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PHILOSOPHIAE DOCTOR (PHD) THESIS 2010:34
ANDERS KARLSSON

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In vivo STUDIES OF RESPIRATORY PHYSIOLOGY IN ARCTIC CHARR (*Salvelinus alpinus*) AND ATLANTIC COD (*Gadus morhua*) EXPOSED TO AQUACULTURE RELEVANT LEVELS OF HYPOXIA, HYPEROXIA AND HYPERCAPNIA

In vivo RESPIRASJONSFYSIOLOGI HOS RØYE OG TORSK: EFFEKTER AV
AKVAKULTURRELEVANTE NIVÅER AV HYPOKSI, HYPEROKSI OG HYPERKAPNI

ANDERS KARLSSON

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(*Salvelinus alpinus*) and Atlantic cod (*Gadus morhua*)
exposed to aquaculture relevant levels of hypoxia, hyperoxia
and hypercapnia

In vivo respirasjonsfysiologi hos røye og torsk:
Effekter av akvakulturrelevante nivåer av hypoksi, hyperoksi og hyperkapni

Philosophiae Doctor (PhD) Thesis

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Paper I

Paper II

Paper III

Paper IV

Papers I-IV have individual page numbers.

Summary

Fish in aquaculture will be exposed to water oxygen and carbon dioxide levels that they do not normally encounter in the wild. These unnatural environmental conditions may be challenging to fish as they may lack the proper coping mechanisms to deal with them. The main objectives of this thesis are to investigate how and to what extent Atlantic cod (*Gadus morhua*) and Arctic charr (*Salvelinus alpinus*) are able to cope with these conditions. All the experiments were performed using *in vivo* methods to measure blood parameters in Arctic charr and Atlantic cod.

The experimental work in this thesis was performed at three different locations with different experimental facilities. The first experiment was carried out at the aquaculture research facility of Hólar University College in Sauðárkrókur, Iceland. In this experiment, Arctic charr were exposed to mild levels of sub- (hypoxia) and supersaturation of water oxygen (hyperoxia) over the course of four days using circular plastic tanks (Paper I). The experiments for Papers II and III were performed at the Marine Research Station of the Norwegian Institute for Water Research at Solbergstrand, Norway, using fibreglass tanks purposely designed for performing experiments with individual fish. In these experiments cod were cannulated in the caudal artery and recovery was followed for two weeks (Paper II), or cod were allowed to recover for 3-4 days and then exposed to varying levels of hyperoxia (Paper III). The final experiment was done at the Tromsø Aquaculture Research Station at Ringvassøy, Norway, using a swim tunnel respirometer (Paper IV). In this final experiment, cod were exposed to separate and combined supersaturation of carbon dioxide (hypercapnia) and hypoxia or hyperoxia.

The dorsal aorta cannulation performed on Arctic charr followed a well established cannulation method. However, this method could not be utilized to cannulate Atlantic cod. The caudal artery cannulation procedure developed during the work presented in this thesis proved to be a useful tool for performing *in vivo* experiments on Atlantic cod. A comparatively long lasting stress response was observed following cannulation of Atlantic cod. A delayed and equal magnitude stress response was observed in metomidate sedated cod compared to unsedated cod. This response indicates that in experiments where a recovery time of less than three days is necessary metomidate sedation should not be used. The use of cannulated Atlantic cod had large benefits compared to sacrificial sampling for certain blood parameters, indicating that sacrificial sampling is inadequate when sampling blood for analysis of plasma pH or K^+ concentration.

The main findings reported in this thesis are that Arctic charr and Atlantic cod do not decrease their arterial partial pressure of oxygen (p_aO_2) when exposed to hyperoxia to obtain a steady p_aO_2 similar to that observed in normoxia. Instead, their p_aO_2 follows the water partial pressure of oxygen (p_wO_2). This may make the Arctic charr and Atlantic cod vulnerable to oxidative stress with consequent negative effects on fish health and welfare. In Paper III the proposed increase in oxidative stress in hyperoxia compared to normoxia was confirmed. This

indicates that water oxygenation in aquaculture of Atlantic cod and Arctic charr should be limited to keep p_wO_2 at normoxic levels. The dependency of p_aO_2 on p_wO_2 indicates that there are no safe levels of hyperoxia in farming of Atlantic cod and Arctic charr; all levels of hyperoxia are likely to cause increased oxidative stress due to increased p_aO_2 . When exposed to hypercapnia, Atlantic cod increased ventilation and consequently p_aO_2 was closer to p_wO_2 in hypercapnia compared to that in normal water CO_2 conditions (normocapnia). Hypercapnia proved to be a more potent ventilatory stimulant than variations in p_wO_2 at aquaculture relevant levels. The increased p_aO_2 associated with hypercapnia may result in Atlantic cod being more vulnerable to oxidative stress when exposed to combined hypercapnia and hyperoxia than cod exposed to hyperoxia alone. This combined situation is especially common in on-land aquaculture facilities where oxygenation is in frequent use. A strict control of oxygen levels in the tank water should efficiently reduce this problem.

Sammendrag

Fisk i oppdrett blir eksponert for nivåer av oksygen og karbondioksid i vannet som de vanligvis ikke opplever under naturlige forhold. Dette skjer fordi det er vanlig å tilsette ekstra oksygen til vannet for å kunne produsere mer fisk per tilgjengelig vannvolum i landbasert oppdrett. Dette unaturlige miljøet kan skape problemer for fisken hvis de ikke har måter å håndtere de nye miljøbetingelsene på. Hovedmålene med denne doktorgradsoppgaven var å undersøke i hvilken grad torsk (*Gadus morhua*) og røye (*Salvelinus alpinus*) klarer å håndtere de unaturlige miljøbetingelsene de blir utsatt for i intensiv oppdrett.

Alle eksperimentene ble utført med *in vivo*-metoder for å måle blodparametre hos torsk og røye. Eksperimentene som blir presentert i denne oppgaven ble utført på tre forskjellige lokaliteter med ulike forsøksfasiliteter. Det første eksperimentet ble utført på forsøksanlegget for akvakultur til Hólar vitenskapelige høyskole i Sauðárkrókur på Island. I dette eksperimentet ble røye eksponert for moderate nivåer av under- (hypoksi) og overmetning av oksygen i vannet (hyperoksi) i runde plasttanker over en periode på fire dager (Paper I). Eksperimentene som ligger til grunn for Paper II og III ble utført på Norsk Institutt for Vannforskning sin marine forskningsstasjon på Solbergstrand i glassfiberkar spesielt designet for forsøk med individuelle fisk. I disse forsøkene ble torsk kateterisert og fulgt i to uker for å undersøke hvordan og hvor fort de henter seg inn etter operasjon, eller eksponert til varierende grad av hyperoksi etter 3-4 dager innhenting fra operasjon. Det siste eksperimentet (Paper IV) ble gjort på Havbruksstasjonen i Tromsø på Ringvassøy i en kombinert svømmetunnel og respirometer. Her ble torsk eksponert for overmetning av karbondioksid (hyperkapni) og hypoksi eller hyperoksi, enten alene eller i kombinasjon.

