot{M}O_{2rout}$ of hypoxia-incubated embryos here are similar to the eggs incubated in hypoxia in Chapter 2 (50% DO; ~23% lower $\dot{M}O_{2rout}$). It is likely that the reduced routine $\dot{M}O_2$ in hypoxia-incubated embryos is due to metabolic depression, which reduces metabolic oxygen demands during periods of oxygen limitation (similar to Chapter 2). Similar reductions in mass-specific $\dot{M}O_2$ have been reported in hypoxia-incubated rainbow trout embryos when measured in hypoxia (50% DO) prior to hatching (Miller et al., 2008). Here, however, at 10 days pre-hatch, routine $\dot{M}O_2$ was no longer reduced in cyclical-hypoxia incubated eggs, despite the routine $\dot{M}O_2$ of embryos incubated in moderate hypoxia remaining lower than normoxia-incubated embryos (Fig 3.3E, F). Reductions in routine $\dot{M}O_2$ in cyclical hypoxia incubated embryos may not be maintained throughout development due to daily cycles of normoxia exposure causing rapid adjustments in metabolic energy demands depending on oxygen availability. In rainbow trout, mass-specific $\dot{M}O_2$ was reduced after 30 min in 50%

DO prior to measurement (Miller et al., 2008). In Arctic charr, metabolic depression measured via calorimetry occurs over 8 h when exposed to anoxia, but metabolic rate can return to normal levels in 24-36 h when normoxia is re-established (Gnaiger et al., 1987). In the present study, embryos and alevins were removed from the cyclical hypoxia treatment for respirometry measurements when oxygen levels had been above 80% DO for up to 6 hours. Temporal effects of hypoxia exposure on metabolism in salmonid embryos may have obscured the effects of cyclical hypoxia due to sampling in the normoxic period of the daily oxygen cycle. Consideration of the temporal effects of hypoxia on salmon embryo metabolism in future studies would help to elucidate the differential responses of embryos to constant and cyclical hypoxic regimes.

Despite elevated routine $\dot{M}O_2$ in normoxia-incubated embryos at eyed-egg and 10 days pre-hatch, routine $\dot{M}O_2$ and O_{2crit} at 3 days pre-hatch were similar between normoxia, moderate hypoxia and cyclical hypoxia incubation treatments (Figs. 3.3A-F & 3.4). This may be due to limitations in oxygen diffusion through the egg chorion and perivitelline that restricts the absolute $\dot{M}O_2$ of normoxia-incubated eggs. Indeed, the increasing metabolic oxygen demand of unhatched embryos associated with growth is not met by an associated increase in oxygen uptake capacity, resulting in lower hypoxia tolerance (increasing O_{2crit}; Figs. 3.3 & 3.4) (Rombough, 1988a). Therefore, it is plausible in the present study that limitations in oxygen diffusion through the egg capsule and perivitelline may ultimately limit the absolute $\dot{M}O_2$ that is attainable in normoxic conditions, therefore explaining why routine $\dot{M}O_2$ was higher in normoxia compared to moderate hypoxia incubated embryos at eyed-egg and 10 days pre-hatch, but similar at 3 days pre-hatch.

The egg oxygen diffusion barrier is removed during hatching, and consequently the O_{2crit} in nearly all alevins fell below 25% DO at 10 days post-hatch (Fig. 3.4D). As a result, O_{2crit} of nearly all alevins was below the cyclical and moderate hypoxia treatment levels, and aerobic metabolism should not be limited. Somewhat surprisingly, moderate hypoxia incubated alevins had a higher routine $\dot{M}O_2$ compared to normoxia-incubated alevins (Fig. 3.3F, H). Increased routine $\dot{M}O_2$ may have been associated with increased energetic requirements of the cardio-respiratory system to maintain oxygen uptake. Indeed, rainbow trout and Arctic charr alevins incubated in hypoxia (~38 and 25% DO, respectively) from hatching onwards increased heart and ventilation rates (Holeton, 1971; McDonald and McMahon, 1977).

Branchial ventilation and cardiac pumping can account for up to 15% of resting oxygen uptake rate in rainbow trout (Farrell and Steffensen, 1987). Therefore, increased energetic costs of oxygen uptake associated with an increase in ventilation or heart rate during hypoxia exposure may explain the ~7% increase in routine $\dot{M}O_2$ in response to moderate hypoxia.

Conclusions

Here, we found that constant severe hypoxia (25% DO) severely decreases growth and development of Atlantic salmon, ultimately resulting in near 100% mortality by 113 DPF. In contrast, the survival of cyclical hypoxia (100-25% DO) incubated embryos was not reduced despite reduced growth, developmental rate and delayed hatching. Sub-lethal impacts of hypoxia may disadvantage fry following emergence from the redd, because fry that are smaller or emerge from the redd later are at higher risk of predation, at a competitive disadvantage compared to early-emerging conspecifics, and remain smaller as they develop (Einum and Fleming, 2000; Roussel, 2007; Skoglund and Barlaup, 2006). Ultimately, reduced growth and a competitive disadvantage may delay smoltification and migration to the sea (Metcalfe et al., 1990). Reduced growth and development associated with temporal and spatial variability in oxygen levels within aquaculture incubation systems may also increase size variation within a cohort of the same age, potentially impairing subsequent performance and influencing management decisions (Pennell and McLean, 1996; Seppä et al., 1999).

Chapter 4: Developmental hypoxia has negligible effects on long-term hypoxia tolerance and aerobic metabolism of Atlantic salmon (*Salmo salar*)

All of the research within this chapter has been published as:

Wood, A. T., Clark, T. D., Andrewartha, S. J., Elliott, N. G. and Frappell, P. B. (2017). Developmental hypoxia has negligible effects on long-term hypoxia tolerance and aerobic metabolism of Atlantic salmon (*Salmo salar*). Physiol. Biochem. Zool. 90, 494-501.

Abstract

Exposure to developmental hypoxia can have long term impacts on the physiological performance of fish due to irreversible plasticity. Wild and captive-reared Atlantic salmon (*Salmo salar*) can be exposed to hypoxic conditions during development, and continue to experience fluctuating oxygen levels as juveniles and adults. Here, we examine whether developmental hypoxia impacts subsequent hypoxia tolerance and aerobic performance of Atlantic salmon. Individuals at 8°C were exposed to 50% (hypoxia) or 100% (normoxia) dissolved oxygen saturation (DO; as % of air saturation) from fertilisation for ~100 days (800 degree days) and then raised in normoxic conditions for a further 15 months. At 18 months post-fertilisation aerobic scope was calculated in normoxia (100% DO) and acute (18 h) hypoxia (50% DO) from the difference between the minimum and maximum oxygen consumption rates ($\dot{M}O_{2min}$ and $\dot{M}O_{2max}$, respectively) at 10°C. Hypoxia tolerance was determined as the DO at which loss of equilibrium (LOE) occurred in a constantly decreasing DO environment. There was no difference in $\dot{M}O_{2min}$, $\dot{M}O_{2max}$ or aerobic scope between fish raised in hypoxia or normoxia. There was some evidence that hypoxia tolerance was lower (higher DO at LOE) in hypoxia-raised fish compared with those raised in normoxia, but the magnitude of the effect was small (12.52% DO vs. 11.73% DO at LOE). Acute hypoxia significantly reduced aerobic scope by reducing $\dot{M}O_{2max}$, while $\dot{M}O_{2min}$ remained unchanged. Interestingly, acute hypoxia uncovered individual-level relationships between DO at LOE and each of $\dot{M}O_{2min}$, $\dot{M}O_{2max}$ and aerobic scope. We discuss our findings in the context of developmental trajectories and the role of aerobic performance in hypoxia tolerance.

Introduction

Developmental plasticity in response to environmental stressors can have long-term consequences for individual performance due to impacts on developmental trajectories (Burggren and Reyna, 2011; Garland et al., 2017; Monaghan, 2008; Mueller et al., 2015a). For example, a hypoxic developmental rearing environment can impact cardiovascular development and regulation in fish (Bagatto, 2005; Miller et al., 2011; Pelster, 2002). Such responses to environmental conditions during development are of increasing concern in the current era of environmental change where hypoxic events are predicted to become more widespread (Altieri and Gedan, 2015; Diaz and Rosenberg, 2008).

Salmonid embryos and yolk-sac alevins often experience hypoxia during development both in wild redds and in aquaculture hatcheries. The availability of oxygen to the embryos is affected by many factors including ambient dissolved oxygen levels, water flow characteristics, embryo density, redd depth, sediment type and developmental stage (Ciuhandu et al., 2007; Dhiyebi et al., 2013; Greig et al., 2007; Ingendahl, 2001; Miller et al., 2008; Youngson et al., 2004). While hatchery water conditions are typically monitored and controlled, oxygen levels within incubators can still become hypoxic in crowded conditions (McLean and Lim, 1985). Localised hypoxic zones can develop within commercial systems (e.g. Heath stack tray incubators) during later developmental stages (Appendix A). Wild and cultured salmonids are also likely to experience fluctuating dissolved oxygen levels during post-larval rearing. For example, salmonids reared in marine sea-cage aquaculture can experience significant dissolved oxygen variations over temporal and spatial scales (Burt et al., 2012; Johansson et al., 2006; Oppedal et al., 2011).

Hypoxia can limit aerobic capacity due to reduced oxygen supply, which can decrease activity levels, growth and survival if threshold oxygen levels are breached (Pedersen, 1987; Wang et al., 2009). Fish that are able to maintain a higher maximum oxygen consumption rate ($\dot{M}O_{2max}$) over a range of oxygen levels may have a higher aerobic performance in low oxygen conditions. Hypoxia tolerance may also impact survival when dissolved oxygen levels fall below the critical oxygen tension (O_{2crit}) required to maintain standard metabolic rate (herein termed minimum oxygen consumption rate; $\dot{M}O_{2min}$). Survival below O_{2crit} is dependent on anaerobic performance and metabolic depression, and most fish cannot survive for prolonged periods under such conditions (Claireaux and Chabot, 2016).

Fish show a range of responses to developmental hypoxia. For example, rainbow trout (*Oncorhynchus mykiss)* have lower maximum sustained swimming speeds at 65 to 110 days post-fertilisation after being reared from 0 to 57 days post-fertilisation in hypoxic conditions of 34% dissolved oxygen (DO; % of air saturation) (Johnston et al., 2013). Moreover, $\dot{M}O_{2min}$ below the O_{2crit} of 4 day post-fertilisation zebrafish (*Danio rerio*) was higher for individuals exposed to 5% DO for four hours at 24 and 36 hours post-fertilisation, but $\dot{M}O_{2min}$ returned to control levels after approximately 6 months in normoxia, indicating that the response was plastic and reversible (Robertson et al., 2014). On the other hand, exposure to 40% DO from 30 to 38 days post-hatch did not change the DO at loss of equilibrium (LOE) of 1 year old European sea bass (*Dicentrarchus labrax)* compared with animals reared in normoxia (Vanderplancke et al., 2015). Clearly, a greater research effort is required to decipher any consistent, long-term physiological responses to developmental hypoxia in fish.

Here, we investigated the long term impacts of embryonic and larval developmental oxygen levels (50% DO vs. 100% DO) on the aerobic performance and acute hypoxia tolerance of captive-reared Atlantic salmon (*Salmo salar*). At 18 months post-fertilisation, $\dot{M}O_{2min}$, $\dot{M}O_{2max}$ and aerobic scope were measured in normoxia and acute hypoxia (50% DO) to determine any lasting impacts on aerobic performance. The dissolved oxygen level at loss of equilibrium (DO at LOE) was measured as an indicator of hypoxia tolerance. We also tested for inter-individual relationships between hypoxia tolerance and each of $\dot{M}O_{2min}$, $\dot{M}O_{2max}$ and aerobic scope. We hypothesised that developmental hypoxia may result in an increase in hypoxia tolerance and aerobic performance ($\dot{M}O_{2max}$) 18 months post-fertilisation due to irreversible phenotypic plasticity causing an increase in oxygen transport capacity.

Methods

Embryonic and larval incubation

Initial stages of the experiments were conducted at the SALTAS aquaculture facility, Wayatinah, Tasmania, Australia. Four half-sibling Atlantic salmon families were created by fertilising eggs from four females with milt from one male using captive bred broodstock reared in freshwater at SALTAS. Half-sibling families were used to reduce potential variability in metabolic measurements and hypoxia tolerance between families (Anttila et al., 2013). Fertilised eggs were randomly allocated to two replicate mesh isolation baskets (18 x

14.5 x 5.5 cm) per treatment with each replicate incubated in separate Heath trays (39 x 32 x 5.5 cm; Marisource, USA). Each Heath tray held four isolation baskets from this study and an unrelated parallel study, with approximately 1750 eggs per basket (7000 eggs per Heath tray).

Eggs were incubated from fertilisation through hatching (~520 degree days; DD, ~65 days) to near yolk-sac absorption (800 DD, ~100 days) at 7.98 ± 0.15°C (± SD) at either 100.0 ± 1.8% dissolved oxygen saturation (DO; as % of air saturation, Control) or 50.1 ± 0.8% DO (Hypoxia) in drum-screened raw river water in a flow-through system. Hypoxia exposure of 50% DO was chosen as a suitably stressful treatment based on previous studies of salmonids (Polymeropoulos, 2013; Rombough, 2007). The Heath tray system was modified to ensure consistent exposure to the experimental conditions by supplying each tray with an independent water supply at a rate of 10 L min^{-1}. The temperature and DO of the two treatments were controlled by an OxyGuard Pacific monitoring system (OxyGuard, Denmark) with a submersible heater and nitrogen or oxygen injection into 200 L treatment sumps. The nitrogen injection system failed on three occasions throughout the 100 day incubation period, such that DO rose to normoxic levels for a maximum continuous period of 41 h prior to being rectified. Nonetheless, eggs in the hypoxia treatment spent 97% of their incubation between 45 and 55% DO (grand mean 51.1 ± 7.6% DO when including nitrogen system failures). Eggs and alevins were reared according to best industry practice which includes formalin treatments of 1.5-2 mL L^{-1} for 15 min three times weekly from 70 DD to 340 DD to prevent fungal growth, and removal of dead eggs and alevins from 280 DD to 950 DD. Additionally, at the eyed stage (280 DD) eggs were physically agitated to assist the removal of dead embryos.

Post-larval rearing

From ~100 days post-fertilisation (800 DD) all treatment groups were returned to normoxic conditions, and at 4 months post-fertilisation (980 DD) all fish were transferred from Heath trays into 50 L tanks for their first feeding. The two replicate isolation baskets were combined into one tank for each treatment. The two 50 L tanks were contained within a semi-closed recirculating system at SALTAS, supplied with drum-screened river water at ambient temperature. Dissolved oxygen was maintained above 90% by supplementary oxygenation controlled by an OxyGuard Atlantic oxygen monitoring system (OxyGuard, Denmark). Fish were held in 50 L tanks from 4 months (980 DD) to 8 months post-fertilisation, after which

they were transferred into two 500 L tanks on the same recirculating system until 17 months post-fertilisation. Ambient temperature followed seasonal cycles and ranged from approximately 5 to 15°C. While replicate tanks were logistically impossible during this 'on farm' phase of the experiment, all fish were exposed to the same recirculating water and there was no evidence that this approach represented a confounding factor in the results (e.g., mortality was negligible and growth rates were the same between tanks).

Fish were transferred to CSIRO Laboratories (Hobart, TAS, Australia) at 17 months post-fertilisation, 37 days prior to experiments. Fish were internally PIT tagged 20 days prior to experiments and allowed to recover for 7 days prior to the treatment groups being mixed equally between two 200 L tanks within a recirculating freshwater filtration system. Temperature was maintained at ~10°C and DO at 90-100% by aeration. Water quality was monitored daily and maintained between 0 – 0.23 mg L^{-1} NH_3-N (ammonia), 0 – 0.2 mg L^{-1} NH_2-N (nitrite), 7.15 – 7.7 pH and 20 – 55 mg L^{-1} $CaCO_3$ (alkalinity). Fish were fed to satiation daily with commercial pellet food for 13 days prior to commencing respirometry and until completion of all experiments (except prior to experimental protocols; see below).

Respirometry

The oxygen consumption rates ($\dot{M}O_2$; ~aerobic metabolic rates) of individual fish (~105 g) were measured in 4.05 L (total volume) intermittent-flow respirometers containing freshwater and using practices outlined in Clark et al. (2013). Briefly, each respirometer consisted of a plastic chamber with an o-ring sealed lid through which water was continuously circulated by an external recirculation loop (13 mm diameter tubing) and inline pump to ensure mixing. Fresh water was introduced into each respirometer during each flush cycle by a large submersible pump connected to each respirometer with PVC tubing, and flushed out through a polyethylene standpipe vented 10 cm above the water surface to prevent back-flow. A timer-controlled solenoid valve regulated water flow from the flush pump to produce a 12.5:7.5 min flush:seal cycle which continuously repeated throughout all $\dot{M}O_2$ measurements unless stated otherwise. Ten respirometry chambers were submerged in a single water bath that was maintained at ~10°C with a digitally controlled electric submersible titanium heater (Aqua Logic, California, USA). Dissolved oxygen was maintained at either 100% by aeration, or at 50% by injecting nitrogen (controlled by an OxyGuard Atlantic oxygen monitoring system). Oxygen concentration within each respirometry chamber was measured

at 5 s intervals using an optical oxygen sensor sealed in the recirculation loop and connected to a four channel FireSting O_2 optical oxygen meter (Pyro Science, Germany).