Kateteriseringen av dorsal aorta i røye fulgte en utprøvd metode for kateterisering av laksefisk. Denne metoden kunne ikke benyttes på torsk. I løpet av doktorgradsarbeidet ble det derfor utviklet en metode for å kateterisere kaudalarterien hos torsk for og kunne ta gjentatte blodprøver så uforstyrret som mulig. Kateteriseringsmetoden viste seg å være verdifull til bruk i *in vivo*-studier med torsk men hadde en lengre innhentingstid etter operasjon enn forventet. Hos torsk som ble gitt et sløvende middel (metomidat) før bedøvelse ble stressresponsen forsinket, men hadde lik styrke, sammenlignet med torsk som ikke fikk metomidat. Derfor bør ikke metomidat brukes for å sløve torsk i forsøk der en innhentingstid på under tre døgn er nødvendig. Å bruke kateterisert fisk har store fordeler framfor å avlive fisk før blodprøvetaking pga. stresset fisken påføres under håndteringen forbundet med avliving og prøvetaking. Plasma pH og konsentrasjon av K^+ -ioner viste seg å være spesielt lett påvirkelige for denne typen stress og analyser av disse parametrene i blodprøver tatt fra håndtert fisk kan derfor ha store iboende feil.

Hovedfunnene i denne doktorgradsoppgaven viser at torsk og røye ikke nedregulerer sitt arterielle partialtrykk av oksygen (p_aO_2) i hyperoksi for å nå et p_aO_2 -målnivå tilsvarende det de har under normoksiske forhold (normalmettet vann). Deres p_aO_2 følger isteden partialtrykket av oksygen i vannet (p_wO_2). Økt p_wO_2 kan derfor medføre økt oksidativt stress i

fisken på grunn av økt produksjon av frie radikaler grunnet det økte oksygentrykket i fisken. I Paper III ble det påvist økt oksidativt stress hos torsk under hyperoksi. Hyperoksi øker derfor risikoen for negative konsekvenser av betydning for fiskevelferd og fiskehelse. Eksperimentene viste at det ikke kan fastsettes noe trygt nivå av hyperoksi, det vil si opp til et nivå der fisken motvirket økningen i p_wO_2 og beholdt p_aO_2 på det samme nivået som i normoksiske forhold. Når torsk ble eksponert for hyperkapni økte torskens pustefrekvens og volum. Dette førte til økt p_aO_2 sammenlignet med vann som var normalmettet med karbondioksid (normokapni). Hyperkapni var en sterkere pådriver for pusting hos torsk enn oksygennivå på nivåer som er relevante for oppdrett. Det økte p_aO_2 -nivået som er forbundet med hyperkapni kan føre til at torsk som eksponeres for kombinert hyperkapni og hyperoksi opplever mer oksidativt stress enn om de var utsatt for hyperoksi alene. Denne effekten vil være spesielt vanlig i landbasert akvakultur der det tilsettes oksygen til vannet, og en økt biomasse fører til økt karbondioksid i vannet. Ved å holde oksygennivåene i karene på et normoksisk nivå og samtidig sikre god utlufting av karbondioksid vil en kunne fjerne dette problemet.

Papers

Paper I

Karlsson, A., Rosseland, B. O., Thorarensen, H. and Kiessling, A. Changes in arterial oxygen tension and blood physiological status in resting and unrestrained Arctic charr *Salvelinus alpinus* exposed to mild hypoxia and hyperoxia. *Journal of Fish Biology*. Accepted for publication pending approval of journal style revision.

Paper II

Karlsson, A., Rosseland, B. O., Massabuau, J. C. and Kiessling, A. Pre-anaesthetic metomidate sedation affects recovery from caudal artery cannulation in Atlantic cod (*Gadus morhua*). Submitted to *Fish Physiology and Biochemistry*.

Paper III

Karlsson, A., Heier, L. S., Rosseland, B. O., Salbu, B. and Kiessling, A. Changes in arterial PO_2 , physiological blood parameters and intracellular antioxidants in free-swimming Atlantic cod (*Gadus morhua*) exposed to varying levels of hyperoxia. *Fish Physiology and Biochemistry*. Published online.

Paper IV

Karlsson, A., Aas-Hansen, Ø., Evensen, T. H., Damsgård, B., Rosseland, B. O., Kiessling, A. and Toften, H. Hypercapnia overrides moderate variations in water oxygen tension in controlling ventilation and blood physiology in Atlantic cod (*Gadus morhua*).

Abbreviations

p_wO_2 : Partial pressure of oxygen in water, measured in kPa

p_aO_2 : Partial pressure of oxygen in arterial blood, measured in kPa

p_wCO_2 : Partial pressure of carbon dioxide in water, measured in kPa

p_aCO_2 : Partial pressure of carbon dioxide in arterial blood, measured in kPa

DA: Dorsal aorta

CA: Caudal artery

CV: Caudal vein

VA: Ventral aorta

HPV: Hepatic portal vein

Hypoxia: Water oxygen level below 100 % of air saturation

Normoxia: Water oxygen level near 100 % of air saturation

Hyperoxia: Water oxygen level above 100 % of air saturation

Normocapnia: Water carbon dioxide level near 100 % of air saturation

Hypercapnia: Water carbon dioxide level above 100 % of air saturation

PS: Post surgery

NIVA-MFS: Marine Research Station of the Norwegian Institute for Water Research at Solbergstrand, Norway

SAS: Statistical Analysis Software

Synopsis

Introduction

Atlantic cod and Arctic charr in the wild

In the wild, fish live more or less adapted to their natural habitats. The habitats of fishes vary greatly across the globe with regards to temperature, pressure, light intensity, seasonality, salinity, pH and saturation of gases. Some species live in very stable environments in terms of the environmental factors mentioned, while other species inhabit constantly changing environments. To thrive in these different habitats fish have evolved different methods for coping with their environments. Some involve specializations to suit their environment in particular, while other adaptations enable fish to cope with different habitats and make them capable of invading new territory where there is less competition. In this way, fish now occupy most marine and freshwater systems despite large variations in environmental conditions. Among the variable conditions are differences in water gas content, the most important being oxygen (O₂) and carbon dioxide (CO₂). In cold climates, fairly stable O₂ and CO₂ conditions are most common. These waters are usually well saturated with O₂ and contain small amounts of CO₂ compared to the levels that are commonly found in warm climates. In warmer climates the conditions are often different, especially in waters with large amounts of algae. The flexibility of the algae metabolic scope creates fluctuating environmental conditions during the course of each day since they consume CO₂ and produce O₂ during the daylight hours, and consume O₂ and produce CO₂ when it is dark. This creates a situation with high CO₂ levels (hypercapnia) and low pH and O₂ levels (hypoxia) during the early morning, and low CO₂ levels and high pH and O₂ levels (hyperoxia) during the late afternoon/evening. An animal that have evolved under these conditions may respond differently to this environment than one that has evolved under much more stable environmental conditions.

Atlantic cod (*Gadus morhua*) and Arctic charr (*Salvelinus alpinus*) are two species which inhabit cold waters ranging from brackish to full strength sea water (cod) and fresh water to full strength sea water (charr). Cod and charr inhabit environments which are normally near fully saturated with O₂ and have low CO₂ levels. When cod and charr do experience changes in O₂ or CO₂ levels, they are usually small, and in the case of O₂, mostly in the direction of low oxygen levels (hypoxia). The periods of hypoxia may occur during winter when ice cover lakes (charr) or in water layers that have not been in contact with air for long periods of time (cod and charr). If cod and charr do encounter unfavourable O₂ or CO₂ levels they nearly always have the option to move away from the unfavourable conditions and in that way avoid them.

Aquaculture and the use of oxygenation

Extensive and early intensive aquaculture relied on high specific water flow (L/kg/min) and the natural O₂ level of the water in order to supply their stock with sufficient levels of O₂ for good health and growth. However, in modern land-based aquaculture production, the high biomass and low specific water flow have made addition of O₂ to the water the norm. This practice is common due to the opportunity to produce more fish per volume of water available to the farm when adding O₂. Thus, available water volume is for many Atlantic salmon (*Salmo salar*) smolt growers the primary factor which is limiting production volume. The addition of O₂ to the water invariably produces hyperoxic conditions, to a small or large extent depending on the way O₂ is introduced to the water (e.g. water inlet or tank internal), the amount of O₂ added and the amount that is consumed by the fish. This creates a situation which Atlantic cod and Arctic charr will not usually experience in the wild, and they are unable to move away from the hyperoxic conditions since they are confined to the tank.

The use of oxygenation in aquaculture is by far most common in on-land facilities. In Norway, these facilities typically produce juveniles (Atlantic cod, Atlantic halibut (*Hippoglossus hippoglossus*) and Arctic charr) or smolts (Atlantic salmon and rainbow trout (*Oncorhynchus mykiss*)) for later on-growing in sea cages (cod, halibut, salmon and trout) or larger, land-based tank systems (charr). Thus, it is up to and including the juvenile life stage that most farmed fish will be exposed to hyperoxia, with the exception of species where on-land on-growing is common. Juvenile fish are often more sensitive than adult fish in terms of impact of environmental stressors. Thus, it is in their most sensitive phase that farmed fish are likely to experience hyperoxia. The increased biomass per water volume that oxygenation allows also creates another diversion from the natural habitats of Atlantic cod and Arctic charr; increased biomass leads to increased CO₂ production, resulting in a hypercapnic environment. The high biomass in the tanks will consume O₂ and produce CO₂ at varying rates throughout the course of a day, largely determined by feeding regime and temperature. This creates a situation where the fish may experience conditions that range from almost no CO₂ to moderate hypercapnia, combined with O₂ levels which fluctuate from hypoxia to hyperoxia.

Hyperoxic conditions have been shown to cause altered behaviour (Espmark and Bæverfjord, 2009), but also increased growth rates (Hosfeld et al., 2008) in Atlantic salmon, indicating both negative and positive effects of oxygenation in aquaculture. However, the extensive Water Quality Project (WQ-project 1999-2006) in Norway (Rosseland et al., 2005; Rosten et al., 2007) and other research projects on intensive fish farming (Stefansson et al., 2007; Bjercknes et al., 2008) all concluded that the modern intensive farming has led to more diseases and often reduced growth and deformities in fish. These negative effects have been attributed to the low specific water flow and the extended use of oxygen, and consequent hyperoxia and hypercapnia, common in intensive aquaculture in Norway.

Hypoxia, hyperoxia and hypercapnia: effects on fish physiology *in vivo*

Of the respiratory gases, water partial pressure of O₂ (p_wO₂) has been attributed a larger influence on gas exchange and ventilation than water partial pressure of CO₂ (p_wCO₂) (Randall, 1982; Graham, 2006). Thus, physiological responses of fish to varying p_wO₂ levels has been the most frequently studied. In particular, hypoxia has been a prevailing topic of past studies, possibly because hypoxia occurs more commonly in temperate and cold water environments compared to hyperoxia. Typical responses to hypoxia in water breathing fishes include lowered arterial partial pressure of O₂ (p_aO₂), increased ventilation rate/ventilatory stroke volume (hyperventilation) (Kinkead et al., 1991) and consequently lowered arterial partial pressure of CO₂ (p_aCO₂) (Gilmour and Perry, 1994) compared to conditions where p_wO₂ is close to 100 % saturated (normoxia). Although less studied, data on teleost responses to high oxygen levels are also available. Typical physiological responses to hyperoxia are the opposite of what is usually observed in hypoxia; increased p_aO₂, reduced ventilation rate/ventilatory stroke volume (hypoventilation) and consequently increased p_aCO₂ (Thomas et al., 1983; Gilmour and Perry, 1994). Lowered arterial blood haematocrit and plasma ion concentrations (Na⁺ and Cl⁻), and increased plasma pH have also been reported in hyperoxia exposed Atlantic salmon (Kristensen et al., 2010). Additionally, hyperoxia has been shown to induce changes in oxidative stress detoxification responses in both Atlantic salmon and Atlantic cod (Olsvik et al., 2005; Olsvik et al., 2006), and in rainbow trout liver cells *in vitro* (Finne et al., 2008).

Although past studies have focused mainly on the effects of p_wO₂, some studies have investigated how p_wCO₂ affects the ventilation and respiratory physiology of fish (see review by Gilmour (2001)). Until quite recently, the changes in ventilation observed in hypercapnia were believed to be secondary effects of reduced oxygen availability. Due to lowered blood pH in short term hypercapnia, the consequent Bohr effect was believed to be the underlying reasons for any effects seen on ventilation in hypercapnia (see review by Gilmour (2001)). However, the presence of CO₂/H⁺ receptors in fishes have been reported during the last decade (Burlison and Smatresk, 2000; McKendry et al., 2001; Milsom, 2002; Perry and Gilmour, 2002), indicating a direct effect of p_wCO₂ or H⁺ on the respiration and gas exchange of fishes. Hypercapnia has also been reported to reduce growth rate in Atlantic salmon smolts prior to seawater transfer (Hosfeld et al., 2008).

The combined effects of hypercapnia and hyperoxia or hypoxia on the respiratory physiology of fish have not been investigated in great detail in the past, but a few studies have been carried out with different fish species. Soncini and Glass (2000) reported alleviation of hypercapnia induced hyperventilation when combined with hyperoxia in the common carp (*Cyprinus carpio*), supporting the view that hypercapnia induced hyperventilation is only an effect of reduced blood O₂ carrying capacity associated with increased p_aCO₂ and reduced plasma pH (Bohr effect). However, Thomas et al. (1983) reported the opposite result in rainbow trout; combined hyperoxia and hypercapnia increased ventilation compared to hyperoxia alone. Thomas (1983) studied the blood physiology of rainbow trout exposed to combined hypoxia and hypercapnia, indicating exposure-time dependent changes in p_aO₂, haematocrit and plasma pH and HCO₃⁻ as the trout adapted to the combined hypoxic and

hypercapnic conditions. Combined hypercapnia and hypoxia or hyperoxia qualify as a multiple stressor scenario, capable of inducing additive, synergistic or antagonistic effects on fish (Salbu et al., 2005). This array of conceivable outcomes makes the effects of combined exposure to hypercapnia and hypoxia or hyperoxia hard to predict.