The rate of declining DO (% min^{-1}) during each 7.5 min sealed cycle was determined by least-squares regression and $\dot{M}O_2$ (mg O_2 min^{-1}) was calculated using equation (4.1).

$$\dot{M}O_2(mg\ O_2\ min^{-1}) = \frac{\Delta\ DO}{\Delta\ t} \times \left(P_B - (P_S \times RH)\right) \times \beta_{O_2} \times vol \times 0.2094 \quad (4.1)$$

where DO is the fractional dissolved oxygen saturation, t is time in minutes, P_B is barometric pressure (kPa), P_S is the calculated saturation vapour pressure of water (kpa; Antoine equation), h is the fractional relative humidity, βO_2 is the oxygen capacitance of water (~0.5375 mg L^{-1} kpa^{-1}; see Dejours (1981)) and vol is the volume of the respirometry chamber minus fish volume in L (assuming 1 kg wet fish mass = 1 L volume).

Metabolic measurements were conducted over 8 consecutive days (10 fish per day), alternating daily between measurements in normoxia and hypoxia. Approximately equal numbers of fish were randomly selected from each holding tank for each incubation treatment group. Each fish was used for respirometry only once. Fish were fasted for at least 18 hours prior to respirometry and were identified via PIT tag and weighed (OHAUS Scout Pro digital balance, OHAUS Corporation, USA) before being transferred to respirometry chambers. Once sealed in the chambers, the fish either remained in normoxic water or they recovered from handling for 3 hours before the DO was reduced to 50% over 1-2 hours. Oxygen consumption rates were measured using a 12.5:7.5 min flush:seal cycle for 14.5-16 hours with no disruption to ensure minimum $\dot{M}O_2$ ($\dot{M}O_{2min}$) was reached. Minimum $\dot{M}O_2$ was determined for each individual as the mean of the lowest six $\dot{M}O_2$ measurements during the 14.5-16 hour resting period (40-70 $\dot{M}O_2$ measurements, depending on individual), excluding any $\dot{M}O_2$ value outside ± 2 SD of the mean (Clark et al., 2013; Norin et al., 2014). Given that fish were measured for at least 14.5 h in a post-absorptive state, it was assumed that $\dot{M}O_{2min}$ provided a reasonable estimation of standard metabolic rate (Chabot et al., 2016).

Maximum metabolic rate ($\dot{M}O_{2max}$) was subsequently measured in each fish using an exhaustive exercise protocol. Fish were individually transferred from their respirometer to a 33 L cylindrical tank receiving water from the respirometry water bath at either 100% or 50%

DO. Each fish was chased for 2 minutes by hand, tapping the tail as necessary to encourage continuous swimming and all fish ceased continuous burst swimming during the 2 minute protocol. At the end of exercise, each fish was immediately (within 15 s) placed back into the same sealed respirometer from which it came. The oxygen decline in the respirometers was measured until DO had decreased by a maximum of 15%, and $\dot{M}O_{2max}$ was calculated from the steepest slope in any ~5 minute period during this time (e.g., see Norin and Clark (2016)). Upon completion of all $\dot{M}O_{2max}$ measurements, all fish were returned to their respective holding tanks for at least 6 days until required for hypoxia tolerance experiments. At the completion of $\dot{M}O_{2max}$ measurements on 5 of 8 days background respiration was measured in empty chambers. Background respiration was determined to be negligible and uncorrected fish $\dot{M}O_2$ values were used for subsequent analysis.

Acute hypoxia tolerance

The hypoxia tolerance of groups of 17-22 salmon (approximately half from each developmental treatment group) was tested in four hypoxia challenges conducted across 4 days. After being fasted for 18 hours, fish were placed in a 200 L flow-through (0.1 L min^{-1}) tank of freshwater at 10°C and recovered from handling overnight at >90% DO. DO was measured at 5 s intervals using an optical oxygen sensor (FireSting O_2 oxygen meter, Pyro Science, Germany). In the morning, the DO was decreased at a rate of 4-4.5% min^{-1} until 45% DO was reached, and then at 0.3-0.35% min^{-1} thereafter, by bubbling nitrogen at a controlled rate. The two different rates of DO decline were used to decrease the length of the experiment while still allowing a precise measure of the DO at loss of equilibrium (LOE) for each individual. Loss of equilibrium was defined as the DO where a fish could no longer maintain balance and their ventral surface was visible for 5 seconds. At LOE, individuals were identified by PIT tag and transferred to a recovery tank. At the completion of each run the fish were killed by anaesthetic overdose (Aqui-S, New Zealand), and their length and mass measured.

Statistical analyses

Statistical analyses were performed using R (R Core Team, 2016). Differences in $\dot{M}O_{2min}$, $\dot{M}O_{2max}$ and aerobic scope between developmental treatment groups (Normoxia, Hypoxia) and acute measurement DO (50%, 100%) were tested using two-way ANCOVA (Type III SS) including fish mass as the covariate. For $\dot{M}O_{2max}$ and aerobic scope there was a

significant interaction involving the covariate which prevented robust comparisons of main effects. As such, log-log transformations of continuous variables were performed to meet the assumptions of an ANCOVA requiring homogeneity of slopes for between-group comparisons. Comparisons of mean DO at LOE between developmental treatment groups (Normoxia, Hypoxia) and acute measurement DO (50%, 100%) were carried out using a two-way ANOVA (Type III SS). Linear regressions of each of mass-specific $\dot{M}O_{2min}$, $\dot{M}O_{2max}$ and aerobic scope against DO at LOE were calculated separately for each acute measurement DO group (50%, 100%).

Results

Mass

Mean fish mass did not differ between developmental treatment groups and averaged 104.5 ± 2.7 g and 107.5 ± 2.8 g at the $\dot{M}O_2$ and LOE measurements, respectively ($\dot{M}O_2$: $F_{(1,78)} = 3.07$, $P = 0.084$, LOE: $F_{(1,75)} = 2.18$, $P = 0.14$).

Metabolic rates

Positive linear relationships existed between fish mass and each of $\dot{M}O_{2min}$, $\dot{M}O_{2max}$ and aerobic scope (Fig. 4.1A-C). Developmental oxygen level did not influence $\dot{M}O_{2min}$, $\dot{M}O_{2max}$ or aerobic scope when measured in either 50% DO or 100% DO (Fig. 4.1, $\dot{M}O_{2min}$: $F_{(1,75)} = 0.437$, $P = 0.51$, $\dot{M}O_{2max}$:$F_{(1,75)} = 3.48$, $P = 0.066$, aerobic scope: $F_{(1,75)} = 2.48$, $P = 0.12$). Thus, $\dot{M}O_2$ values of developmental groups are combined herein but are presented separately in figures for clarity.

The $\dot{M}O_{2max}$ and aerobic scope of fish measured at 50% DO were ~50% and 70% lower, respectively, than fish which were measured in 100% DO (Fig. 4.1B, E, $\dot{M}O_{2max}$: $F_{(1,75)} = 1343.1$, $P < 0.001$, Fig. 4.1C, F, aerobic scope: $F_{(1,75)} = 633.7$, $P < 0.001$). However, $\dot{M}O_{2min}$ was unaffected (Fig. 4.1A, D, $\dot{M}O_{2min}$: $F_{(1,75)} = 0.158$, $P = 0.69$). There was a significant interaction between fish mass and measurement DO (50% or 100% DO) for $\dot{M}O_{2max}$ and aerobic scope; the slopes of the relationships between mass and each of $\dot{M}O_{2max}$ and aerobic scope (but not $\dot{M}O_{2min}$) were reduced in 50% DO compared with 100% DO (Fig. 4.1B, C, $\dot{M}O_{2max}$: $F_{(1,72)} = 49.02$, $P < 0.001$, aerobic scope: $F_{(1,72)} = 34.8$. $P < 0.001$). Nevertheless, log-log transforming the relationships for $\dot{M}O_{2max}$ and aerobic scope against mass revealed that the scaling exponent (slope) was not different between 50% and 100% DO levels. That is, the

relative but not the absolute decrease in $\dot{M}O_{2max}$ and aerobic scope with acute hypoxia was proportional across body mass (inset in Fig. 4.1B, C, $\dot{M}O_{2max}$: b = 0.893, P < 0.001; aerobic scope: b = 0.759, P < 0.001).

Acute hypoxia tolerance

When combining $\dot{M}O_2$ measurements for both DO measurement groups, DO at LOE was slightly but significantly higher in the hypoxia-incubated developmental group (12.52 ± 0.27 % DO) compared to the normoxia-incubated group (Fig. 4.2, 11.73 ± 0.27 % DO; $F_{(1,75)}$ = 4.11, P = 0.046). There was no relationship between fish mass and DO at LOE ($F_{(1,72)}$ = 0.063, P = 0.80). Additionally, the $\dot{M}O_2$ measurement DO (50% DO or 100% DO) had no subsequent effect on DO at LOE (50%: 11.81 ± 0.27 % DO, 100%: 12.47 ± 0.27 % DO, $F_{(1,75)}$ = 2.83, P = 0.096).

Relationship between $\dot{M}O_2$ and hypoxia tolerance

When $\dot{M}O_2$ was measured in normoxia there was no relationship between DO at LOE and $\dot{M}O_{2min}$, $\dot{M}O_{2max}$ or aerobic scope (Fig. 4.3, $\dot{M}O_{2min}$: $F_{(1,37)}$ = 0.12, R^2 = 0.003, P = 0.73, $\dot{M}O_{2max}$: $F_{(1,37)}$ = 0.016, R^2 = 0.00, P = 0.90, aerobic scope: $F_{(1,37)}$ = 0.065, R^2 = 0.001, P = 0.80). Interestingly, measuring $\dot{M}O_2$ in hypoxia revealed significant relationships between DO at LOE and each of $\dot{M}O_{2min}$, $\dot{M}O_{2max}$ and aerobic scope (Fig. 4.3, $\dot{M}O_{2min}$: $F_{(1,36)}$ = 7.77, R^2 = 0.18, P < 0.01, $\dot{M}O_{2max}$: $F_{(1,36)}$ = 12.73, R^2 = 0.26, P = 0.001, aerobic scope: $F_{(1,36)}$ = 24.18, R^2 = 0.40, P < 0.001).

Discussion

The present study is one of only a few measuring the long term impacts of developmental hypoxia on fish following an extended period of normoxia after incubation. There was no evidence to suggest that developmental hypoxia exposure during embryonic and larval incubation affects long term aerobic performance in Atlantic salmon and only weak evidence for an effect on acute hypoxia tolerance.

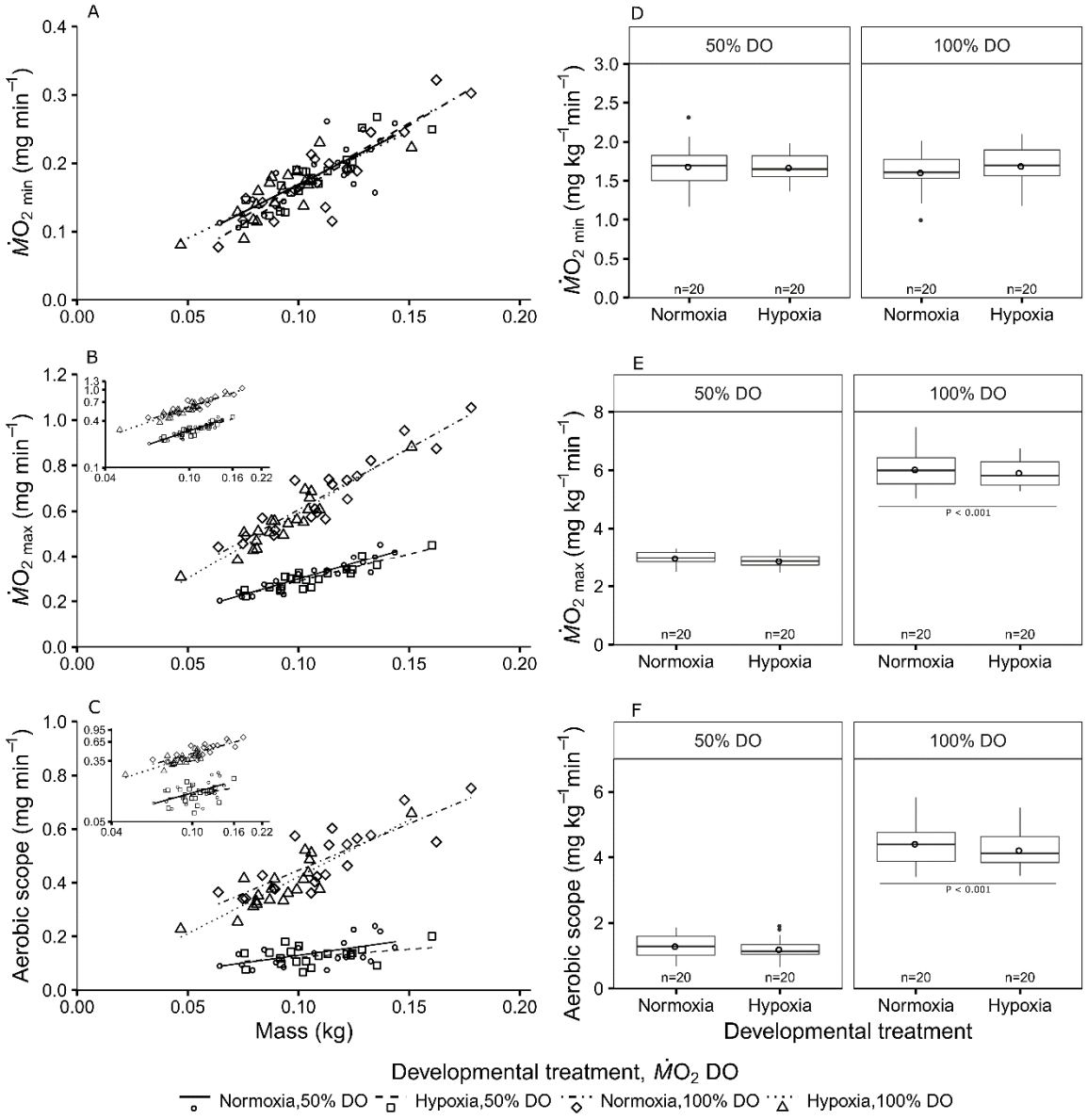

Figure 4.1: (A-C) The relationship between fish mass and $\dot{M}O_{2min}$ (A), $\dot{M}O_{2max}$ (B), aerobic scope (C) for each developmental oxygen treatment and $\dot{M}O_2$ DO measurement level. Data points represent individuals and lines represent a linear regression of each treatment group. Inset plots are log-log transformed axes with regression lines representing scaling of metabolic attributes with fish mass (D-F). The effect of developmental oxygen treatment (Normoxia, Hypoxia) and $\dot{M}O_2$ DO measurement level (100% DO, 50% DO) on $\dot{M}O_{2min}$ (D), $\dot{M}O_{2max}$ (E), aerobic scope (F). There was no difference in $\dot{M}O_{2min}$, $\dot{M}O_{2max}$ or aerobic scope between developmental treatment groups within the 100% and 50% DO measurement levels. Additionally, $\dot{M}O_{2min}$ was not affected by $\dot{M}O_2$ DO measurement level. However, $\dot{M}O_{2max}$ and aerobic scope were higher in the 100% DO measurement group ($P<0.001$, denoted on graphs). The solid central line is the median, the box denotes 25th and 75th percentile, whiskers extend to the highest or lowest value within 1.5*inter-quartile range and filled circles are outliers beyond that range. Mean values are represented by open circles.

The $\dot{M}O_{2min}$, $\dot{M}O_{2max}$ or aerobic scope did not differ between groups of Atlantic salmon raised in hypoxia or normoxia during development when measured in normoxia or hypoxia ~18 months post-fertilisation. Given the link between aerobic metabolic attributes and performance traits, it is possible that our findings translate to parameters beyond what we have measured here. For example, it is possible that performance traits which are related to $\dot{M}O_{2max}$ and aerobic scope (e.g., maximum swimming speed) and those related to $\dot{M}O_{2min}$ (e.g., growth and behavioural traits) may also suffer no long term impacts of developmental hypoxia, but these ideas remain to be thoroughly investigated (Metcalfe et al., 2016; Norin et al., 2016; Reidy et al., 2000).

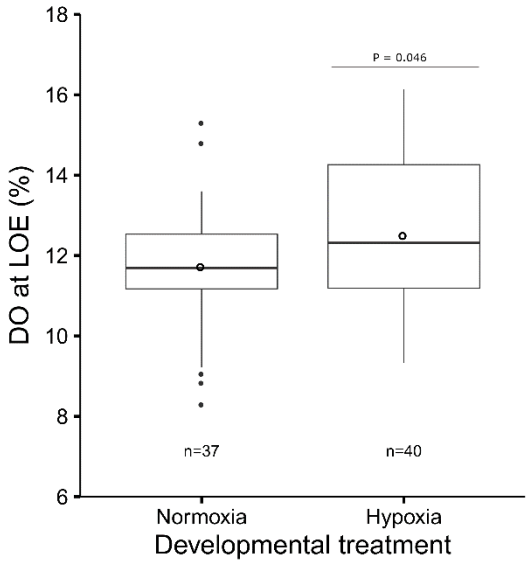

Figure 4.2: The effect of developmental oxygen treatment (Normoxia, Hypoxia) on DO at LOE (%). The DO at LOE of the hypoxia developmental treatment group was higher than those incubated in normoxia (P = 0.046). The solid central line is the median, the box denotes 25th and 75th percentile, whiskers extend to the highest or lowest value within 1.5*inter-quartile range and filled circles are outliers beyond that range. Mean values are represented by open circles.