In salmonids such as the Atlantic salmon and rainbow trout, p_aO_2 is largely dependent on p_wO_2 and is generally found in the range of 60-80 % of p_wO_2 in hypoxia, normoxia and hyperoxia (Perry and Reid, 1992; Gilmour and Perry, 1994; Kristensen et al., 2010). Other fishes such as the American eel (*Anguilla rostrata*), the common carp and the Wels catfish (*Silurus glanis*) have resting, normoxic p_aO_2 levels which are lower than that of salmonids (Forgue et al., 1989; Perry and Reid, 1992; Takeda, 1993). The Wels catfish and the American eel respond to changes in p_wO_2 similarly to salmonids in that they alter ventilation when necessary (Forgue et al., 1989; Perry and Reid, 1992). However, the Wels catfish alters its ventilation to such an extent that its p_aO_2 does not change across a wide range of p_wO_2 levels; from quite severe hypoxia (3 kPa) into extreme hyperoxia (39 kPa) its p_aO_2 is maintained at ca 2 kPa (Forgue et al., 1989). The low p_aO_2 in the Wels catfish corresponds well to that of other water breathing animals such as molluscs and crustaceans, and has been proposed to be a protective mechanism deployed by the organism in order to protect its tissues from oxidative stress (Massabuau, 2001). In the Wels catfish, the low p_aO_2 is combined with a low 50 % O_2 saturation level of haemoglobin (P_{50}) of ~0.6 kPa pO_2 (Albers et al., 1981; Forgue et al., 1989), securing O_2 delivery to tissues despite a very low p_aO_2 . This strategy is likely very beneficial to fishes which inhabit environments with very fluctuating, and often hypoxic, p_wO_2 levels. Species which are largely unable to move to a more favourable environment in the wild, such as molluscs, will also have advantages of utilizing this strategy. The Atlantic cod p_aO_2 has been reported to respond similarly to hypoxia and normoxia as in the salmonids (Kinkead et al., 1991). However, the response of Atlantic cod p_aO_2 to hyperoxia has not been previously described and no accounts of Arctic charr p_aO_2 levels have been reported.

Objectives

In aquaculture, Arctic charr and Atlantic cod are commonly exposed to hyperoxia, hypoxia or hypercapnia, and even combined hypercapnia and mild hypoxia or hyperoxia. The possibility of increased oxidative stress in hyperoxia (Olsvik et al., 2005; Olsvik et al., 2006; Stefansson et al., 2007; Finne et al., 2008) and unknown effects of combined hypercapnia and hypoxia or hyperoxia on the physiology of these species present a knowledge gap with regards to how these species should be treated in aquaculture. Thus, the main objectives of this thesis were to investigate how the p_aO_2 levels in Atlantic cod and Arctic charr respond to varying and aquaculture-relevant levels of p_wO_2 , and to identify if this response causes increased oxidative stress in the Atlantic cod. A third objective was to identify how the Atlantic cod ventilation and blood physiology responded to combined levels of hypercapnia and hyperoxia or hypoxia.

Methods

Experimental design

The experiment in Paper I was carried out at the aquaculture research facility of Hólar University College in Sauðárkrókur, Iceland, using their stock of Arctic charr. The charr were kept unrestrained in individual, 60 cm diameter, tanks with a continuous water flow of ca 1 L/min during the experiment. The charr were cannulated in the dorsal aorta (DA) and exposed to mild hypoxia, mild hyperoxia and two normoxic control treatments: day 1, normoxia; day 2, hyperoxia (~120 %); day 3, normoxia; day 4, hypoxia (~80 %). The experimental O₂ treatments were created in an open aired header tank and all the charr received the same treatment at the same time. Blood was sampled at the end of each 24-hour treatment and analyzed for pO₂ and other blood parameters in order to evaluate whether Arctic charr have a p_aO₂ that is dependent on p_wO₂.

All the experiments in Papers II and III were carried out at the Marine Research Station of the Norwegian Institute for Water Research (NIVA-MFS) at Solbergstrand, Norway. The cod used in these experiments originated from a commercial cod farm (Profunda AS) which produces cod juveniles for on-growing in other facilities. The cod used in the experiments were left over fish (ca 700 g) from their brood stock production. The cod were kept in a 6 m diameter holding tank at NIVA-MFS from delivery until experimentation (5-16 weeks). The experimental tank setup was identical to the one described below, except that the lamp pictured was situated directly above the tank, as opposed to in one corner on the picture, and the water level was slightly raised to be closer to the shelter (Figure I).

In Paper II, Atlantic cod were either pre-anaesthesia sedated with metomidate or not sedated, and then cannulated in the caudal artery (CA). Recovery was followed up to and including day 14 post surgery by sampling of blood from the CA cannula. The blood was analysed for an array of parameters including acid-base and stress parameters in order to evaluate recovery time and best sedation practice for CA cannulation of Atlantic cod.

In Paper III, Atlantic cod were cannulated in the CA and exposed to four hyperoxic O₂ treatments and normoxic controls over the course of 8 days: day 1, normoxia; days 2-3, hyperoxia (~120 %); days 4-5, normoxia; day 6, hyperoxia (~160 %); day 7, hyperoxia (190 %); day 8, hyperoxia (~200 %). Blood was sampled from the CA cannula at the end of each treatment and analyzed for pO₂ and other blood parameters to determine if the p_aO₂ of Atlantic cod was dependent on p_wO₂ in hyperoxia. Blood was also analyzed for glutathione to determine oxidative stress level in cod in each treatment. Liver and head kidney were sampled at the end of the experiment and compared to a control to identify possible effects of intermittent hyperoxia on the oxidative stress level in these tissues. As in Paper I, the O₂ treatments were created using an open aired header tank supplying all the experimental tanks simultaneously. Therefore, all cod except the control always received the same water treatment.

The experiment described in Paper IV was conducted at the Tromsø Aquaculture Research Station at Ringvassøy, Norway, in cooperation with Nofima Marin. Atlantic cod were cannulated in the CA and later fitted with a tag for measurement of ventilation parameters before being placed in a swim-tunnel respirometer. The cod were exposed to all possible combinations of three CO₂ levels (low, medium, high) and three O₂ levels (hypoxia, normoxia, hyperoxia), yielding a total of nine treatments including the control (low CO₂, normoxia). Blood was sampled from the CA cannula at the end of each treatment and analyzed for pO₂ and pCO₂, acid-base parameters, glucose and ions. Ventilation was also recorded to investigate the ventilatory responses of Atlantic cod to separate and combined hypercapnia and hypoxia or hyperoxia and put these results in context with the blood physiology.

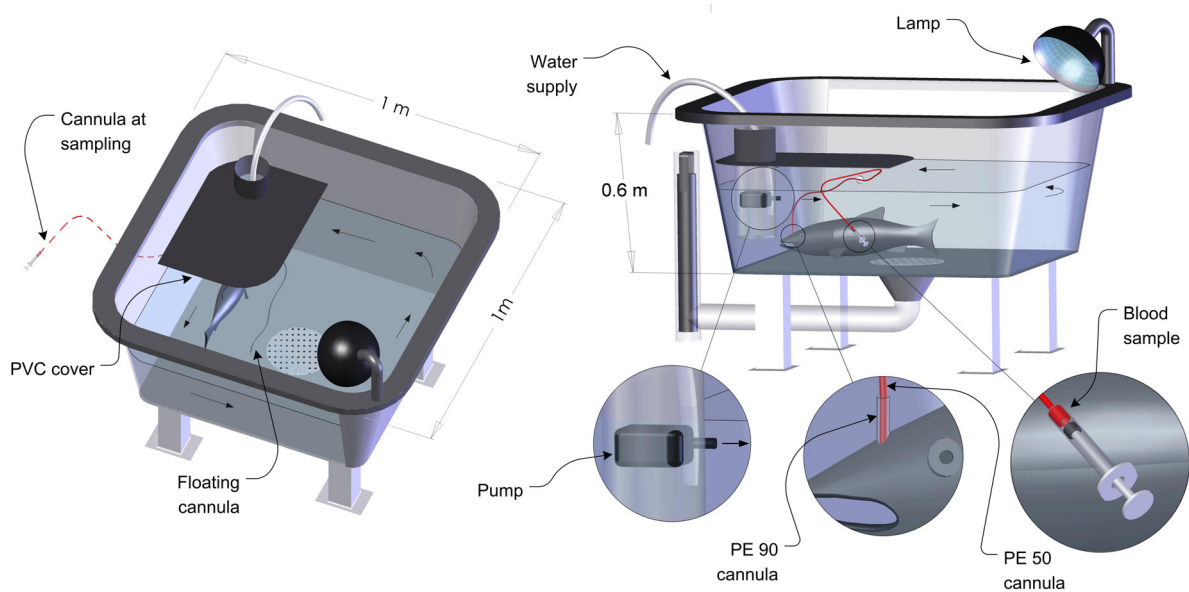


Figure I. Tanks for experiments with individual, cannulated fish. After Djordjevic et al. (2010).