Somewhat counterintuitively, we found some evidence that salmon exposed to developmental hypoxia had decreased acute hypoxia tolerance (higher DO at LOE) compared to those raised in normoxia (Fig. 4.2). Nonetheless, while the acute hypoxia tolerance of the two developmental incubation groups was significantly different (P = 0.046), the magnitude of the effect was small (12.52% DO vs. 11.73% DO). For our experiment where DO was decreased at 0.33 % min^{-1} this represents a difference in mean time to LOE of ~2.4 minutes. Such a difference may be negligible, as the median time to LOE can vary substantially (up to

114.8 minutes) between families of Atlantic salmon (Anttila et al., 2013). Thus, it is unclear what real world impacts an effect of such small magnitude would have on fish performance and survival given the spatial and temporal DO variability often experienced in wild and aquaculture environments.

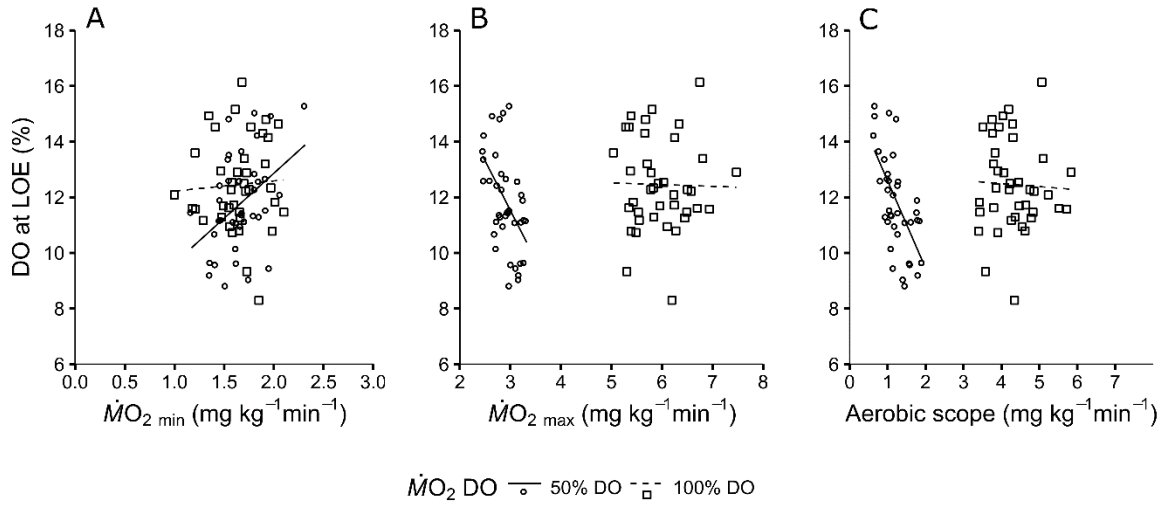

Figure 4.3: The relationship between metabolic attributes $\dot{M}O_{2min}$ (A), $\dot{M}O_{2max}$ (B) and aerobic scope (C) with DO% at LOE for each $\dot{M}O_2$ DO level (100% DO, 50% DO). In 50% DO relationships exist between DO at LOE and each of $\dot{M}O_{2min}$ (y = 3.23x + 6.40), $\dot{M}O_{2max}$ (y = -3.57x + 22.25) and aerobic scope (y = -3.17x + 15.73; see text for statistics). In 100% DO there was no relationship between DO at LOE and each of $\dot{M}O_{2min}$, $\dot{M}O_{2max}$ and aerobic scope (P > 0.73 in all cases). Data points represent individuals and lines represent a linear regression of each treatment group.

The concept of developmental trajectories proposes that exposure to an environmental stressor such as hypoxia can cause plasticity during development which may permanently impact the phenotype of an animal or be reversible following a return to normal conditions (Burggren and Reyna, 2011). While our results cannot address whether the metabolic phenotype was unchanged during development or was affected and subsequently returned to normal, evidence for plasticity of $\dot{M}O_2$ in salmonids during and following developmental hypoxia exposure is mixed. The $\dot{M}O_{2min}$ of rainbow trout embryos was not affected during developmental hypoxia exposure in one study (Miller et al., 2008), but recent work suggests an increase in $\dot{M}O_{2min}$ in response to developmental hypoxia in Atlantic salmon alevins (Polymeropoulos, 2013). A longer term study did not measure $\dot{M}O_2$ but found that maximum sustainable swimming speed (U_{crit}) of rainbow trout was lower for up to 48 days following

developmental hypoxia (Johnston et al., 2013). Also, developmental hypoxia has been shown to affect blood properties, heart rate programming and muscle development in salmonids, although the longevity of these changes is unknown (Bianchini and Wright, 2013; Matschak et al., 1997; Miller et al., 2011). Given the evidence for cardiovascular plasticity in salmonids exposed to developmental hypoxia, it is possible that fish exposed to developmental hypoxia in the present study underwent plastic changes during development but transitioned back to a 'normal' phenotype once returned to normoxic conditions. Moreover, it is unclear whether hypoxia during critical windows of embryonic and larval development or at other post-larval life stages may be more influential to long-term phenotypic trajectories than chronic hypoxia during embryonic and larval incubation (Burggren and Reyna, 2011).

We measured a marked decrease in $\dot{M}O_{2max}$ (50%) and aerobic scope (70%) but not $\dot{M}O_{2min}$ in both developmental groups when measured in acute hypoxia (50% DO, 18 h) compared to normoxic conditions (Fig. 4.1). Since the $\dot{M}O_{2min}$ of fish in the present study was not restricted at 50% DO, this provides evidence that the O_{2crit} was below 50% DO. Indeed, O_{2crit} has been reported as approximately 39% DO at 12°C in Atlantic salmon (Remen et al., 2013) and 13.1% DO at 10°C in rainbow trout (Ott et al., 1980). The $\dot{M}O_{2max}$ decreased by ~50% in 50% DO compared to normoxia (Fig. 4.1B, E), which is similar to a study of juvenile rainbow trout (Svendsen et al., 2012). Interestingly, it has been shown recently that $\dot{M}O_{2max}$ is affected differently in individuals exposed to hypoxia depending on their $\dot{M}O_{2max}$ in normoxia. Fish with a relatively low $\dot{M}O_{2max}$ are proportionally less affected than those with a high $\dot{M}O_{2max}$ (Norin et al., 2016).

The relationships between DO at LOE and each of the metabolic attributes (Fig. 4.3) are likely to be driven by how $\dot{M}O_{2max}$ and $\dot{M}O_{2min}$ interact to determine O_{2crit}. Indeed, O_{2crit} is thought to play a role in determining DO at LOE. The O_{2crit} of an individual is often defined as the oxygen level below which $\dot{M}O_{2min}$ can no longer be maintained and fish begin oxyconforming. Similarly, O_{2crit} is also thought to coincide with the oxygen level where declining $\dot{M}O_{2max}$ intersects with $\dot{M}O_{2min}$, which implicates both metabolic parameters in determining O_{2crit} (Claireaux and Chabot, 2016; Claireaux et al., 2000; Svendsen et al., 2012). Individual salmon in the present study exhibited a lower DO at LOE when they possessed a higher $\dot{M}O_{2max}$ and aerobic scope and a lower $\dot{M}O_{2min}$, suggesting that O_{2crit} is dependent on $\dot{M}O_{2max}$ and $\dot{M}O_{2min}$ and contributes to driving the threshold level of DO before LOE. However, this pattern was only evident for $\dot{M}O_{2max}$ measured in hypoxic conditions. This

may be because $\dot{M}O_{2max}$ is not necessarily limited by oxygen availability in normoxic conditions and therefore inter-individual differences in the capacity of fish to take up oxygen may not be fully revealed when measuring $\dot{M}O_{2max}$ in normoxia (Lefrançois and Claireaux, 2003; Svendsen et al., 2012). However, limited oxygen availability does not explain why we found a relationship between $\dot{M}O_{2min}$ at 50% DO and DO at LOE but not for $\dot{M}O_{2min}$ at 100% DO, because $\dot{M}O_{2min}$ is not reduced until below O_{2crit}. An explanation for this may be that individual fish have differential responses to environmental fluctuations depending on the trait being measured, as seen in barramundi (*Lates calcarifer)* (Norin et al., 2016). Nevertheless, because we did not measure the response of each individual in both normoxia and hypoxia, it remains uncertain whether $\dot{M}O_{2min}$ measurements in hypoxia truly reveal a differential relationship between $\dot{M}O_{2min}$ and DO at LOE at the individual level.

We found that developmental hypoxia had a negligible effect on the long-term aerobic performance and hypoxia tolerance of Atlantic salmon despite evidence of immediate and enduring cardiovascular alterations in response to developmental hypoxia in other fish species (Johnston et al., 2013; Miller et al., 2011; Yaqoob and Schwerte, 2010). As hypoxic conditions continue to become more prevalent in aquatic systems (Altieri and Gedan, 2015; Diaz and Rosenberg, 2008), it is increasingly important to understand the likely intra- and inter-specific responses of aquatic taxa. Future research will help to understand whether developmental hypoxia has immediate and universal effects on organismal performance and if so, how long these changes are sustained once benign conditions are re-established.

Chapter 5: Hypoxia during incubation does not affect the acclimation of aerobic capacity and haematology in Atlantic salmon during re-exposure to hypoxia

Abstract

Hypoxia in aquatic ecosystems is becoming increasingly prevalent, potentially reducing fish performance and survival by limiting the oxygen available for aerobic activities. Fish acclimate to hypoxia via a variety of short- and long-term physiological modifications in an attempt to maintain aerobic performance. In particular, hypoxia exposure during early development may result in long-term cardio-respiratory modifications that affect hypoxia acclimation capacity. We incubated Atlantic salmon (*Salmo salar*) in normoxia (~100% DO), moderate hypoxia (~63% DO) or cyclical hypoxia (100-25% DO daily) from fertilisation until 113 days post-fertilisation prior to rearing all groups in normoxia for a further 8 months. Subsequently, subsets of each group were acclimated to hypoxia (50% DO) for up to 44 days prior to haematology, aerobic metabolic rate and hypoxia tolerance measurements. Hypoxia exposure during incubation (fertilisation to 113 days post-fertilisation) did not affect the haematology, aerobic performance or hypoxia tolerance of juvenile salmon in later life. Juveniles acclimated to hypoxia increased aerobic capacity (maximum aerobic metabolic rate and aerobic scope) when measured at 50% DO but not at 100% DO, despite associated increases in blood oxygen carrying capacity (increased haematocrit and haemoglobin concentration). However, increased aerobic capacity in hypoxia was not associated with improvements in hypoxia tolerance (critical oxygen level and DO at loss of equilibrium). Our results suggest that Atlantic salmon possess a considerable capacity to physiologically acclimate to hypoxia by improving aerobic capacity in low oxygen conditions, however, acclimation capacity is not affected by hypoxia exposure during incubation.

Introduction

Aquatic hypoxia is increasing in prevalence throughout marine and freshwater systems, largely due to nutrient enrichment from anthropogenic sources (Diaz and Rosenberg, 2008). The majority of aquatic organisms, including most species of fish, are obligate water-breathers and therefore must endure or escape areas with low dissolved oxygen (DO). Salmonids may experience fluctuating oxygen levels well below 20% DO (% air saturation) during embryonic and larval development in under-gravel redds due to variations in water flow rates, embryo density and developmental stage (Ciuhandu et al., 2007; Dhiyebi et al., 2013; Greig et al., 2007; Ingendahl, 2001; Miller et al., 2008). Once free-swimming, salmonids may again encounter hypoxia in freshwater lakes and marine ecosystems due to expanding hypoxic zones and decreasing global aquatic oxygen content (Diaz and Rosenberg, 2008; Jenny et al., 2016; Keeling et al., 2010; Schmidtko et al., 2017). Given the critical role of oxygen in driving performance and fitness, it is increasingly important to understand the responses and limits of aquatic organisms in low DO environments.

Oxygen uptake rate (aerobic metabolic rate; $\dot{M}O_2$) and critical oxygen level (O_{2crit}) measurements are useful tools for assessing the extent to which aquatic animals can endure low DO environments. Aerobic scope (the difference between minimum metabolic rate, $\dot{M}O_{2min}$ and maximum metabolic rate, $\dot{M}O_{2max}$) represents the capacity for oxygen uptake rate to increase beyond baseline levels and support energy-demanding activities such as digestion and swimming (Claireaux and Chabot, 2016; Fry, 1971). A decrease in oxygen availability decreases $\dot{M}O_{2max}$ and aerobic scope, therefore decreasing the potential for fish to undertake oxygen-demanding processes. When environmental oxygen concentration declines below the O_{2crit} (the DO below which $\dot{M}O_{2min}$ can no longer be maintained), fish must either decrease their metabolic energy requirements or meet energy demands via anaerobic metabolism to maintain physiological homeostasis (Richards, 2009). If DO remains below O_{2crit} then loss of equilibrium (LOE) and death can ensue (Claireaux and Chabot, 2016). Thus, the aerobic scope and O_{2crit} of individuals provides insight into fish performance in low DO environments. Furthermore, the acclimation capacity of these traits may play some role in predicting population-level responses to increasingly hypoxic environments.

Hypoxia exposure of fish typically induces physiological acclimation that can increase oxygen uptake capacity, thereby improving hypoxia tolerance and aerobic performance. Fish

immediately respond to hypoxia by releasing red blood cells via the contraction of the spleen, followed by erythropoietin-induced red blood cell formation during prolonged hypoxia exposure (Boutilier et al., 1988; Gallaugher and Farrell, 1998; Lai et al., 2006; Tervonen et al., 2006). As a result, blood-oxygen carrying capacity of fish increases during hypoxia exposure through increases in haematocrit (via increased number of red blood cells) and haemoglobin concentration ([Hb]). Improvements in blood-oxygen carrying capacity can be associated with improvements in hypoxia tolerance. For instance, increased blood-oxygen carrying capacity via increased haematocrit and [Hb] during hypoxia exposure was associated with a reduction in O_{2crit} and DO at loss of equilibrium in killifish (*Fundulus heteroclitus*) (Borowiec et al., 2015). Increases in acute hypoxia tolerance following hypoxia acclimation have also been reported in Atlantic salmon (*Salmo salar*; decreased DO at loss of equilibrium), zebrafish (*Danio rerio*; increased time to death at low DO) and brook trout (*Salvelinus fontinalis*; increased time to death at low DO) (Anttila et al., 2015; Rees et al., 2001; Shepard, 1955). However, physiological responses to hypoxia acclimation are not consistent across all studies. Hypoxia acclimation of rainbow trout (*Oncorhynchus mykiss*) did not affect U_{crit}, haematocrit or [Hb] despite increased blood-O_2 affinity and decreased red blood cell ATP (Bushnell et al., 1984). Similarly, haematocrit and [Hb] of steelhead trout (*Oncorhynchus mykiss)* was not affected by at least 17 weeks of acclimation to 40% DO (Motyka et al., 2017). Thus, there may be species- and environment-dependent factors affecting physiological responses to hypoxia acclimation that have not been clearly elucidated.

It is possible that some of the disagreement between studies of hypoxia tolerance in fish stems from developmental plasticity, which is the ability of an individual to develop a range of phenotypes depending on its incubation environment. The resulting phenotype that develops in response to environmental conditions during early development may have positive or negative long-term consequences for fitness by altering an organism's developmental trajectory (Bateson et al., 2014; Beldade et al., 2011; Burggren and Reyna, 2011; Gilbert, 2012). For example, the U_{crit} of rainbow trout exposed to developmental hypoxia was lower than that of trout exposed to developmental normoxia after both groups had been subsequently held for 48 days in normoxia (Johnston et al., 2013). However, acute hypoxia tolerance (DO at loss of equilibrium) in Atlantic salmon and European seabass (*Dicentrarchus labrax*) exposed to developmental hypoxia was unaffected compared with normoxia-incubated individuals after more than 6 months in normoxia (Chapter 4;

Vanderplancke et al., 2015). Additionally, $\dot{M}O_{2min}$ was unaffected in zebrafish after 6 months in normoxia following developmental hypoxia, and $\dot{M}O_{2min}$, $\dot{M}O_{2max}$ and aerobic scope of Atlantic salmon and European seabass were unaffected after 6 months or more in normoxia following post-developmental hypoxia (Chapter 4; Robertson et al., 2014; Zambonino-Infante et al., 2017). While the long-term effects of early developmental hypoxia may not be apparent in environmentally benign conditions, it is plausible that developmental hypoxia may instead affect the animal's future capacity to acclimate to changing DO environments (Beaman et al., 2016). Indeed, temperature during early development can determine the long-term acclimation capacity of U_{crit} in zebrafish (*Danio rerio*), and $\dot{M}O_{2min}$ and aerobic scope in mosquitofish (*Gambusia holbrooki*) (Scott and Johnston, 2012; Seebacher et al., 2014). Therefore, it is possible that developmental hypoxia could similarly influence long-term hypoxia acclimation capacity.

Here, we measured haematology, aerobic capacity and hypoxia tolerance to compare the hypoxia acclimation capacity of juvenile Atlantic salmon following exposure to either normoxia (~100% DO), constant hypoxia (~63% DO) or cyclical hypoxia (100-25% DO daily) during early development. We hypothesised that early developmental hypoxia will increase hypoxia acclimation capacity later in life via larger and more rapid physiological responses upon re-exposure to hypoxia. These responses may include more pronounced increases in blood oxygen carrying capacity via increased haematocrit and [Hb] associated with increases in $\dot{M}O_{2max}$, aerobic scope and hypoxia tolerance (O_{2crit} and DO at LOE).

Materials and methods

Developmental incubation

The Atlantic salmon in this chapter were exposed to the normoxia, moderate hypoxia and cyclical hypoxia incubation treatments described in Chapter 3. In brief, salmon embryos and alevins (yolk-sac larvae) were exposed from 4 hours post-fertilisation until 113 days post-fertilisation (DPF; ~910 degree days) to one of three treatments (2 replicate Heath trays per treatment): 99.6 ± 1.6% DO at 8.05 ± 0.20°C (normoxia; mean ± SD), 63.0 ± 3.3% DO at 8.03 ± 0.16°C (moderate hypoxia), or 24 hour cyclical hypoxia between 100% and 25% DO (66.0 ± 22.7% DO; 8.12 ± 0.19°C; Fig. 3.1).

Post-incubation rearing

At 113 DPF, ~600 alevins were transferred from each Heath tray to ~42 L glass aquaria (L x W x H = 44 x 30 x 30 cm or 60 x 24 x 30 cm) containing normoxic freshwater supplied from the semi-closed recirculation system described in Chapter 3. Each replicate Heath tray was transferred to an individual tank, resulting in 2 replicate tanks per developmental treatment group (6 tanks in total). Water temperature was increased from 8°C to 10°C over a two hour period and DO increased to 90-100% from incubation treatment levels (cyclical hypoxia treatment already > 90% DO at transfer). All tanks were fed daily with an equal ration of commercial Atlantic salmon pellet feed (Skretting, Tasmania, Australia). Fish were maintained under these conditions for ~33 weeks and stocking density throughout rearing was maintained below 18 g L^{-1} by periodically culling randomly selected individuals.

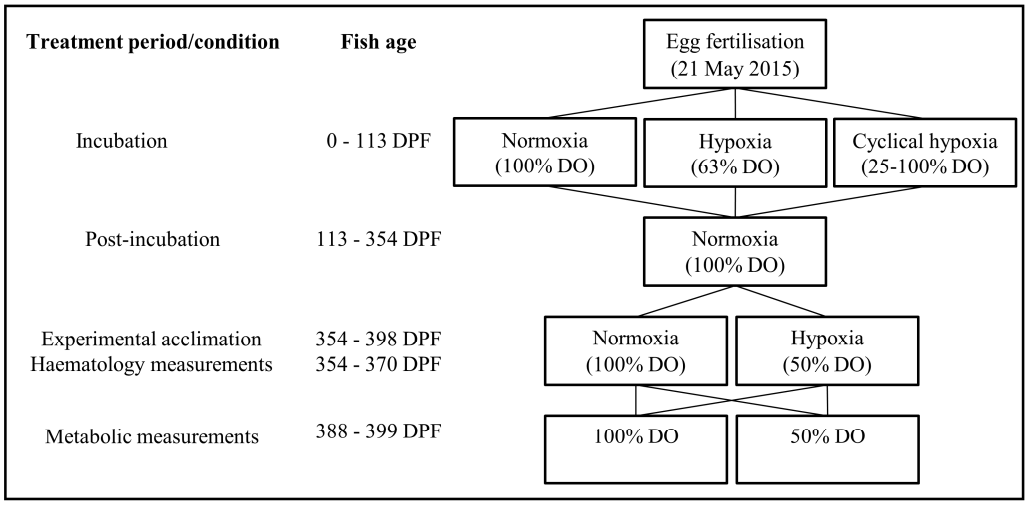

Figure 5.1: Experimental and measurement oxygen exposures and timing.

Experimental acclimations

At 342 DPF, the fish were distributed between two 6-tank freshwater aquarium systems, maintaining 2 replicate tanks per developmental treatment within each aquarium system as during post-incubation rearing (Fig. 5.1). The fish were initially stocked at a density of ~7 g L^{-1} at ~10 °C and acclimated until 354 DPF in normoxic conditions before being exposed to either 97.8 ± 2.2% DO at 10.3 ± 0.3 °C (normoxia) or 51.2 ± 3.4% DO at 10.3 ± 0.3 °C (hypoxia). Water was recirculated to each 6-tank system from separate 200 L sumps at 3 L min^{-1} for each tank and each 200 L sump was recirculated at 3-5 L min^{-1} from a common semi-closed recirculating filtration system. Dissolved oxygen in the normoxia system was

maintained with vigorous aeration, and DO in the hypoxic treatment system was maintained by nitrogen injection into the 200 L sump controlled by an Oxyguard Pacific oxygen monitoring system (Oxyguard, Denmark). Water temperature was maintained at ~10°C by a heat exchanger controlled by a central building management system. Water quality was monitored on at least 3 days per week and maintained at 0 – 0.18 mg L^{-1} NH_3-N (ammonia), 0.07-0.18 mg L^{-1} NH_2-N (nitrite), 7.15 – 7.75 pH and 35 – 60 mg L^{-1} $CaCO_3$ (alkalinity). Fish were maintained under the acclimation conditions for ~33 days and fed to satiation once per day with a commercial pellet feed (as above).

Haematology during experimental acclimations

Haematocrit and haemoglobin concentration ([Hb]) were measured in 53-58 hypoxia-acclimated fish at 0 d (pre-treatment), 1, 7 and 14 d of exposure to hypoxia, and in 54-60 normoxia-acclimated fish at 0, 7 and 14 d following establishment of the treatments. Approximately equal numbers of fish were randomly selected from each replicate tank. Each fish was euthanised by anaesthetic overdose (Aqui-S, Lower Hutt, New Zealand) for 10-20 min prior to blood sampling. Blood was obtained by transecting the caudal peduncle using a sterilised scalpel blade. The potentially contaminated blood that initially pooled was removed with paper towel. Blood for analysis was sampled directly from the blood pooling on the transected caudal peduncle into a 0.8 mm diameter heparinised microcapillary tube (Drummond Scientific Co., USA) for haematocrit measurement, and a HemoCue Hb201+ microcuvette (HemoCue, Ängelholm, Sweden) for haemoglobin measurement. Haematocrit was calculated as the percentage of packed red cell volume in whole blood following centrifugation at ~4000 RCF for 4 min using a microhaematocrit centrifuge (SpinCrit, USA). Blood [Hb] determined by the HemoCue (following 7 min of incubation in the microcuvette; see Clark et al. (2008)) were adjusted using a calibration equation for Atlantic salmon blood (Andrewartha et al., 2016). Mean corpuscular haemoglobin concentration (MCHC) was calculated from [Hb] and haematocrit using equation (5.1).

$$MCHC = \frac{[Hb]}{(Haematocrit \div 100)} \quad (5.1)$$

Respirometry setup

Aerobic metabolic rate ($\dot{M}O_2$: oxygen uptake rate) of individual fish (10.18 ± 0.24 g) was measured in 552 mL (total volume) intermittent-flow respirometer with constant mixing. Each respirometer consisted of a plastic chamber with an o-ring sealed lid. Oxygen concentration was measured at 5 s intervals using an optical oxygen sensor connected to a four channel FireStingO2 optical oxygen meter (PyroScience, Germany). The oxygen sensor for each respirometer was mounted within the outflow pipe of a small submersible pump inside the respirometer, which shielded the sensor from contact with the fish and ensured respirometer water and oxygen concentration remained constantly mixed and homogenous. Fifteen respirometry chambers were submerged in a single water bath housed in a temperature-controlled room and supplied with freshwater from a common sump. The sump temperature was maintained at ~10°C with a submersible titanium heater (Aqua Logic, California, USA) and dissolved oxygen was maintained at either 100% by vigorous aeration, or at 50% by nitrogen injection controlled by an Oxyguard Atlantic oxygen monitoring system (Oxyguard International, Denmark). Water for all respirometers was periodically replaced via a flush pump with a single timer-controlled solenoid valve that controlled water flow.

Table 5.1: Incubation treatment, acclimation treatment and respirometry measurement DO group combinations and sample sizes for Atlantic salmon (*Salmo salar*) respirometry measurements.

Incubation treatment	Acclimation treatment	Respirometry measurement DO (%)	Sample size
Normoxia	Normoxia	100	19
Normoxia	Normoxia	50	14
Normoxia	Hypoxia	100	13
Normoxia	Hypoxia	50	19
Constant hypoxia	Normoxia	100	20
Constant hypoxia	Hypoxia	50	16
Cyclical hypoxia	Normoxia	100	19
Cyclical hypoxia	Hypoxia	50	19

Respirometry protocol

Respirometry measurements commenced 33 days after establishing the experimental acclimation treatments (388 DPF) and were completed by 44 days post-exposure (Fig. 5.1). Fish were fasted for at least 18 hours prior to respirometry. Fish were measured from a total of eight incubation/acclimation treatment group combinations, which are outlined in Table

5.1 as 'incubation treatment' + 'acclimation treatment' + 'DO at respirometry measurements'. Note that the normoxia incubation + normoxia acclimation + 50% DO measurement, and the normoxia incubation + hypoxia acclimation + 100% DO measurement groups were included to test the acute responses of fish to a change in DO immediately upon removal from their acclimation conditions. Each respirometry trial was conducted over one day at either 50% or 100% DO, so that all respirometers in a trial could be maintained under the same DO conditions.

Fish were randomly selected from each incubation + acclimation treatment group, ensuring that approximately equal numbers were selected from each replicate acclimation treatment tank (Table 5.1). Individual fish were transferred to respirometers (at respective acclimation conditions) and allowed at least 60 min to settle prior to undergoing an exhaustive exercise protocol to induce maximum metabolic rate ($\dot{M}O_{2max}$) as described previously (Clark et al., 2013). Briefly, fish were individually transferred from the respirometers to a 44 L cylindrical exercise tank (69 cm diameter x 12 cm water depth) and chased by hand for 60 seconds using tail tapping to encourage burst swimming. All fish stopped burst swimming within 60 seconds, indicating they had reached exhaustion, but were forced to continue exercising maximally for the entire time period. The exercise tank was constantly replenished with water from the water bath containing the respirometers to ensure constant DO and temperature. Following the exercise protocol the fish were immediately (within 15 s) sealed in their respirometers and DO was measured every 5 s until it dropped by a maximum of 15% DO, at which point the respirometer was flushed. After the final $\dot{M}O_{2max}$ measurement for each run was complete, all respirometers were set to a 10:10 min flush:seal cycle for at least 13 hours (including overnight) to determine post-absorptive resting metabolic rate ($\dot{M}O_{2min}$).

Loss of equilibrium, O_{2crit} and post-respirometry haematology

At completion of the $\dot{M}O_{2min}$ measurements, the respirometers were sealed and oxygen was allowed to decline as it was consumed by the fish. Each fish was constantly monitored and at loss of equilibrium (LOE; ventral surface of fish visible for at least 5 seconds), the DO was recorded and the chamber was set to continuously flush for at least 10 min while the fish recovered equilibrium. The time elapsed between sealing the chamber and LOE varied between 51 and 416 minutes. The critical oxygen tension (O_{2crit}; the DO below which $\dot{M}O_{2min}$ cannot be maintained) always occurred at a higher DO than LOE. Following the recovery of

equilibrium, each fish was removed from the respirometer, a blood sample was taken for haematocrit and [Hb] measurements (as described above) and the chambers were sealed for at least 40 min to measure background respiration.

Additionally, O_{2crit} and DO at LOE were measured in a subset of fish using a modified approach to reduce CO_2 and metabolite build-up in the respirometers and investigate any influences on O_{2crit} and DO at LOE. In the modified approach, DO was gradually decreased via nitrogen injection over a 90 min period while simultaneously measuring $\dot{M}O_2$ during a 10:10 min flush:seal cycle. The respirometers were sealed at 100, 90, 80, 70 and 60% DO for $\dot{M}O_2$ measurements, and DO in the respirometry sump was decreased to the next measurement level during each 10 min sealed cycle by injecting nitrogen gas. Once DO decreased to 50% DO, the chambers were sealed and DO allowed to decline via fish respiration until loss of equilibrium and sampled as described above.

Data analysis and statistics

Oxygen uptake rate ($\dot{M}O_2$, mg O_2 min^{-1}) was calculated from the rate of declining DO using equation (4.1), described in Chapter 4. The mean background respiration of all chambers for each run was subtracted from all fish $\dot{M}O_2$ measurements for that run. Background respiration did not exceed 15% of fish $\dot{M}O_2$ for all runs except 4 and 5, where background respiration was approximately 37% and 32% of fish $\dot{M}O_2$, respectively. For the resting period of the respirometry protocol a slope between 360 and 510 s long was used for each sealed event to calculate $\dot{M}O_2$. Minimum $\dot{M}O_2$ ($\dot{M}O_{2min}$) for each individual was calculated as the mean of the lowest four $\dot{M}O_2$ values (from a total of 40-47 measurements per fish), after excluding exceedingly high or low values that were outside ± 2 SD of the mean of the lowest four values (Clark et al., 2013). Post-exercise $\dot{M}O_2$ was calculated from the steepest 60 s declining DO slope following exhaustive exercise, and $\dot{M}O_{2max}$ was calculated from the steepest slope obtained at any point throughout the ~15 h respirometry protocol. Aerobic scope was calculated for each individual as $\dot{M}O_{2max}$ - $\dot{M}O_{2min}$.

For the duration of the LOE trial, $\dot{M}O_2$ was calculated for each consecutive 360 s interval. The critical oxygen level (O_{2crit}) was calculated using the calcO_2Crit function within the fishMO2 R package with our measurements of $\dot{M}O_{2min}$ and $\dot{M}O_2$ in a declining oxygen environment (Chabot, 2016; Claireaux and Chabot, 2016). In brief, the function calculates

O_{2crit} by fitting a linear regression to the $\dot{M}O_2$ values measured below the lowest DO where the $\dot{M}O_2$ is greater than the fifth percentile of all $\dot{M}O_{2min}$ measurements calculated previously (pivotDO). The O_{2crit} is then determined as the DO where the linear regression intercepts $\dot{M}O_{2min}$. To suit our experiment, pivotDO was calculated from $\dot{M}O_2$ values measured at >40% DO for fish that commenced at 50% DO and at >80% DO for fish that commenced at 100% DO. Our calculations used $\dot{M}O_{2min}$ determined via the abovementioned approaches rather than using the calcSMR function included within the fishMO2 package.

In addition, acute hypoxia tolerance was defined as the cumulative oxygen deficiency between O_{2crit} and loss of equilibrium. Oxygen deficiency was calculated from a function between time and DO below O_{2crit} before loss of equilibrium (DO_{def}; equation (5.2)) (Claireaux and Chabot, 2016).

$$DO_{def} = \sum_{t=0}^{t=end} \left(\frac{O_{2crit} - DO_t}{60} \right) \quad (5.2)$$

where t=0 is time when DO drops below O_{2crit}, t=end is time when the fish loses equilibrium and time increment = 1 min. For example, 1 hour spent at 1% DO below O_{2crit} is equivalent to a DO_{def} of 1.

All statistical analyses were performed using R and the packages lme4 , lsmeans and car (Bates et al., 2015; Fox and Weisberg, 2011; Lenth, 2016; R Core Team, 2016). Differences in $\dot{M}O_{2min}$, $\dot{M}O_{2max}$, aerobic scope, O_{2crit}, DO at LOE and DO_{def} between incubation treatment, acclimation treatment and measurement treatment groups were tested using ANCOVA (Type III SS) with fish mass as a covariate or using ANOVA (Type III SS) where there was not a significant relationship with fish mass. Where significant covariate interactions were detected, the independent variable and covariate (mass) were log-transformed to satisfy the statistical assumptions of ANCOVA and allow robust group comparisons. Differences in fish mass between incubation treatment groups, acclimation treatment groups and measurement treatment groups were tested using ANOVA (Type III SS). The effect of incubation and acclimation DO on [Hb], haematocrit and MCHC was tested using a linear mixed effects model with tank as the random effect and P values computed using Kenward-Roger approximations with Type III SS. Pairwise comparisons were conducted using least square

means and either the Tukey method (ANCOVA, ANOVA) or Kenward-Roger approximations (linear mixed effects model). Means are reported as mean ± SEM unless otherwise stated and comparisons of means from ANCOVA analyses calculated using least square means.

Results

Changes in haematology during acclimation

Haemoglobin concentration ([Hb]), haematocrit and MCHC within the normoxia or hypoxia acclimation treatments were not affected at any time-point by the previous incubation treatment (i.e. constant or cyclical hypoxia or normoxia; Fig. 5.2). Prior to commencing the hypoxia acclimation (0 days), [Hb] (89.3 ± 1.0 g L^{-1}), haematocrit (50.0 ± 0.4%) and body mass (5.84 ± 0.23 g) were similar between the groups destined for normoxia and hypoxia acclimation ($P = 0.1403$, $P = 0.9570$ and $P = 0.1979$, respectively, Fig. 5.2A-D). By chance, at day 0 MCHC was ~10% higher in the group destined for normoxia acclimation vs. hypoxia acclimation (188.0 ± 2.1 vs. 170.0 ± 2.2 g L^{-1}, respectively, $P = 0.0001$, Fig. 5.2E, F).

Mean haemoglobin concentration of hypoxia-acclimated fish increased ~13% from 0 days to 1 day post-exposure ($P < 0.0001$) and was elevated above normoxia-acclimated fish at 1 and 2 weeks (both $P < 0.0001$, Fig. 5.2C, D). Haematocrit was similar within the hypoxia acclimation group at 0 days and 1 day post hypoxia exposure ($P > 0.05$), however, it was ~ 6-8% higher at 1 and 2 weeks relative to 0 days ($P = 0.0075$ and $P < 0.0001$ respectively, Fig. 5.2B) and ~9-13% higher relative to normoxia-acclimated fish at 1 and 2 weeks ($P = 0.0012$ and $P = 0.0208$ respectively, Fig. 5.2A, B). Consequently, MCHC initially increased by ~ 13% after 1 day in hypoxia ($P < 0.0001$) but at 1 and 2 weeks returned to levels similar to pre-hypoxia exposure (0 days) and normoxia-acclimated fish ($P > 0.05$, Fig. 5.2E, F). Notably, fish acclimated to normoxia were larger at two weeks of acclimation than those acclimated to hypoxia (9.38 ± 0.55 g vs. 7.53 ± 0.40 g, respectively, $P = 0.0021$).