Cannulation procedures and experimental conditions

The DA cannulation is one of the longest standing and most frequently used cannulation methods available; it has been used since the early 1960's (Conte et al., 1963; Smith and Bell, 1964). To this day the surgical technique is largely the same as it was used throughout the 1960's (Holeton and Randall, 1967a; Holeton and Randall, 1967b; Randall et al., 1967; Stevens and Randall, 1967a; Stevens and Randall, 1967b) and 1970's (Soivio et al., 1975). However, some alterations to the surgery procedure itself and, likely more important, to the anaesthetic procedures used during surgery have been made by Kiessling and co-workers from the 1990's. The use of more advanced anaesthetic techniques incorporating not only anaesthesia, but also pre-anaesthesia sedation and analgesia have most likely improved welfare during and after surgery, and reduced recovery time (Kiessling et al., 1995; Kiessling et al., 2003; Sunde et al., 2003). Recent studies have also incorporated the use of tanks purposely designed for experiments with individual, cannulated fish; the goal being to create an environment practically void of environmental stressors while at the same time allowing the fish to behave and move more freely than in traditional "black box" type setups (Djordjevic et al., 2010; Kristensen et al., 2010). This has been done using vibration reducing materials in tank stands and floor material, sheltering the experimental room from external disturbances and, likely most important, the personnel doing the blood sampling are trained to be as quiet and unobtrusive as possible when sampling. At the same time the fish are provided with a shelter to "hide" under and a current to swim against. This puts the fish in a position where the sampler has unhindered access to the cannula in order to obtain a blood sample, while the fish is undisturbed by the sampler (Figure I). This tank setup was used for all experiments in Papers II and III.

While the DA cannulation technique is an easily performed and commonly used procedure, it cannot be used on all species of teleosts, mainly due to anatomical differences between the species. The DA is formed by the merging of the left and right gill efferent arteries of the first and second gill arches (Figure II). In salmonids, this arterial junction is located between the second and third gill arch (Fig. II), while other teleosts have the junction in less accessible positions. In the case of the Atlantic cod, the arterial junction that creates the DA is situated so far back in the roof of the mouth that a cannula would have to be positioned in, or very close to, the pharyngeal sphincter (visual inspection by dissection). A cannula placed in this position would undoubtedly cause a disturbance and consequent stress, pain or suffering for the cod, yielding an animal unfit for experiments both from an experimental and ethical point of view. Additionally, the cannulation procedure itself would be difficult to perform due to the narrow working space of the inner buccal cavity. Previous studies in respiratory physiology of Atlantic cod used occlusive cannulation of the afferent and efferent vessels of one gill arch to access the DA and ventral aorta (VA); effectively removing one gill arch (Axelsson and Nilsson, 1986; Axelsson and Fritsche, 1991). The occlusive nature of this procedure has possible implications for fish welfare, especially in long term experiments as the risks of infection and tissue necrosis would dramatically increase with time. Also, due to the removal of one of eight gill arches, respiratory physiology may be affected by the procedure. The DA and the occlusive DA/VA cannulation procedures were determined to be

too invasive to use in the experiments presented in this thesis and the caudal artery (CA) was investigated as a possible vessel to cannulate.

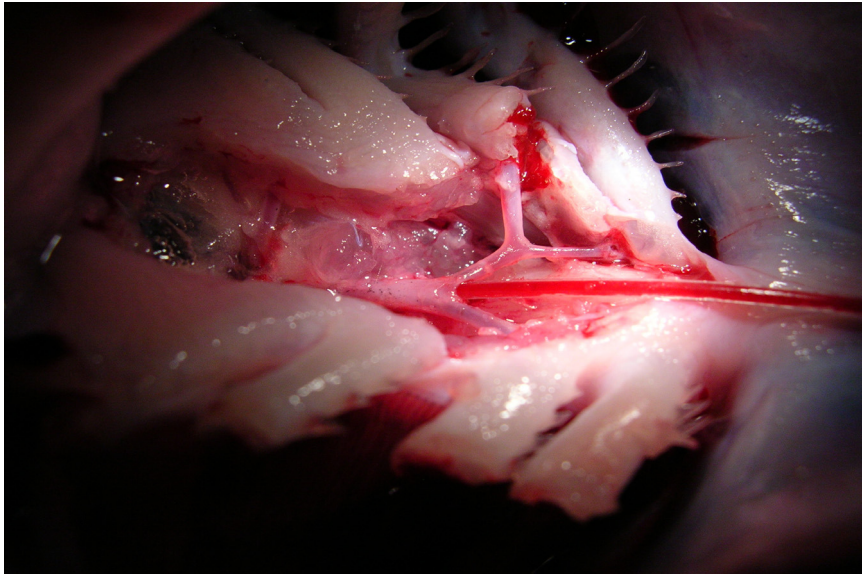


Figure II: Exposed dorsal aorta of Atlantic salmon (*Salmo salar*) with cannula (photo: A. Karlsson).

The CA is the same blood vessel as the DA; the vessel is the main artery in teleosts and extends from directly behind the gills, along the underside of the spine, and all the way to the base of the caudal fin (Thorarensen et al., 1991). A previous study reporting the use of CA cannulation used open surgery, i.e. a large incision was made through skin and muscle tissue to reach the spine before a cannula was inserted into the CA (Forgue et al., 1989). For the experiments in this thesis, a different and less invasive approach was chosen; a “closed” type surgery which was methodically adapted from the DA cannulation described by Djordjevic et al. (2010). The cannulation procedure is described in detail in Paper II. The CA is situated inside a cavity created by the bones protruding from the vertebrae. This space is shared with another blood vessel; the caudal vein (CV). Thus, the possibility of cannulating the CV is always present and all CA cannulated fish must be dissected post-experimentation to determine if the cannulation was successful and consequently if the blood samples acquired are valid. This procedure was performed in all experiments where CA cannulation was used.

Statistical methods

Multiple sampling of blood from the same individuals over time creates problematic effects in statistics, as it is a prerequisite in most statistical analyses that all samples are independent. This is clearly not the case in repeated measurements from the same individuals. However, the sampling of individuals over time also allows for the use of much fewer animals than traditional group-based experiments, with consequent benefits to experimental animal welfare in way of reducing the number of experimental animals. Additionally, the use of repeated sampling of individuals can refine experiments by reducing inter individual variation to a large degree, since the same individuals are used in all samplings. Reduction of the number of animals used and refinement of experimental setups are two of the three R's (reduction, refinement and replacement) in the three R's principle of experimental animal welfare (Russell and Burch, 1959). None the less, the potential problems with repeated measurements must be dealt with in the statistical analysis.