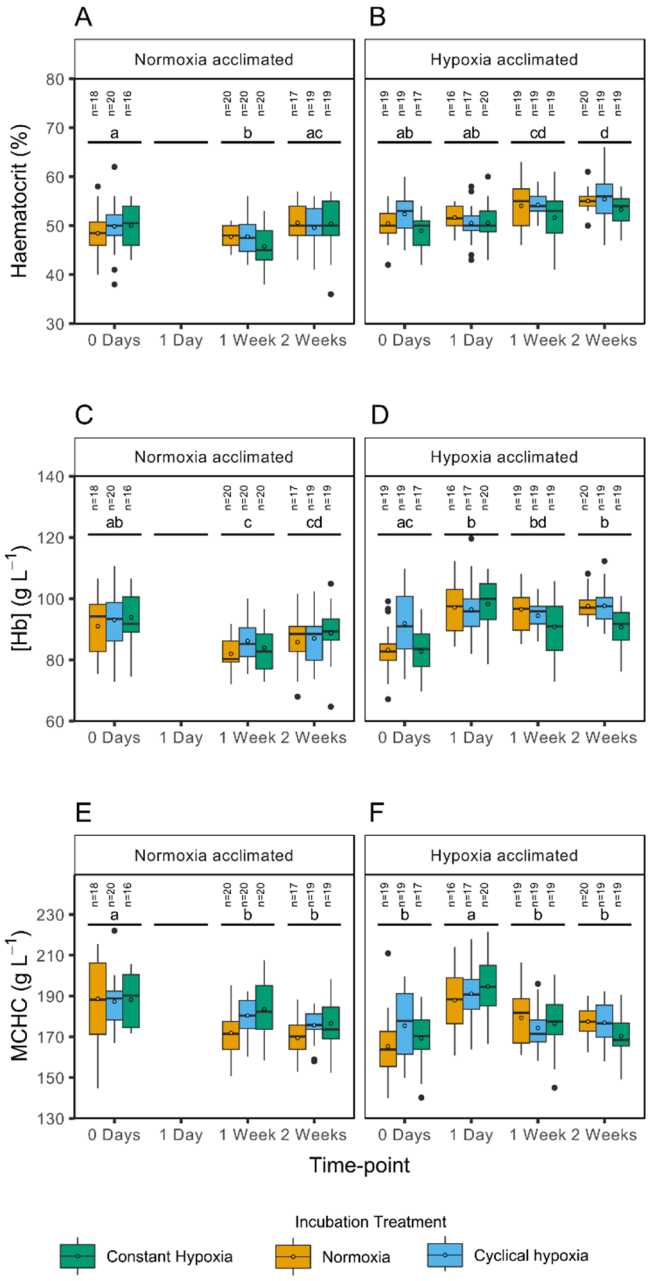

Figure 5.2: Boxplots of haematocrit (A, B), [Hb] (C, D) or MCHC (E, F) in normoxia and hypoxia-acclimated Atlantic salmon (*Salmo salar*) incubated in normoxia, constant hypoxia (~63% DO) or cyclical hypoxia (100-25% DO daily). Fish acclimated to normoxia were not measured at day 1 of the acclimation period. The solid central line is the median, the box denotes 25th and 75th percentile, whiskers extend to the highest or lowest value within 1.5*inter-quartile range and filled circles are outliers beyond that range. Mean values are represented by open circles. Different lowercase letters represent significant differences between time-points across hypoxia and normoxia acclimation treatments (averaged across incubation treatment; Tukey, $P < 0.05$).

Metabolism, hypoxia tolerance and haematology following acclimation

The mass of fish used in metabolic experiments (after ~5 weeks of acclimation) was similar between incubation, acclimation and measurement treatment groups (13.41 ± 0.88 g). Incubation treatment had no effect on $\dot{M}O_{2min}$, $\dot{M}O_{2max}$, aerobic scope, O_{2crit}, DO at LOE or DO_{def} of fish when acclimated and measured in either hypoxia or normoxia (Table B.1, Figs. B.1 & B.2). As such, the incubation treatment groups were combined for subsequent analyses to test for effects of acclimation and measurement treatment conditions.

Minimum $\dot{M}O_2$ was ~16% higher (comparison of least-square means) in fish measured in 50% DO compared with fish measured in 100% DO, while acclimation treatment had no effect (P = 0.0003 and 0.6373, respectively. Fig. 5.3A & Table B.2). Exposure to 50% DO during respirometry caused general reductions in $\dot{M}O_{2max}$ and aerobic scope compared with measurements conducted at 100% DO. However, when measured at 50% DO hypoxia-acclimated fish had ~23% higher $\dot{M}O_{2max}$ and ~52% higher aerobic scope than normoxia-acclimated fish (both P < 0.0001; Fig. 5.3B, C). Interestingly, $\dot{M}O_{2max}$ and aerobic scope were similar between hypoxia-acclimated and normoxia-acclimated fish when measured at 100% DO (P = 0.9251 and P = 0.8812 respectively; Fig. 5.3B, C).

A positive relationship existed between mass and O_{2crit} following log transformation of the data (P < 0.0001), which was similar between acclimation treatment groups (P = 0.4728, Fig. 5.4A & Table B.2). However, this relationship was different between measurement DO groups (P = 0.0073, Fig. 5.3A & Table B.2), whereby the slope was greater for fish that commenced measurement at 100% DO compared with fish that commenced measurement at 50% DO. Notably, despite a difference in mass fish that were sealed in respirometers at 100% DO had a higher O_{2crit} than those normoxia-acclimated fish for which DO was lowered to 50% prior to sealing the respirometers (29.3 ± 1.0 vs. 24.9 ± 1.2% DO, lsmeans; $F_{(1,29)}$ = 7.11, P = 0.0124; Fig. 5.5A).

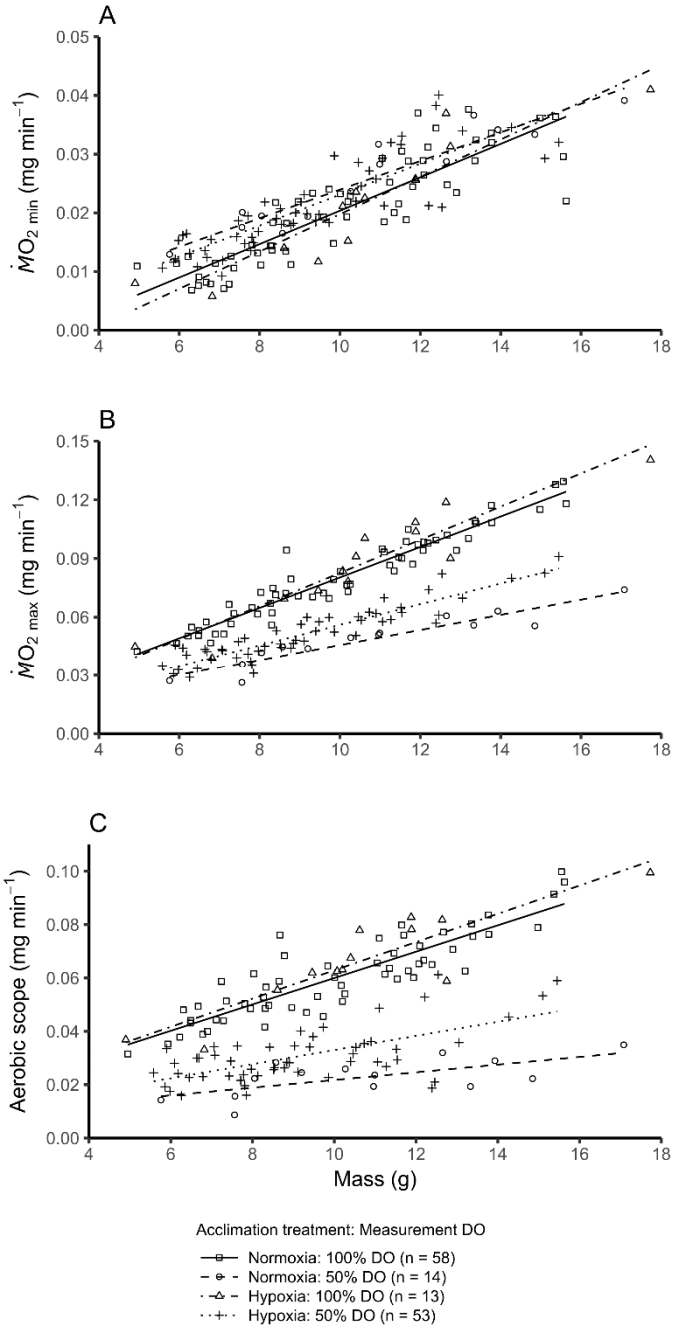

Figure 5.3: Relationships between mass and $\dot{M}O_{2min}$ (A), $\dot{M}O_{2max}$ (B) or aerobic scope (C) in Atlantic salmon (*Salmo salar*) acclimated to either hypoxia or normoxia and then measured in 50% or 100% DO. Data points represent individuals and lines are linear relationships for each acclimation + measurement group combination.

The DO at LOE also did not differ between acclimation groups, but it was lower for fish that were measured in 50% DO compared with 100% DO ($P < 0.0001$, Fig. 5.4C, D & Table B.2). Moreover, DO at LOE was similar for normoxia-acclimated fish regardless of whether the respirometers were sealed at 100% DO or reduced to 50% DO via nitrogen injection prior to

sealing ($F_{(1,28)}$ = 0.017, P = 0.8981). Conversely, DO_{def} was lower for fish acclimated to hypoxia compared with normoxia (P = 0.0187), but similar between fish measured in 50% DO vs. 100% DO (P = 0.374, Fig. 5.4B & Table B.2). Normoxia-acclimated fish that were sealed in respirometers at 100% DO had a higher DO_{def} than those for which DO was lowered to 50% prior to sealing the respirometers (7.1 ± 1.0 vs. 3.9 ± 1.1, Fig. 5.5B, $F_{(1,28)}$ = 4.63, P = 0.0402).

Following respirometry, [Hb] and haematocrit were similar for all acclimation and measurement treatment groups ([Hb]: 96.38 ± 0.79 g L^{-1}, haematocrit: 55.16 ± 0.44%, P > 0.05, Table B.2). However, an interaction existed for MCHC between acclimation and measurement treatment groups, whereby fish acclimated to hypoxia and measured in 100% DO had a higher MCHC than those acclimated to normoxia and measured in 100% DO (184.0 ± 2.7 g L^{-1} vs. 172.1 ± 2.2 g L^{-1}, respectively, P = 0.0404, Table B.2). When measured in 50% DO, MCHC was similar between fish acclimated to either normoxia or hypoxia (179.3 ± 3.5 g L^{-1} vs. 174.1 ± 2.1 g L^{-1}, P > 0.05).

Discussion

Despite the potential for incubation conditions to influence the developmental trajectory of fishes, there were no lasting effects of incubation hypoxia on the physiological phenotype (including acclimation capacity) of juvenile Atlantic salmon (Figs. B.1 & B.2) (Johnston et al., 2013; Scott and Johnston, 2012; Seebacher et al., 2014). In contrast, we demonstrated that the severity of reduction in aerobic capacity when measured at 50% DO in the juvenile life stage can be mitigated following ~33 days of hypoxia acclimation (Fig. 5.3C). This compensatory capacity in aerobic performance may be critical to maintain high performance in the diverse environments that salmonids occupy throughout their lifecycle. Interestingly, the improved aerobic capacity of hypoxia-acclimated fish in 50% DO did not translate to improved performance in 100% DO (Fig. 5.3C).

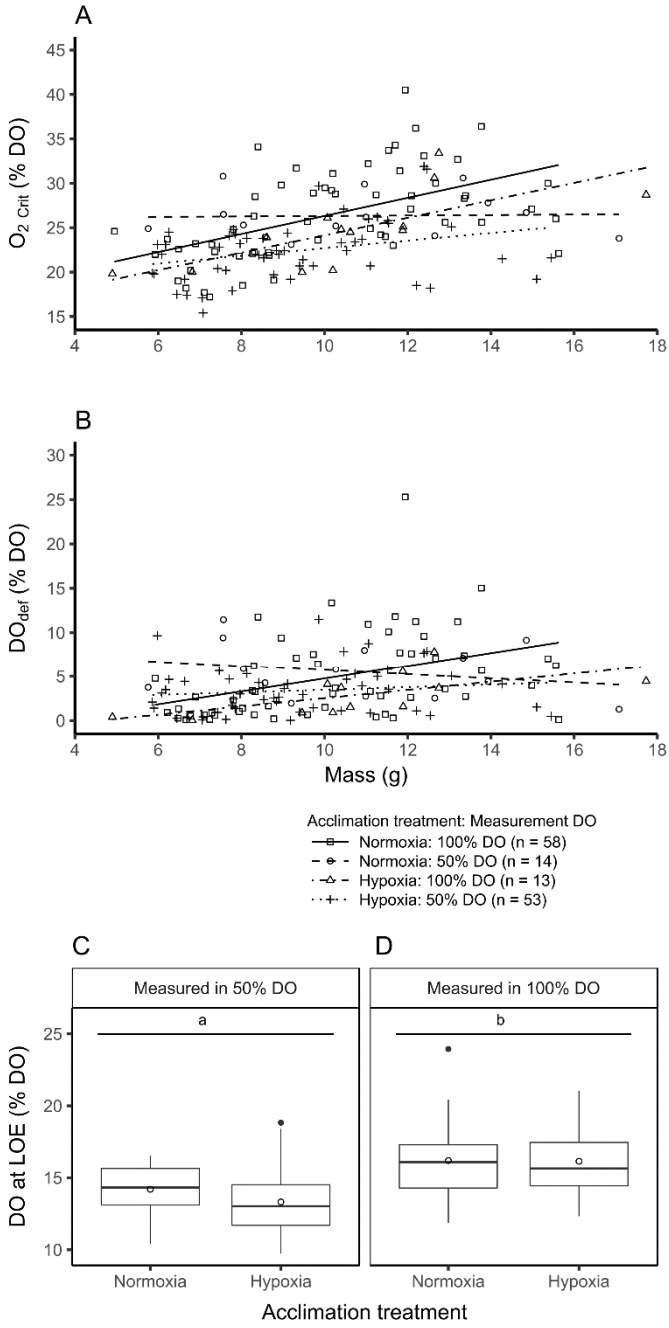

Figure 5.4: Relationships between mass and O_{2crit} (A) or DO_{def} (B), along with boxplots of DO at LOE (C, D) in Atlantic salmon (*Salmo salar*) acclimated in hypoxia or normoxia and then measured in 50% or 100% DO. For scatterplots (A, B), data points represent individuals and lines are linear relationships for each treatment group combination. For boxplots (C, D) the solid central line is the median, the box denotes 25th and 75th percentile, whiskers extend to the highest or lowest value within 1.5*inter-quartile range and filled circles are outliers beyond that range. Mean values are represented by open circles. Different lowercase letters indicate significant differences between groups (C, D; Tukey, $P < 0.05$).

Long term effects of incubation hypoxia

Most of the relatively few studies that have investigated how the incubation environment impacts long-term acclimation capacity in fish have focused on temperature. Indeed, incubation temperature has been reported to determine the long-term acclimation capacity of U_{crit} in zebrafish as well as $\dot{M}O_{2min}$ and aerobic scope in mosquitofish (Scott and Johnston, 2012; Seebacher et al., 2014). It is hypothesised that an evolutionary advantage to alter long-term acclimation capacity in response to the incubation environment only exists if the developmental environment provides reliable information about future environments (Beaman et al., 2016). This may help to explain the lack of incubation effects observed in the present study. The DO levels experienced by salmon eggs and alevins in under-gravel redds are not influenced by the same mechanisms as the DO in the freshwater and marine ecosystems inhabited by juveniles and adults (Jenny et al., 2016; Miller et al., 2008; Schmidtko et al., 2017; Youngson et al., 2004). Therefore, the DO concentrations experienced in under-gravel redds are unlikely to provide reliable information about the DO conditions experienced in later life.

Our results corroborate previous studies reporting no long-term effects of developmental hypoxia on aerobic performance or hypoxia tolerance of Atlantic salmon or European seabass or $\dot{M}O_{2min}$ in zebrafish (Robertson et al., 2014; Vanderplancke et al., 2015; Wood et al., 2017; Zambonino-Infante et al., 2017). However, some evidence exists that incubation hypoxia can cause long-term reductions in aerobic performance of fish. For example, incubation hypoxia reduced the U_{crit} of rainbow trout when measured 50 days post–incubation (Johnston et al., 2013). In the context of the aforementioned studies, our results suggest that the reported impacts of incubation hypoxia on $\dot{M}O_2$ are not sustained during long term rearing in normoxic conditions.

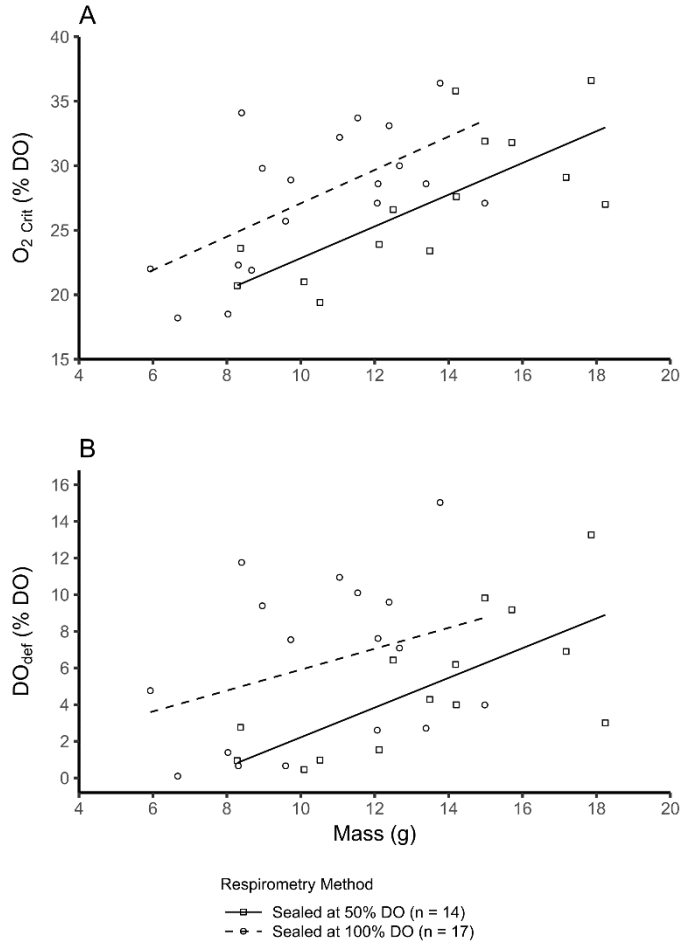

Figure 5.5: Relationships between mass and O_{2crit} (A) or DO_{def} (B) in Atlantic salmon (*Salmo salar*) during two methods of lowering DO (sealed in respirometers at 100% DO or lowering DO to 50% using nitrogen prior to sealing). Data points represent individual fish and lines are linear relationships for each treatment group.

Hypoxia acclimation and blood-oxygen carrying capacity

Proportional increases in haematocrit and [Hb] at 1-2 weeks of hypoxia exposure are likely due to an increase in erythropoietin-induced red blood cell formation in an attempt to increase oxygen delivery (Fig. 5.2) (Lai et al., 2006). However, an increase in [Hb] at 1 day of hypoxia acclimation that was not associated with increased haematocrit was unexpected (Fig. 5.2). Notably, our blood sampling technique, where fish were sampled after anaesthetic overdose, is likely to have caused splenic contraction and adrenergic induced red blood cell swelling caused by exercise- and anaesthesia-related stress (Hill and Forster, 2004; Nikinmaa and Salama, 1998; Pearson and Stevens, 1991; Yamamoto, 1987). Therefore, haematocrit values are estimates of maximal rather than baseline or routine levels. While this may preclude comparisons with studies that have used more rapid sampling approaches it does not

impact comparisons across the treatment groups used here (Clark et al., 2011; Gallaugher and Farrell, 1998).

Hypoxia acclimation and aerobic metabolic capacity

Hypoxia acclimation did not influence $\dot{M}O_{2min}$ when measured in normoxia or hypoxia, although fish measured in 50% DO exhibited a slightly higher $\dot{M}O_{2min}$ (Fig. 5.3A). The latter finding contrasts with Chapter 4, where $\dot{M}O_{2min}$ of juvenile Atlantic salmon was not affected when measured in 50% DO and contrasts with a reported decrease in $\dot{M}O_{2min}$ in rainbow trout exposed to ~50% DO for 24 h (Boutilier et al., 1988). As such, we view this finding with some caution and suggest that increased activity levels in response to acute hypoxia may be responsible for the apparent elevation in $\dot{M}O_{2min}$. In any event, the lack of reduction in $\dot{M}O_{2min}$ at 50% DO is consistent with our finding that the O_{2crit} of all individual salmon used here is below 50% DO (Fig. 5.4A).

Interestingly, we found that ~33-44 days of hypoxia acclimation increased $\dot{M}O_{2max}$ and aerobic scope of fish when measured in hypoxia (50% DO), but not when measured in normoxia (Fig. 5.3B, C). As far as we are aware, only one previous study has documented a similar finding, whereby goldfish acclimated to hypoxia for 48 hours had a higher $\dot{M}O_{2max}$ and aerobic scope when measured in hypoxia but not in normoxia (Fu et al., 2011). Increased aerobic scope of hypoxia-acclimated goldfish when measured in hypoxia was associated with an increase in U_{crit}, indicating that aerobic activities are likely to improve when oxygen uptake capacity is increased. Indeed, increased $\dot{M}O_{2max}$ for hypoxia-acclimated fish when measured in 50% DO but not in 100% DO (Fig. 5.3) indicates that oxygen uptake rate is only enhanced in hypoxic conditions. This may be driven by a leftward shift of the oxygen dissociation curve to enable better oxygen loading at the gills in hypoxia, because hypoxia acclimation can be associated with a decrease in allosteric modulators like blood cell ATP and GTP (Rutjes et al., 2007; Tetens and Lykkeboe, 1981; Tetens and Lykkeboe, 1985; Wood and Johansen, 1972; Wood and Johansen, 1973). Providing some support for that possibility, blood-O_2 affinity increased while haematocrit and [Hb] were maintained in rainbow trout acclimated to hypoxia for 2 weeks (Bushnell et al., 1984). To further elucidate the differential impacts of hypoxia-acclimation on $\dot{M}O_{2max}$ when measured in hypoxia or normoxia, focus should be paid to the mechanisms that determine oxygen uptake and metabolic energy demands in fish.

Notably, O_{2crit} and DO_{def} (but not DO at LOE) were lower when the DO in the respirometers was lowered to 50% using nitrogen injection compared with fish that were subjected to closed respirometry from 100% DO (Fig. 5.5). Similar findings for shiner perch (*Cymatogaster aggregata*) suggest that a build-up of CO_2 and metabolites may reduce the hypoxia tolerance of fish (Snyder et al., 2016). However, measuring hypoxia and normoxia acclimation treatment groups in both 50% and 100% DO allows robust comparisons between acclimation groups. Hypoxia acclimation did not improve O_{2crit} or DO at LOE despite the increased $\dot{M}O_{2max}$ and aerobic scope of hypoxia-acclimated fish in 50% DO, suggesting that hypoxia tolerance is not strongly linked with aerobic capacity (Fig. 5.4). The lack of improvement in hypoxia tolerance of juvenile Atlantic salmon following hypoxia acclimation contrasts with previous studies on zebrafish (time to death), Atlantic salmon (DO at LOE), barramundi (O_{2crit}) and killifish (DO at LOE, O_{2crit}), but is similar to studies on Atlantic salmon (O_{2crit}) and snapper (*Pagrus auratus*, O_{2crit}) (Anttila et al., 2015; Borowiec et al., 2015; Collins et al., 2016; Cook et al., 2013; Rees et al., 2001; Remen et al., 2013). While the factors influencing the acclimation capacity of hypoxia tolerance require further attention, it is clear that any potential link between hypoxia tolerance and aerobic capacity is not strong (Cook et al., 2013).

Conclusions

Salmonids exposed to constant hypoxia (~63% DO) or cyclical hypoxia (100-25% DO daily) during early development in under-gravel redds will not experience long-term physiological improvements once they have left the redd. Thus, the impacts of hypoxia in the redd are likely to be more immediate, such as decreased growth and development, and delayed hatching (Chapter 3; Hamor and Garside, 1976; Matschak et al., 1997; Miller et al., 2011). However, hypoxia acclimation reveals marked plasticity in aerobic capacity during the juvenile life stage, which may help to support the energetic requirements of juveniles in environmentally variable freshwater rearing habitats. While increased haematocrit and [Hb] play some role in improving aerobic capacity during hypoxia acclimation, the lack of improvement in aerobic capacity of hypoxia-acclimated fish under normoxic conditions suggests that other factors are at play. Our findings suggest that there may be little evolutionary advantage in Atlantic salmon modifying their hypoxia acclimation capacity in response to their incubation environment. Although, the oxygen levels used here are also not

sufficiently severe to cause long-term impairment to physiological function. Instead, salmonids possess sufficient physiological plasticity to acclimate to the unpredictable environmental conditions encountered throughout their complex lifecycle.

Chapter 6: General discussion

This thesis is one of the first long-term studies of Atlantic salmon (*Salmo salar*) physiological performance when incubated in hypoxia during early development. We found that salmon are most susceptible to hypoxic conditions prior to hatching but can defend against moderate or cyclical hypoxia (50% DO, 63% DO or 100-25% DO daily) by reducing metabolic demands (Chapters 2 & 3). Constant severe hypoxia (~27% DO) breaches the threshold oxygen level against which embryos can defend, resulting in abnormal development and high mortality (Chapter 3). Physiological responses to hypoxia during early development are temporary, and not maintained through to the juvenile stage (Chapters 4 & 5). Nevertheless, regardless of developmental conditions, juvenile salmon possess a considerable capacity for hypoxia acclimation that improves aerobic scope during subsequent hypoxia exposures (Chapter 5). While salmon may experience hypoxia throughout many of their life stages, they possess an innate capacity to withstand periods of hypoxia that may negate any advantage of long-term physiological modifications during early development.

Metabolic responses during hypoxia incubation

Atlantic salmon are most sensitive to hypoxia prior to hatching (Chapters 2 & 3). Hypoxia reduced growth and development prior to hatching in Chapter 2, and Chapter 3 showed that growth and developmental rate were specifically reduced prior to the eyed-egg stage. Reduced growth and developmental rate were associated with lower $\dot{M}O_2$ in hypoxia-incubated salmon, and in most cases associated with lower O_{2crit}. Reduced $\dot{M}O_2$ of hypoxia-incubated salmon has also been reported elsewhere and suggests that embryos can depress their metabolism in hypoxic conditions (Gnaiger et al., 1987; Hamor and Garside, 1979; Miller et al., 2008). Metabolic depression during hypoxia exposure may be advantageous due to the low anaerobic capacity of salmonid embryos and larvae (Ninness et al., 2006). Indeed, despite reductions in $\dot{M}O_2$, overall growth and developmental rate, the survival of incubating salmon was not affected during daily cycles between 100 and 25% DO (Chapter 3). Constant incubation in severe hypoxia (~27% DO) caused deformities and near-complete mortality. Differential effects of cyclical hypoxia and constant hypoxia incubation indicate that there may be temporal limitations in the physiological responses that defend against hypoxia, which are alleviated during bouts of normoxic respite.

After hatching, incubation in moderate (50 or 63% DO) or cyclical (100-25% DO daily) hypoxia did not reduce growth and developmental rate. In addition, hypoxia incubation following hatching did not reduce $\dot{M}O_2$ as it did prior to hatching (Chapters 2 and 3). Conversely, in Chapter 3 salmon incubated in moderate hypoxia slightly increased $\dot{M}O_2$ at 10 days post-hatch, however, it is unclear if the increase in $\dot{M}O_2$ was associated with increased tissue oxygen requirements and/or increased activity in the respirometers. This finding was not consistent across studies, with a lack of increase in $\dot{M}O_2$ of hypoxia-incubated salmon following hatching in Chapter 2. Nevertheless, the results of Chapter 2 and 3 suggest that the capacity of the oxygen transport cascade was not improved following hatching, because O_{2crit} in hypoxia-incubated fish was similar to normoxia-incubated fish.

Oxygen uptake measurements of alevins in Chapters 2 and 3 were conducted up until approximately 2 weeks post-hatch, leaving the possibility of further physiological responses in the following incubation period until the fry stage. The period of post-hatch development between 2 weeks post-hatch and the fry stage (that was not measured in this thesis) represents a window of potential variability in aerobic performance for future investigation due to the increasing reliance on the cardio-respiratory system for oxygen transport. Indeed, the proportion of oxygen uptake by alevins via the gills and cardio-respiratory system has been reported to steadily increase from ~25% at hatching to greater than 60% by the fry stage (Rombough and Ure, 1991; Wells and Pinder, 1996). Hypoxia incubation of late-larval stage fish can enhance the cardio-respiratory system, which may improve oxygen uptake as reliance on the cardio-respiratory system increases. For example, hypoxia-incubated Arctic charr (*Salvelinus alpinus*) increased gill lamellae surface area at 47 days post-hatching, and post-hatch zebrafish larvae increased intersegmental muscle vascularisation compared with normoxia-incubated fish (McDonald and McMahon, 1977; Yaqoob and Schwerte, 2010). In contrast, lower maximal sustainable swimming speed in hypoxia-incubated rainbow trout (*Oncorhynchus mykiss*) indicates that hypoxia incubation may also negatively impact the larval cardio-respiratory system (Johnston et al., 2013). The influence (positive or negative) of hypoxia incubation on cardio-respiratory development during late-larval stages is a worthy avenue of future research, because the potential impacts of cardio-respiratory modifications on oxygen uptake may influence performance as salmon leave the redd.

Hypoxia acclimation capacity of juvenile salmon

In Chapters 4 and 5 there was no evidence to suggest that the aerobic physiology of hypoxia-incubated salmon was permanently affected following a period of on-growing in normoxia. Salmon that were incubated in hypoxia had similar aerobic scopes, hypoxia tolerances and haematology when measured in normoxia or acute hypoxia, and following a period of hypoxia acclimation (Chapters 4 and 5). Overall, acute hypoxia exposure reduced aerobic scope, which may negatively affect the capacity to perform simultaneous energy-demanding activities such as growth, swimming and post-exercise recovery (Brett and Blackburn, 1981; Bushnell et al., 1984; Fu et al., 2011; Petersen and Gamperl, 2010). However, juvenile salmon that were acclimated to hypoxia for up to 44 days had a higher aerobic scope than normoxia-acclimated fish when challenged with hypoxia (50% DO, Chapter 5). Increased aerobic scope in hypoxic conditions is likely to reduce the limiting effect that hypoxia has on performance (Fu et al., 2011). The results of Chapter 5 illustrate the innate capacity for physiological acclimation to hypoxia in Atlantic salmon irrespective of the oxygen availability during incubation.

The environment experienced during incubation needs to be a reliable predictor of the future environment in order for favourable permanent physiological modifications during incubation to carry an evolutionary advantage (Beaman et al., 2016). However, physiological responses to the incubation environment are not always beneficial, and may result from abnormal development in sub-optimal conditions (Johnston et al., 2013; Scott and Johnston, 2012). The oxygen level within salmon redds is largely influenced by hydrological events, in particular, groundwater infiltration (Greig et al., 2007). It could be considered unlikely that the oxygen levels in under-gravel redds are a reliable indicator of the oxygen level within streams, lakes and oceans inhabited during later life stages. Indeed, the oxygen level in redds can be more closely aligned with groundwater oxygen levels rather than water within the stream (Greig et al., 2007). Thus, the innate hypoxia acclimation capacity of salmon discussed above is likely to be advantageous for dealing with transient hypoxia bouts, while long-term physiological modifications triggered by redd oxygen levels may not be adaptive because the redd environment is not a reliable indicator of future environments.

The incubation oxygen levels that did not cause mass mortality in this thesis also had no negative long-term implications for aerobic physiology. Severe incubation hypoxia (~27%

DO) caused abnormalities and complete mortality, whereas salmon could survive moderate and cyclical hypoxia (Chapters 2 and 3). Thus, any threshold oxygen level that may cause abnormal cardio-respiratory development without severely impacting survival during incubation must lie between the oxygen levels used in this thesis. Some evidence for the existence of this threshold oxygen level comes from a study in which rainbow trout incubated in hypoxia (~30% DO) had slower maximum sustainable swimming speed (U_{crit}) than normoxia-incubated fish, even once returned to normoxic conditions. Abnormal cardio-respiratory development that is maintained post-incubation could have negative impacts for fish performance, however, additional investigations are required to determine if such threshold oxygen levels exist that can affect long-term aerobic performance in salmon.

Are Tasmanian Atlantic salmon oxy-regulators or oxy-conformers?

The results of this thesis do not support previous reports that the Tasmanian Atlantic salmon population contains both oxy-conforming and oxy-regulating individuals (Barnes et al., 2011a; Barnes et al., 2011b). At both 50% DO and 100% DO, $\dot{M}O_{2min}$ was similar and all fish had the capacity to increase oxygen uptake above $\dot{M}O_{2min}$ (i.e., they had substantial aerobic scope), although aerobic scope was reduced at 50% DO due to a decline in $\dot{M}O_{2max}$ (Chapters 4 and 5). The findings here suggest that all Atlantic salmon measured in this thesis are oxy-regulators, and that $\dot{M}O_{2min}$ is independent of oxygen level above O_{2crit}. It is possible that respirometry techniques played a role in the previous reports identifying salmon as oxy-conformers. Indeed salmon measured in closed static respirometers were either oxy-regulators, oxy-conformers or too variable to be classified either way, whereas salmon that were measured in a well-mixed swim flume were all classified as oxy-regulators (Barnes et al., 2011b). Measurement of $\dot{M}O_2$ in constantly declining oxygen within static closed respirometers may have resulted in variable $\dot{M}O_2$ which may affect classification of fish as either oxy-regulators or oxy-conformers. For example, sporadic activity of fish can make determination of $\dot{M}O_{2min}$ difficult unless measurements are conducted for at least 24 h at a single DO level (Chabot et al., 2016). In addition, $\dot{M}O_2$ measurements in static (unmixed) respirometers may underestimate true $\dot{M}O_2$ due to oxygen stratification, and the build-up of metabolites in a closed respirometer may cause stress and impact $\dot{M}O_2$ (Rodgers et al., 2016; Snyder et al., 2016). Fish $\dot{M}O_2$ may also vary across different activity states within respirometers. For example, Adriatic sturgeon (*Acipenser naccarii*) were classified as oxy-conformers when measured inactive in constantly-recirculated, intermittent-closed

respirometers and oxy-regulators when measured while swimming in a well-mixed swim flume (McKenzie et al., 2007). However, Chapters 4 and 5 show no evidence that salmon were oxy-conforming when measured while inactive or following exhaustive exercise in continuously-recirculated, intermittent-closed respirometers, according to best practice guidelines (Clark et al., 2013; Steffensen, 1989). Care must be taken to ensure that $\dot{M}O_2$ measurements represent the appropriate metabolic state and that respirometry techniques robustly account for the behavioural and physiological characteristics of the fish.