In the papers presented in this thesis, the mixed model procedure for repeated measurements in Statistical Analysis Software (SAS) v. 9.1-9.13 was used where individuals were sampled repeatedly. This analytical procedure is specifically designed for repeated measurements, avoiding the problematic effects of repeated sampling by including individual as a random effect in the model and modelling a covariance structure based on the nature of the experiment and the relationship between the samples. Compound symmetry was used as covariance structure in the statistical analysis of the data in Papers I, III and IV. This covariance structure assumes equal covariance between all treatments or sampling times. A first order heterogeneous covariance structure was used in the statistical analysis of the data in Paper II. This type of covariance structure assumes different covariance between treatments or sampling times based on a time scale; the closer together two treatments are in time, the more related they are and they are more likely to be similar to each other than treatments further away in time. This type of structure is common in longitudinal studies where time is a factor, as is the case in Paper II.

Results and discussion

Summaries of individual papers

Paper I: Changes in arterial oxygen tension and blood physiological status in resting and unrestrained Arctic charr *Salvelinus alpinus* exposed to mild hypoxia and hyperoxia

Arctic charr were cannulated in the dorsal aorta, allowed to recover and exposed to mild hypoxic and hyperoxic conditions. Effects of hypoxia and hyperoxia were assessed by comparison of oxygen and carbon dioxide tensions, plasma cortisol, glucose, electrolytes, acid-base parameters and haematocrit in arterial blood. Arterial oxygen and carbon dioxide tensions and bicarbonate increased with increasing water oxygen tension and the difference in oxygen tension between water and arterial blood did not change with increasing water oxygen tension. In hypoxia, plasma cortisol decreased and glucose increased compared to normoxia, while no change occurred in blood haematocrit or plasma pH, sodium or potassium by water oxygen level. The dependency of Arctic charr arterial oxygen tension on water oxygen tension likely make the charr vulnerable to oxidative stress in hyperoxia. Thus, hyperoxic conditions should be avoided in culture of Arctic charr.

Paper II: Pre-anaesthetic metomidate sedation affects recovery from caudal artery cannulation in Atlantic cod (*Gadus morhua*)

Atlantic cod were either metomidate sedated or not sedated prior to anaesthesia with metacain, and subsequently subjected to a non invasive caudal artery cannulation method never before described for Atlantic cod. Post surgery (PS) recovery was assessed by comparison of plasma cortisol, glucose, electrolytes and acid-base parameters with values in non cannulated cod obtained by sacrificial sampling (reference level). Metomidate sedation caused a delayed stress response in cod, causing plasma cortisol to return to reference levels quicker in unsedated cod (day 2 PS) than sedated cod (day 4 PS) while plasma glucose was elevated in both sedated and unsedated cod up to and including 5 days PS. Plasma potassium (K^+) was elevated and plasma pH was lowered until 24 hours PS, where after both variables were stable and significantly different from reference levels, indicating a stress effect of sacrificial sampling on plasma K^+ and pH. We conclude that caudal artery cannulation can be a useful tool in obtaining repeated blood samples from Atlantic cod. The necessary recovery time from caudal artery cannulation was determined to be six days PS irrespective of pre-anaesthesia sedation status for total recovery. However, depending on the variable studied, shorter recovery times may be used. Omitting metomidate sedation prior to caudal artery cannulation of Atlantic cod should be considered if a recovery time of less than three days is necessary.

Paper III: Changes in arterial PO_2 , physiological blood parameters and intracellular antioxidants in free-swimming Atlantic cod (*Gadus morhua*) exposed to varying levels of hyperoxia

Free-swimming Atlantic cod (*Gadus morhua*) were exposed to water oxygen pressures (P_wO_2) ranging from 18.1 to 41.5 kPa and sampled for blood using an indwelling caudal artery cannula. Arterial blood oxygen pressure (P_aO_2) increased with increasing P_wO_2 ; from 12.0 kPa in normoxia (18.1 kPa) to 34.2 kPa in the highest hyperoxic level tested (41.5 kPa). Blood CO_2 pressure and plasma bicarbonate concentration increased with P_wO_2 , indicating reduced ventilation with increased P_wO_2 . Plasma glucose, sodium and potassium were not affected by water oxygen level.

Blood oxidative stress biomarkers, reduced glutathione, oxidized glutathione and the oxidative stress index (ratio between oxidized and total glutathione), differed intermittently between normoxia and hyperoxia. The oxidative stress index was higher in blood of exposed compared to unexposed control cod. Together with elevated P_aO_2 , these findings suggest increased production of reactive oxygen species and increased oxidative stress in Atlantic cod exposed to hyperoxia.

Paper IV: Hypercapnia overrides moderate variations in water oxygen tension in controlling ventilation and blood physiology in Atlantic cod (*Gadus morhua*)

Atlantic cod (*Gadus morhua*) were fitted with caudal artery cannulas and ventilation registration tags and exposed to separate and combined hypercapnia (0.43 and 0.70 kPa) and hypoxia (15.6 kPa) or hyperoxia (24.7 kPa). Ventilation rate, ventilatory stroke volume, ventilatory minute volume, oxygen consumption, blood gases and relevant blood physiological parameters were measured in all treatments. When exposed separately, ventilation increased in hypercapnia and hypoxia, and decreased in hyperoxia. However, no effects were observed on ventilation with changes in O_2 level under hypercapnic conditions. Oxygen consumption was maintained in hypoxia, and increased in hyperoxia, compared to the control. Oxygen consumption was not affected by hypercapnia.

Arterial blood oxygen tension increased with water oxygen tension, and the difference between water and arterial oxygen tensions was larger in the control treatment than in separate or combined hypercapnia and hypoxia. Arterial CO_2 tension increased, and plasma pH and bicarbonate decreased, in hypercapnia. Plasma glucose was higher in the control treatment than in separate or combined hypercapnia and hypoxia, but not in hyperoxia. Plasma sodium was higher in hyperoxia than in hypoxia and plasma potassium was higher in the 0.70 kPa hypercapnic level than in the control treatment.

We conclude that, at moderate levels, water CO_2 tension is a more potent ventilatory stimulant than water O_2 tension and that the Atlantic cod pO_2 difference always decreased from the control treatment into separate or combined hypercapnia and hypoxia.

Recovery from surgical procedures and stress parameters

The recovery time from CA cannulation was longer in Atlantic cod (Paper II) than what was expected based on previous reports using Atlantic salmon (Eliason et al., 2007; Djordjevic et al., 2010). In particular, plasma glucose responded the most differently from what has been previously reported. It was elevated from the reference level up to and including 5 days post surgery (Paper II) and did not reach a stable level in the entire two week experimental period. In Atlantic salmon there was no response in plasma glucose after DA cannulation (Djordjevic et al., 2010) and the fish were fully recovered within three days after hepatic portal vein cannulation (HPV) (Eliason et al., 2007). Food deprived Atlantic cod (Olsen et al., 2008) have been reported to have longer plasma glucose recovery times than unfed Atlantic salmon (Olsen et al., 2002) subjected to similar stressors. However, the difference in plasma glucose recovery time reported by Olsen et al. (2008) was not in the order of several days as observed in the present study.