Potential long-term implications of hypoxia incubation

This thesis shows that incubating salmon can experience highly dynamic oxygen levels within their incubation environment. The oxygen level measured at certain positions in an aquaculture Heath tray system dropped below 20% DO several times per day (Appendix A), and it is known that oxygen levels in natural redds can drop below 5% DO depending on temporal variation in hydrological conditions (Malcolm et al., 2006; Schindler Wildhaber et al., 2014; Sear et al., 2014). Such hypoxia exposures may severely impact survival, growth and development depending on the timing and severity of exposure. Chapter 3 shows that chronic exposure to severe hypoxia (~27% DO) produces deformities and near-complete mortality, and daily cyclical hypoxia (25% DO) reduces growth, delays development and retards hatching. Evidence suggests there is a threshold oxygen level that impacts survival, for example, embryos did not emerge from natural sea trout redds with a mean oxygen level below 53% DO, suggesting complete mortality caused by low oxygen levels (Ingendahl, 2001). However, sub-lethal oxygen levels in natural redds and aquaculture systems can still disadvantage salmon by reducing growth and development.

Reduced growth, development rate and delayed hatching caused by hypoxia exposure may disadvantage fry once they have been removed from Heath trays (aquaculture) or emerged from under-gravel redds (wild). Spatial hypoxia variation within aquaculture incubation systems may result in size variation of salmon within the same incubation system despite similar fertilisation dates and growth potential. For example in Chapter 3, salmon that were incubated in cyclical hypoxia (25% DO daily) took approximately 8 days longer at 8°C to reach the same size as normoxia-incubated fish. Consistent fish size at transfer from incubation systems to tanks for first feeding is important to ensure optimal feeding and growth (Pennell and McLean, 1996; Rombough, 1985). If there is a large variation within the

population then some fish may be small and underdeveloped at transfer, risking physical damage or localised crowding on the tank bottom, or some fish may be transferred too late when the yolk-sac has already been depleted and growth is compromised (Heming et al., 1982; Koss and Bromage, 1990; Pennell and McLean, 1996). A large size variation in the population may also increase the risk of aggressive interactions and reduce the growth of smaller subordinate fish, resulting in population-level declines in biomass production (Abbott and Dill, 1989; Abbott et al., 1985; Seppä et al., 1999). Given the potential for spatial and temporal variability in oxygen levels within artificial incubation systems to reduce growth, development and survival, it is important for hatchery workers to recognise the oxygen dynamics in incubation systems and the potential consequences during subsequent rearing phases.

Similar to artificial incubation systems, temporal and spatial variability in oxygen levels within and between natural redds may differentially affect individual performance due to reductions in size and delayed emergence associated with hypoxia. Reductions in growth, development and delayed hatching of cyclical hypoxia incubated salmon in Chapter 3 are likely to result in delayed emergence if these conditions were experienced in natural redds. Indeed, Chinook salmon that were incubated in hypoxia developed slower and emerged later than normoxia-incubated fish (Geist et al., 2006). Fry that are smaller and emerge later from redds are likely to be at a competitive disadvantage amongst conspecifics and predators in the stream environment. For example, late-emerging brown trout fry have to migrate further from the redd than early-emerging conspecifics to establish territories due to competitive interactions (Skoglund and Barlaup, 2006). As a result, the fry are at a higher risk of predation or starvation and are smaller at subsequent life stages, which may ultimately delay the season that fish migrate to sea as smolts (Einum and Fleming, 2000; Metcalfe et al., 1990; Roussel, 2007). Varying oxygen levels between redds indicates that redd site selection by spawning salmon could have severe consequences for survival and life history of their offspring (Guillemette et al., 2011; Peterson and Quinn, 1996). For example, redd sites selected by brook trout (*Salvelinus fontinalis*) and Chinook salmon (*Oncorhynchus tshawytscha*) have higher DO and upwelling water flow than nearby non-spawning sites (Geist, 2000; Guillemette et al., 2011). Thus, choosing redd sites that have higher oxygen levels throughout incubation is one way that spawning salmon can contribute to the fitness of their offspring (Guillemette et al., 2011).

Conclusions and future directions

This thesis provides insight into the physiological responses that can alleviate the negative performance impacts of hypoxia that may occur throughout a salmon's life in natural or aquaculture systems. The characteristics and threshold oxygen levels of physiological responses vary throughout incubation and in later life, although hypoxia incubation does not affect long-term physiological performance by altering developmental trajectories. An innate capacity for physiological hypoxia acclimation potentially defends against harmful long-term effects of hypoxia incubation, and may negate any advantages associated with long-term physiological modifications.

The incubation period of salmon remains a critical life stage that may be altered by varying oxygen levels. Continued efforts to identify threshold oxygen levels for physiological modifications are important due to the dynamic oxygen levels within salmonid habitats. In particular, the effects of hypoxia-induced physiological modifications at emergence from the redd remains a research area that is not well understood. Investigating the impacts of incubation environment on aerobic performance in the period before and after redd emergence will help to understand bottlenecks in survival at redd emergence and during first ponding in aquaculture. Furthermore, potential intra- and inter-population genetic variation in physiological performance are worthy of further investigation. Selecting for favourable physiological traits (such as hypoxia tolerance) within aquaculture breeding programs may enhance productivity during periods of suboptimal conditions by improving growth and survival. Additionally, population specific ecological management techniques may help to improve conservation outcomes.

Overall, further understanding of the physiological impacts of varying oxygen levels will assist to improve the sustainability of natural and aquaculture salmonid populations by evidence-based management of natural salmonid habitats and aquaculture practices.

Appendix A: Oxygen measurements in a Tasmanian salmon hatchery

Vertical Heath tray incubation systems are commonly used globally to incubate salmon in hatcheries. Within each Heath tray eggs and alevins are placed on a mesh insert that allows water flow through the eggs and alevins (Fig. A.1B) (Billard and Jensen, 1996). Water cascades vertically, sequentially through up to 16 trays in one stack (Fig. A.1A). Oxygen level generally decreases with oxygen consumption by embryos and alevins as water flows from the top to the bottom of the system (McLean and Lim, 1985). It has been suggested that oxygen levels may vary spatially and temporally within incubation systems depending on the density and developmental stage of the embryos and alevins, however, comprehensive measurements have not been conducted (Britton et al., 1983; McLean and Lim, 1985). To better understand the oxygen conditions within a salmonid Heath tray incubation system, the temporal and spatial variation in oxygen levels were measured at multiple locations for up to 55 days in a Tasmanian salmon hatchery.

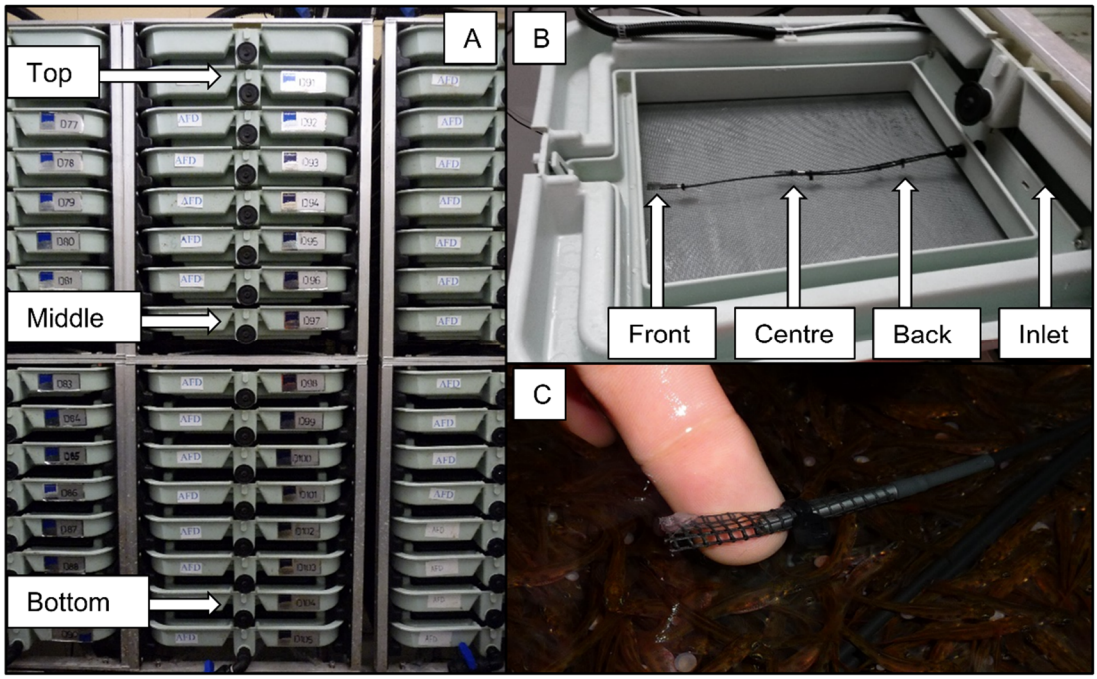

Figure A.1: Dissolved oxygen levels were measured in Heath trays at the top, middle and bottom of a Heath tray stack (A). Oxygen sensors located at the inlet (sensor not visible), back, centre and middle of each individual Heath tray (B). Oxygen sensor and mesh protective barrier, with alevins in the background (C).

Appendix A

Oxygen levels were measured at various locations within Heath trays during 2014 and 2015 at the Salmon Enterprises of Tasmania (SALTAS) hatchery in Wayatinah, Tasmania, Australia. At SALTAS, the water supplied to the Heath tray systems is temperature controlled and supplemented with oxygen in an attempt to prevent sub-optimal oxygen levels. In 2014 dissolved oxygen (DO; % air saturation) was measured in 3 Heath trays at the top, middle and bottom of a stack of 16 Heath trays, whereas in 2015 DO was only measured in the tray at the top of the Heath tray stack (Fig. A.1A). In both years oxygen measurements commenced several days prior to hatching, and were conducted for 7 days in 2014 or 55 days in 2015 (until transfer to tanks as fry). In both years four oxygen sensors were placed in each Heath tray at the inlet (behind mesh insert), and the back, middle and front of the mesh insert tray (within the eggs and alevins; Fig. A.1B). One temperature sensor was place at the inlet of each tray (Fig. A.1). Dissolved oxygen was sampled every 5 s using a 4 channel Firesting optical oxygen sensor system and the mean DO was calculated for each 60 s segment prior to analysis and graphing (Pyroscience, Germany). The oxygen sensors were calibrated at the beginning and end of each measurement period, and did not drift across the measurement period. Each oxygen sensor was protected from contact with the eggs and alevins by a mesh shroud surrounding the tip of the sensor but this did not prevent consistent water flow across the sensor tip (Fig. A.1C). Typical commercial management protocols were applied by hatchery staff throughout all DO measurements.

Overall, oxygen levels ranged from 7.6 – 149.1% DO in 2014 and 10.8 – 215.2% DO in 2015 (Figs. A.2 & A.3). Mean oxygen level at the inlet of the Heath trays decreased as water flowed from the top tray (135.3 ± 8.1% DO; mean ± SD) to the bottom tray (110.6 ± 2.5% DO) in the Heath tray stack (Fig. A.3A, E, I). In 2015, the mean daily minimum oxygen level was lowest at the back of the Heath tray (33.6 ± 28.8 % DO) compared to the inlet (142.9 ± 26.4 % DO), centre (130.5 ± 31.5 % DO) and front (127.6 ± 35.5 % DO). Indeed, the oxygen level at the back of the tray dropped below 50% DO on 46 out of 55 days for between 3 and 492 minutes per day (Figs. A.3 & A.4). In 2014, the mean daily minimum oxygen level was below 100% DO at the back, centre and front of the middle Heath tray, at the back and centre of the bottom Heath tray and only at the centre of the top Heath tray (Fig. A.2).

In 2014, reduced oxygen levels appeared to be related to an air bubble that periodically formed under the mesh insert within Heath trays, which appeared to be related to hatching. In

2015 no air bubble was observed, and low oxygen levels at the back of the Heath tray may have been linked to crowding of alevins.

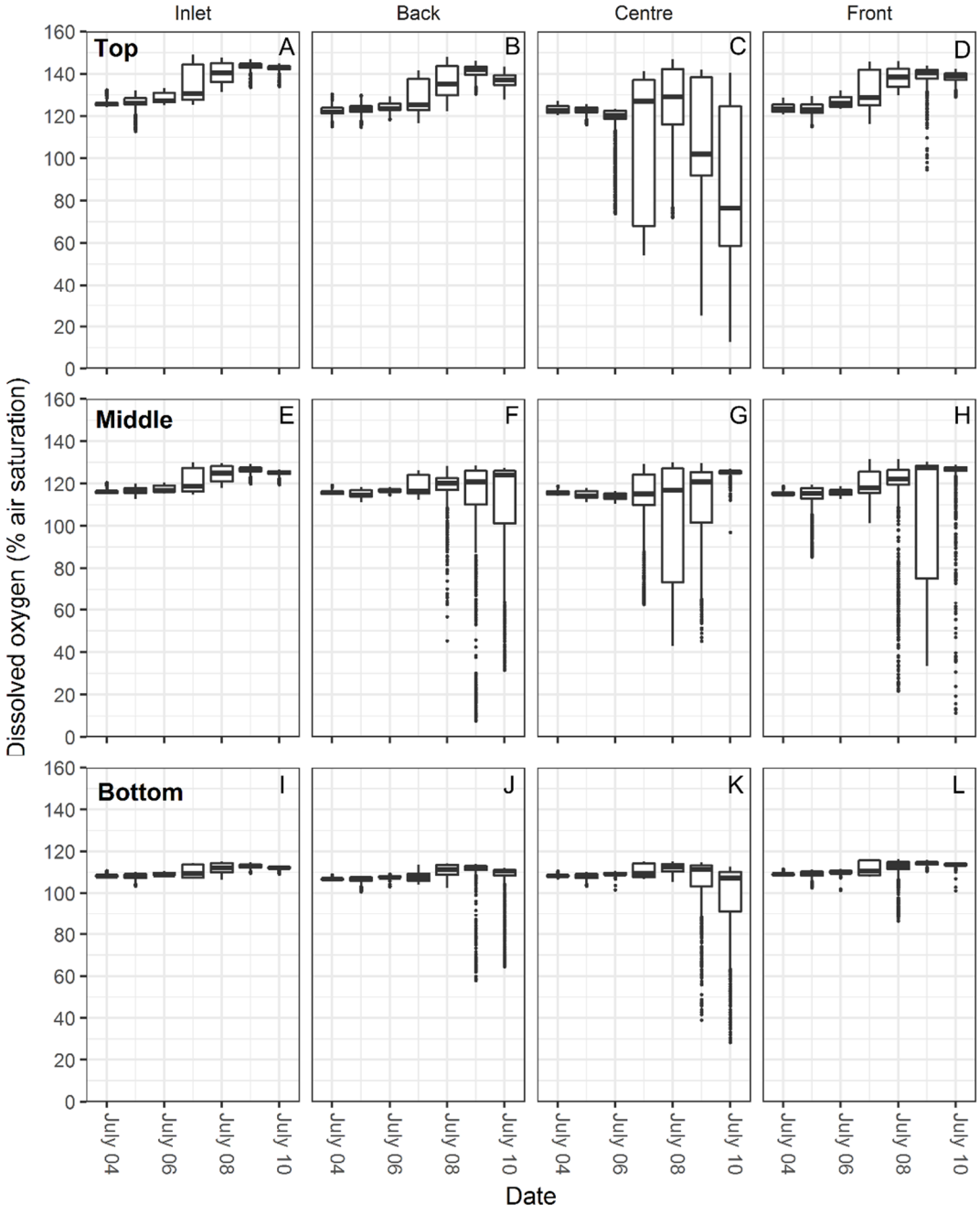

Figure A.2: Boxplots showing daily variation in dissolved oxygen levels (% air saturation) within a Heath tray at either the top (A-D), middle (E-H) or bottom (I-L) of a Heath tray system at a Tasmanian Atlantic salmon (*Salmo salar*) hatchery in 2014. Sensors were located at either the inlet (A, E, I), back (B, F, J), centre (C, G, H) or front (D, H, L) of each individual tray (Fig. A.1). The solid central line is the median, the box denotes 25th and 75th percentile, whiskers extend to the highest or lowest value within 1.5*inter-quartile range and filled circles are outliers beyond that range.