The experiment in Paper III was carried out in between the experiments reported in Paper II. At that time the results from Paper II were not complete and the prolonged recovery time of Atlantic cod was not evident. Thus, the fish were given a recovery time of 3-4 days prior to experimentation, later described in Paper II as insufficient for a full recovery of plasma glucose. This recovery time was based upon previous studies using Atlantic salmon, where a recovery time of three days was sufficient for all blood parameters to recover fully from both DA and HPV cannulations, the latter being a much more invasive procedure than the CA cannulation (Eliason et al., 2007; Djordjevic et al., 2010). The continuously decreasing plasma glucose levels observed in Paper III can therefore be attributed to the insufficient recovery time applied prior to experiment, rather than being an effect of the experimental oxygen treatments. A similar response was also observed to some extent in Paper I. The experiment described in Paper I used Arctic charr which were given a recovery time after DA cannulation of 48 hours, which has proved sufficient for Atlantic salmon (Djordjevic et al., 2010). In spite of that, the plasma cortisol levels were generally higher than what is common in resting salmonids and it decreased during the four day experiment with the lowest level observed in the last O₂ treatment (hypoxia). None the less, these issues were considered to not interfere with the other findings from the experiments in Papers I and III, and likely did not affect them to any large extent.

Metomidate sedation has, to the author's knowledge, not previously been reported to delay stress responses in fish. However, the results presented in Paper II point in this direction and may explain the high resting plasma cortisol levels observed in Paper IV. An average recovery time of six days from cannulation until experimentation was used in Paper IV, which should be sufficient for both plasma glucose and cortisol to recover (Paper II). However, the day prior to experimentation the cod were fitted with a tag for registration of ventilation parameters; essentially undergoing sedation, anaesthesia and a second surgery. The ventilation tag installation and transfer to the swim tunnel respirometer have previously not induced detectable effects in cod after an overnight recovery (B. Damsgård, personal communication). However, in their previous tests, Damsgård and co-workers did not use sedation with metomidate prior to anaesthesia in the tag installation process, but it was used in

the experiment in Paper IV. As the results from Paper II were not available when conducting the experiment, the delayed stress effect associated with metomidate sedation was unknown. Therefore, the increased cortisol levels reported in Paper IV is likely an effect of metomidate sedation in the tag installation procedure, which caused a delayed stress response in Atlantic cod. The observed effect of metomidate sedation on plasma cortisol in Paper IV was not evident for plasma glucose. The glucose levels observed in Paper IV seem to be fully recovered compared to the reference and endpoint plasma glucose levels in Paper II. Additionally, the Atlantic cod plasma glucose levels in Paper IV behaved differently from what was expected; they were significantly higher in the control treatment than in all other treatments except hyperoxia. This result is puzzling since plasma glucose would be expected to increase in the event of a stress response or disturbance, e.g. caused by the experimental hypercapnic, hypoxic or combined hypoxic/hypercapnic treatments (Paper IV). The observed changes in plasma glucose within the time frame of the experiment is an interesting result in itself, especially so since Atlantic cod have been reported to have a slower plasma glucose recovery time following a stressor than Atlantic salmon (Olsen et al., 2008). The finding that Atlantic cod plasma glucose has the ability to respond quickly suggests that the slow plasma glucose removal observed by Olsen *et al.* (2008) and in Papers II and III may not be a general inability to reduce plasma glucose at a quick rate, but rather a prolonged or residual stress effect compared to Atlantic salmon.

The discrepancy between the levels of plasma potassium (K^+) and pH in sacrificially sampled and CA cannulated cod reported in Paper II reveals one of the major advantages of using cannulated fish compared to sacrificial sampling. By using cannulated fish, one can eliminate the variation related to stress responses caused by the netting procedure, i.e. the fish is not cornered or chased around in the tank, netted, lifted out of water, euthanized and then finally sampled for blood. All these procedures may induce serious and potent stress responses in fish, in fact, chasing (combined with lowered water level) is a commonly used procedure to induce stress responses in fish for experimental purposes (Olsen et al., 2008). In addition to a lowered variability and therefore a likelihood of better results from the experiment, the use of cannulation techniques can greatly reduce the number of fish needed in an experiment. In that way the use of cannulation techniques represent improvements in 2 of the 3 R's (reduction and refinement) defined in the 3 R's principle of the use of animals in experiments (Russell and Burch, 1959).

Respiratory blood parameters and ventilation

Atlantic cod (Papers III and IV) and Arctic charr (Paper I) p_aO_2 levels proved to be heavily dependent on p_wO_2 . When p_wO_2 increased, neither cod nor charr maintained p_aO_2 at a steady level, instead, their p_aO_2 increased with increasing p_wO_2 . This result is also reflected in that the pO_2 difference changed little, if at all significant, in response to changes in p_wO_2 across Papers I, III and IV (15.6 - 41.5 kPa). These findings are in line with what has previously been reported for Atlantic cod exposed to hypoxia (Perry et al., 1991), Atlantic salmon in hyperoxia (Kristensen et al., 2010) and rainbow trout in hypoxia and hyperoxia (Gilmour and Perry, 1994). To what extent the p_aO_2 of a fish is dependent on p_wO_2 is likely to be an effect of the affinity of haemoglobin for O_2 of the species, i.e. at what p_aO_2 level is haemoglobin near fully saturated (Perry and Reid, 1992). Since the major part of the blood O_2 content is bound to haemoglobin in the erythrocytes, it is important that the haemoglobin saturation is sufficient to allow normal metabolic activity at p_wO_2 levels which are common for the habitat of the fish. If the fish routinely experiences hypoxic p_wO_2 levels, it will be an advantage to have fully saturated haemoglobin even in hypoxic conditions to allow normal activity in hypoxia. This theory provides an explanation of the difference between dependencies of p_aO_2 on p_wO_2 between different species of fish (Forgue et al., 1989; Perry and Reid, 1992; Kristensen et al., 2010). If a high p_aO_2 level is necessary to fully saturate haemoglobin, the fish will strive to maintain a high p_aO_2 level and vice versa. This theory may to some extent explain the low p_aO_2 reported in the Wels catfish (Forgue et al., 1989). However, it does not explain why the Wels catfish maintains this low p_aO_2 throughout a large range of p_wO_2 levels. If a high p_aO_2 level did not have any problematic effects, it would only aid the fish in delivering more O_2 to the tissues, allowing a higher activity level if necessary or simply diffusing out again if not. Therefore it is likely that fish do experience some negative effects of high p_aO_2 levels and the likely culprit is, as suggested by Massabuau (2001), oxidative stress.