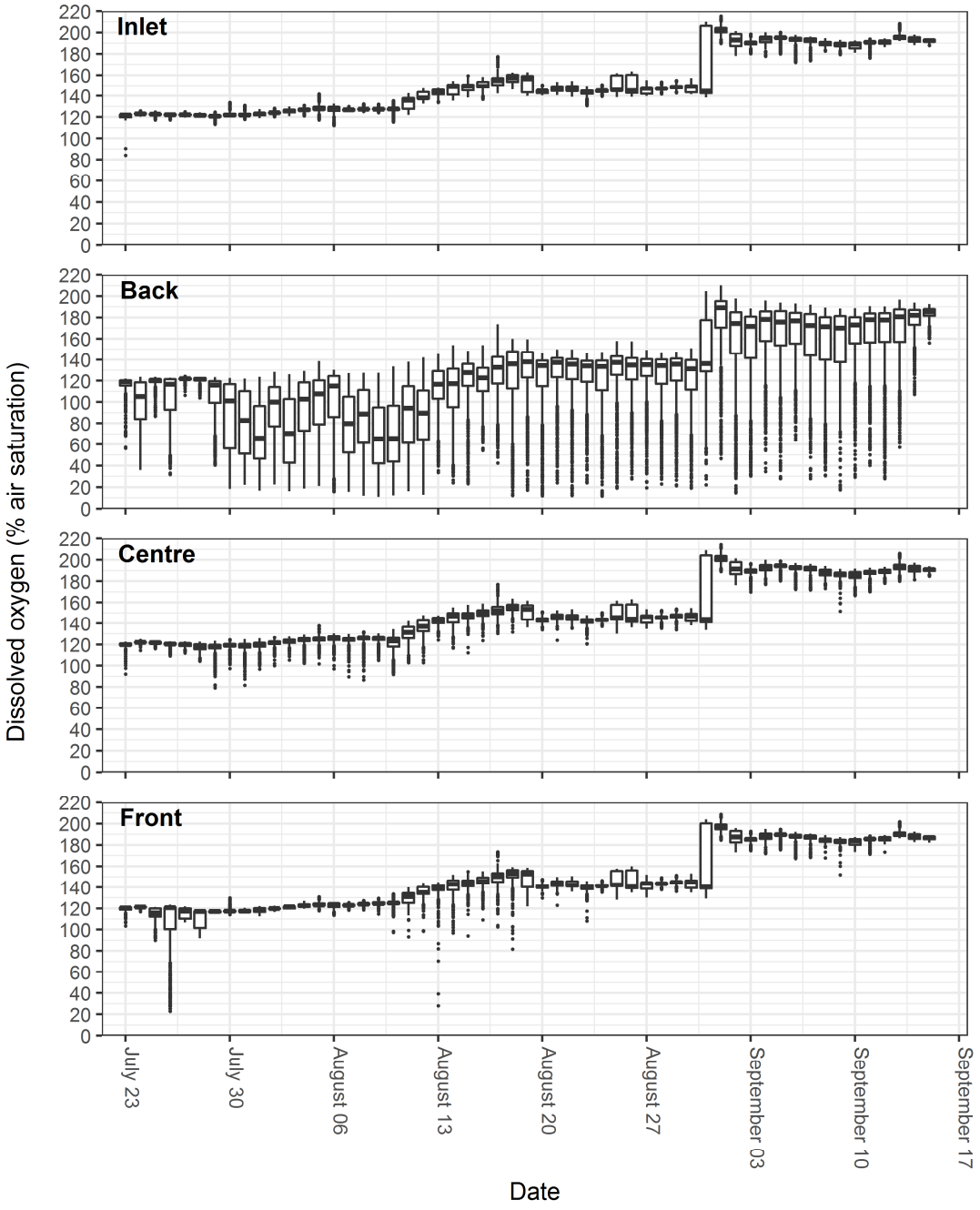

Figure A.3: Boxplots showing daily variation in dissolved oxygen levels (% air saturation) at either the inlet, back, centre or front of a Heath tray at the top of a Heath tray system at a Tasmanian Atlantic salmon (*Salmo salar*) hatchery in 2015. The solid central line is the median, the box denotes 25th and 75th percentile, whiskers extend to the highest or lowest value within 1.5*inter-quartile range and filled circles are outliers beyond that range.

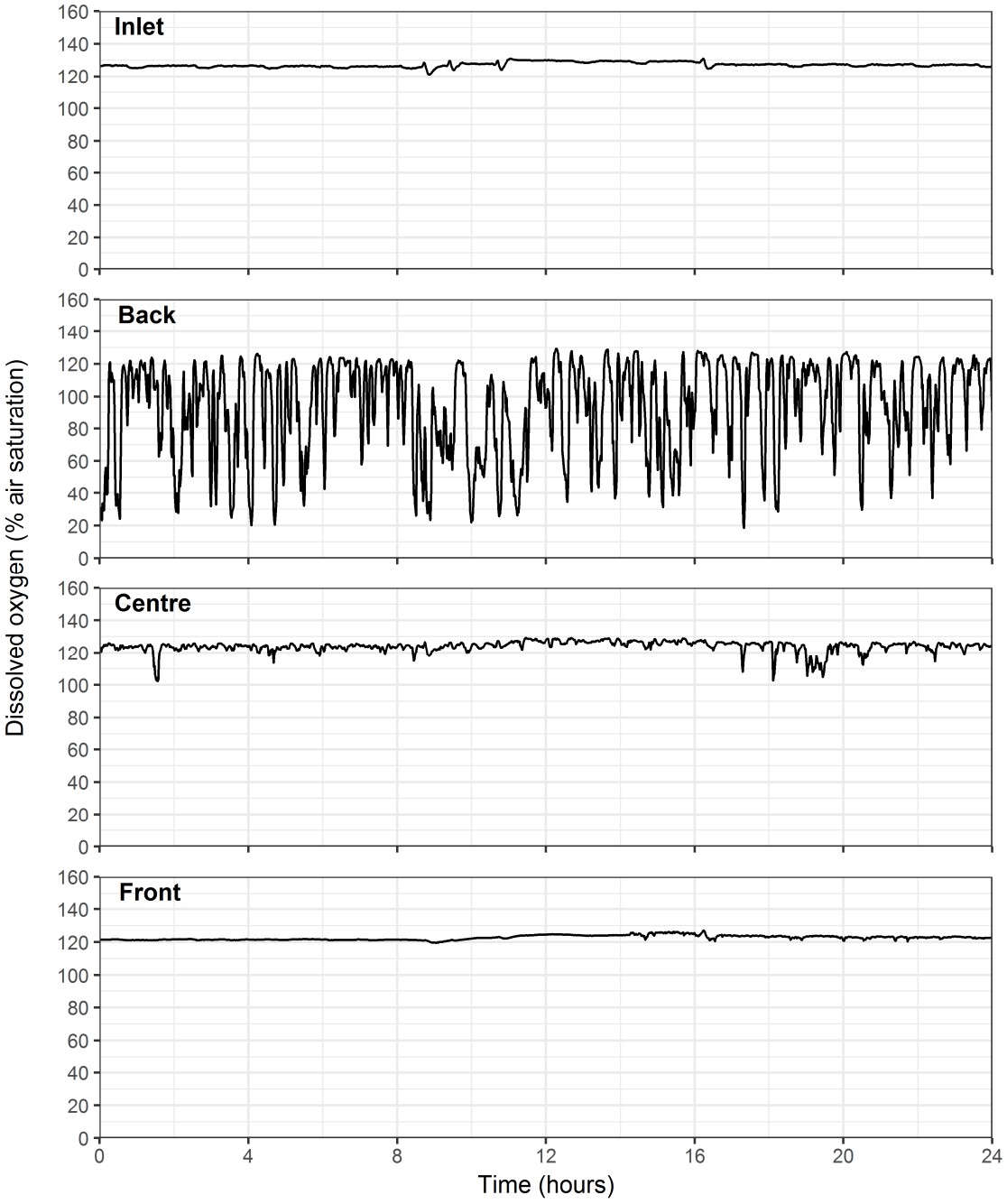

Figure A.4: Dissolved oxygen level (% air saturation) across a representative 24 h period at the inlet, back, centre and front of a Heath tray at the top of a Heath tray system (Fig. A.1) in a Tasmanian Atlantic salmon (*Salmo salar*) hatchery in 2015.

Appendix B: Supplementary figures (Chapter 5)

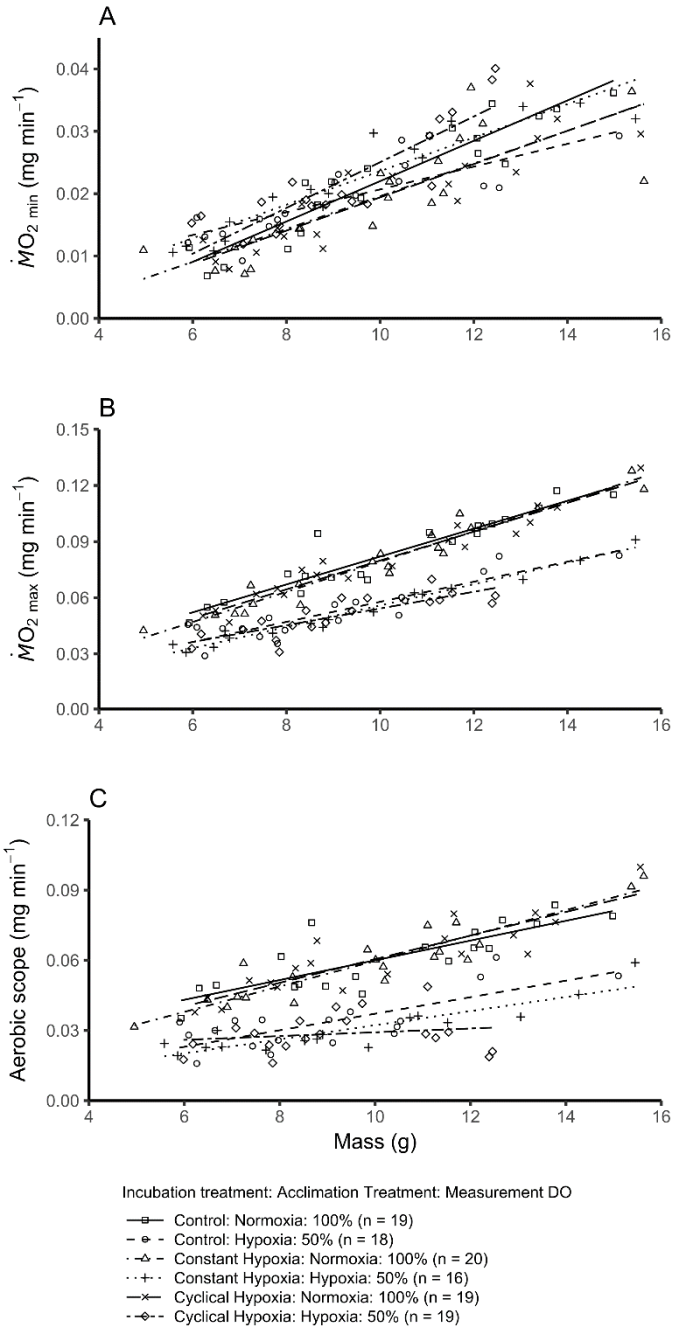

Figure B.1: Relationships between mass and $\dot{M}O_{2min}$ (A), $\dot{M}O_{2max}$ (B) or aerobic scope (C) of Atlantic salmon (*Salmo salar*) incubated in normoxia, constant hypoxia (~63% DO) or cyclical hypoxia (100-25% DO daily), acclimated to normoxia (100% DO) or hypoxia (50%DO) and measured at 100% DO or 50% DO. Individuals are represented by data points and lines are relationships between mass and metabolic parameters for each treatment group.

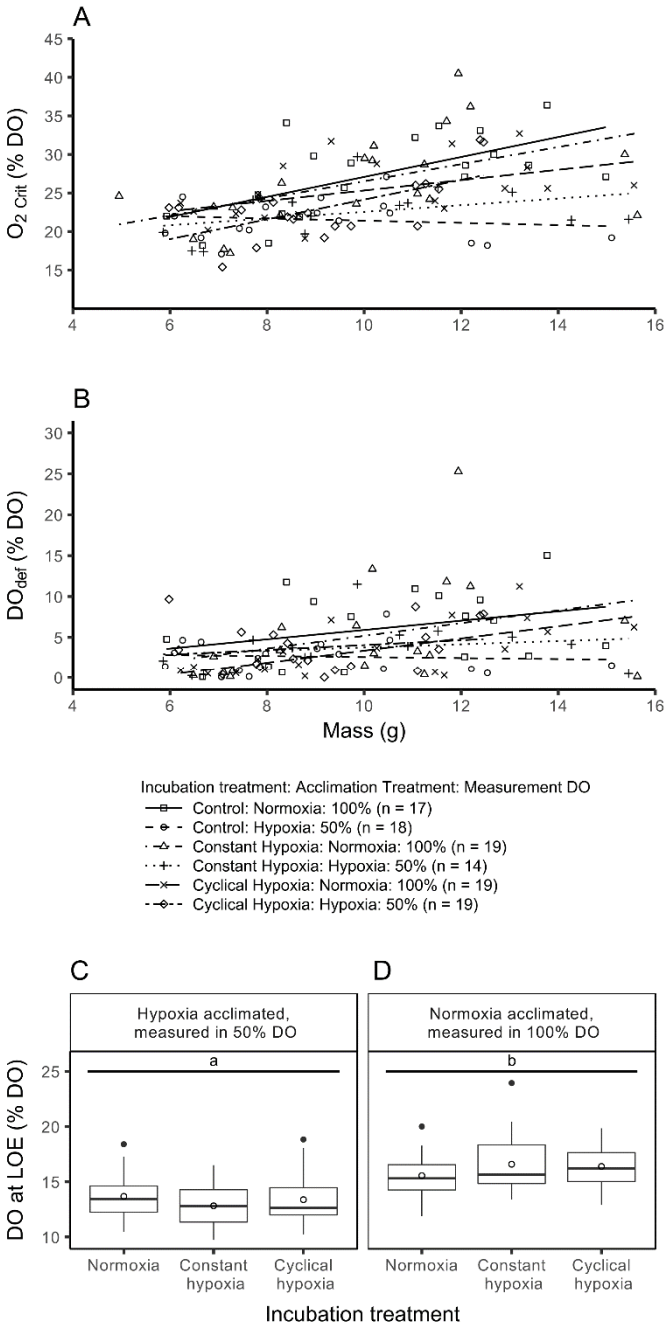

Figure B.2: Relationships between mass and O_{2crit} (A) or DO at LOE (B), boxplots of DO_{def} (C) in Atlantic salmon (*Salmo salar*) incubated in normoxia, constant hypoxia (~63% DO) or cyclical hypoxia (100-25% DO daily), acclimated to normoxia (100% DO) or hypoxia (50%DO) and measured at 100% DO or 50% DO. Data points represent individual fish and lines are linear relationships between mass and metabolic parameters for each incubation + acclimation + measurement DO treatment group combination. Incubation treatment group had no effect on either O_{2crit}, DO at LOE or DO_{def} (Tukey, $P > 0.05$).

Appendix B

Table B.1: Results from ANCOVA (A) and ANOVA (B) testing the effect of incubation treatment and acclimation/measurement treatment on metabolic and haematological parameters. Subscript values indicate group and residual degrees of freedom. Highest order effects ($P < 0.05$) are in bold.

A	$\dot{M}O_{2min}$*		$\dot{M}O_{2max}$*		Aerobic Scope*		O_{2crit}		DO at LOE^		DO_{def}	
	F_{104}	P	F_{104}	P	F_{104}	P	F_{101}	P	F_{101}	P	F_{99}	P
$Mass_1$	342.11	< 0.0001	695.20	< 0.0001	123.59	< 0.0001	25.26	< 0.0001	-	-	9.50	0.0027
$Incubation_2$	0.12	0.8866	1.95	0.1474	1.87	0.1592	0.07	0.9320	0.13	0.8770	0.44	0.6433
$Acclimation_1$	20.34	**< 0.0001**	336.33	**< 0.0001**	274.59	**< 0.0001**	19.66	**< 0.0001**	43.14	**< 0.0001**	2.39	0.1254
Incubation x $Acclimation_2$	2.85	0.0622	0.19	0.8278	1.97	0.1443	1.72	0.1842	1.51	0.2257	2.12	0.1260
B	[Hb]		Haematocrit		MCHC							
	F_{104}	P	F_{88}	P	F_{87}	P						
$Incubation_2$	0.44	0.6447	0.83	0.4397	0.21	0.8087						
$Acclimation_1$	1.35	0.2472	0.83	0.3650	0.12	0.7302						
Incubation x $Acclimation_2$	2.00	0.1405	0.25	0.7812	2.29	0.1073						

*ANCOVA was performed using log transformed independent variable and covariate (mass).
^ANOVA was performed as there was no overall relationship with mass.

Appendix B

Table B.2: Results of ANCOVA (A) and ANOVA (B) testing for effect of acclimation and measurement treatment on metabolic and haematological parameters. The highest order effects (P < 0.05) are in bold. Subscript values indicate group and residual degrees of freedom. Dashes indicate that the covariate interactions are not significant and were removed from the model to conduct between groups comparisons.

A	$\dot{M}O_{2min}$		$\dot{M}O_{2max}$*		Aerobic Scope*		O_{2crit}*		DO at LOE^		DO_{def}	
	F_{133}	*P*	F_{133}	*P*	F_{133}	*P*	F_{127}	*P*	F_{129}	*P*	F_{127}	*P*
$Mass_1$	449.44	< 0.0001	833.31	< 0.0001	151.58	< 0.0001	21.74	< 0.0001	-	-	9.16	0.0030
$Acclimation_1$	0.22	0.6373	24.62	< 0.0001	28.56	< 0.0001	1.33	0.2516	0.89	0.3464	5.68	**0.0187**
$Measurement_1$	13.54	**0.0003**	421.55	< 0.0001	382.78	< 0.0001	6.58	0.0115	24.21	**< 0.0001**	0.80	0.3739
Mass x $Acclimation_1$	-	-	-	-	-	-	0.51	0.4783	-	-	-	-
Mass x $Measurement_1$	-	-	-	-	-	-	7.45	**0.0073**	-	-	-	-
Acclimation x $measurement_1$	< 0.01	0.9271	16.36	**< 0.0001**	18.13	**< 0.0001**	1.02	0.3137	0.72	0.3995	0.14	0.7083
Mass x acclimation x $measurement_1$	-	-	-	-	-	-	0.77	0.3818	-	-	-	-
B	[Hb]		Haematocrit		MCHC							
	F_{133}	*P*	F_{117}	*P*	F_{117}	*P*						
$Acclimation_1$	0.24	0.6218	0.02	0.8708	1.22	0.2712						
$Measurement_1$	0.24	0.6281	1.21	0.2736	0.20	0.6537						
Acclimation x $Measurement_1$	0.67	0.4161	2.45	0.1205	7.72	**0.0064**						

* ANCOVA was performed using log transformed independent variable and covariate (mass).
^ ANOVA was performed as there was no overall relationship with mass

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