The p_aCO_2 and plasma HCO_3^- increased in both Atlantic cod and Arctic charr with increasing p_wO_2 , indicating that charr (Paper I) and cod (Paper III) reduced ventilation in response to hyperoxia. Reduced ventilation and increased p_aCO_2 in response to increased p_wO_2 is a common response in fishes (Thomas et al., 1983; Gilmour and Perry, 1994; and see review by Gilmour, 2001). The assumption that hyperoxia reduced ventilation was reinforced by the results in Paper IV; ventilation rate decreased with increasing p_wO_2 in the range of ~75 to ~120 % water O_2 saturation (15.6-24.7 kPa). It is likely that the effect of p_wO_2 levels on ventilation rate observed in Paper IV also extended to the wider range of p_wO_2 levels used in Paper III, especially when considered in light of the secondary evidence of decreased ventilation observed in Paper III. The p_aCO_2 and plasma HCO_3^- increased with increasing p_wO_2 in Papers I and III, but was not significantly affected by p_wO_2 in Paper IV. Additionally, plasma pH did not change consistently with the p_wO_2 levels used in Papers I, III and IV (15.6 - 41.5 kPa). There was no effect of p_wO_2 on plasma pH in Arctic charr (Paper I), while it increased with p_wO_2 in Atlantic cod (Paper III). In Paper IV, plasma pH was not affected by p_wO_2 alone. These differences were likely due to differences in exposure time and the range of p_wO_2 levels used in the studies. If a wider range of p_wO_2 levels had been used in Papers I and

IV, effects of p_wO_2 on p_aCO_2 , HCO_3^- and pH may have become evident. Thomas (1983) reported changes in blood acid-base status with exposure time in rainbow trout exposed to hypercapnia and hypoxia. This response may explain the differences in acid-base status reported in Papers I, III and IV. While exposure time was 14 minutes in Paper IV, it was 24-48 hours in Papers I and III. This difference may have allowed the fish in Papers I and III to adjust to the environmental conditions and reach a somewhat steady state with regards to acid-base balance, as reported by Thomas (1983). Thus, exposure time may be an important factor in these types of experiments, and future studies should be designed with this in mind as the results may have different implications under different conditions. The results from Paper IV are likely more applicable in an environment where saturations of gases are rapidly changing than in situations where stable conditions are the norm, and vice versa. In an aquaculture setting, either of the studies will be applicable since conditions can be relatively stable or quite fluctuating, depending on feeding regime, temperature and fish density.

Hypercapnia had a pronounced effect on the blood acid-base parameters p_aCO_2 , HCO_3^- and pH in Atlantic cod (Paper IV). This complies well with previous studies in rainbow trout (Thomas, 1983; Gilmour and Perry, 1994) and common carp (Soncini and Glass, 2000). Hypercapnia also increased ventilation; the hyperventilation was not relieved by combined exposure to hypercapnia and hyperoxia. This indicates an effect of hypercapnia alone on the ventilation of Atlantic cod and suggests that, at aquaculture relevant levels, CO_2 is a more potent ventilatory stimulant than O_2 . Combined hyperoxia and hypercapnia tended to increase the p_aO_2 from hyperoxia alone, albeit not significantly (Paper IV). However, the increase from hyperoxia (20.6 ± 0.5 kPa) into combined hyperoxia and hypercapnia (22.2 ± 0.5 kPa) was significant when the adjustment for multiple comparisons was excluded from the analysis ($p=0.0096$). This indicates that combined hyperoxia and hypercapnia likely pose a greater risk than hyperoxia alone in inducing increased oxidative stress in Atlantic cod, with possible negative consequences to fish health and welfare.

Concluding remarks

The high p_aO_2 levels observed in Atlantic cod and Arctic charr in hyperoxia induced oxidative stress defence mechanisms in Atlantic cod. These findings indicate that hyperoxia is an inducer of oxidative stress in fish and that hyperoxia should be avoided in aquaculture. However, this goal may not be possible to accomplish in real life aquaculture, mainly due to the extremely fine line fish farmers have to balance when calculating water supply and oxygen requirements of cod. If the oxygen saturation is kept at less than full saturation, the lost production volume represent an economical burden, and when oxygen saturation is kept at more than full saturation, fish farmers face the risk of imposing health problems and welfare issues on the fish. Deformities and high mortality have previously been suggested to be an effect of hyperoxia in aquaculture (Stefansson et al., 2007) and the findings in this thesis support those suggestions. The combined effects of hyperventilation in hypercapnia and increased p_aO_2 in hyperoxia will likely increase the negative effects on fish welfare and production. In spite of being fairly commonplace in aquaculture, these conditions should be avoided to insure good health and welfare of farmed Atlantic cod and Arctic charr.

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Paper I

1 Changes in arterial oxygen tension and blood physiological status in resting and
2 unrestrained Arctic charr *Salvelinus alpinus* exposed to mild hypoxia and
3 hyperoxia

4
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13
14 Concise running headline: Arctic charr p_aO_2 is dependent on p_wO_2

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16 Abstract

17 Arctic charr *Salvelinus alpinus* arterial blood tensions of oxygen (p_aO_2) and carbon dioxide
18 (pCO_2) increased with increasing water oxygen tension (p_wO_2) while the pO_2 difference (p_wO_2
19 - p_aO_2) did not change with varying p_wO_2 .

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22 Keywords

23 Salmonid; normoxia; dorsal aorta cannulation; oxygen transport; oxygen supersaturation;
24 aquaculture

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28 Oxygenation is commonly used to reduce the water requirements in aquaculture. Increased
29 water oxygen tension (p_wO_2) typically causes increased arterial blood oxygen tension (p_aO_2)
30 in teleosts (Perry *et al.*, 1991; Perry and Reid, 1992; Takeda, 1993; Kristensen *et al.*, 2010).
31 However, not all teleosts respond in this manner; the Wels catfish *Silurus glanis* L.
32 maintained p_aO_2 at 2 kPa in water oxygen levels ranging from 3-40kPa (Forgue *et al.*, 1989).
33 This response is common amongst water breathing animals of other phyla and was later
34 suggested to be a protective mechanism against oxidative stress (Massabuau, 2001). The
35 objective of this study was to investigate the effect of mild hypoxia and hyperoxia on the
36 p_aO_2 , CO_2 tension (pCO_2) and other parameters in arterial blood of resting Arctic charr
37 *Salvelinus alpinus* L.

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40 Nine *S. alpinus*, average weight 793.6 ± 114.6 g and total length 41.0 ± 2.3 cm (\pm
41 standard deviation) were cannulated in the dorsal aorta (Djordjevic *et al.*, 2010), placed in
42 individual round tanks (diameter 60 cm) with a water flow of ~ 1 L min^{-1} and allowed to
43 recover for 48 hours before the experiment commenced. The water quality was well suited to
44 the needs of *S. alpinus*: temperature 10.6-11.0 °C, salinity 9-10, pH 7.50-7.87, conductivity
45 $1.3-1.5$ S m^{-1} , alkalinity $1.3-1.4$ mmol l^{-1} , turbidity 0.12-0.62 FNU, total nitrogen 40-140 μg l^{-1} ,
46 ammonium $<5-16$ μg l^{-1} , nitrate $<1-110$ μg l^{-1} , CO_2 0.20-0.31 mg l^{-1} , organic carbon 0.39-
47 1.1 mg l^{-1} , chloride 4.3-4.9 g l^{-1} , sodium 2.3-2.6 g l^{-1} and calcium 100-120 mg l^{-1} . The light
48 regime was L24:D0 (Light:Dark) throughout the experimental period. The fish were exposed
49 to four consecutive 24-hour oxygen regimes in the following order (approximate % saturation
50 relative to air saturation in brackets): Normoxia1 (100 %), hyperoxia (120 %), normoxia2
51 (100 %) and hypoxia (80 %). The treatments were achieved by diffusing oxygen (O_2 ,
52 hyperoxia) or nitrogen (N_2 , hypoxia) into an open header tank which supplied the

53 experimental tanks. Arterial blood and tank water was sampled at the end of each 24-hour
54 oxygen treatment. The p_aO_2 and p_wO_2 were immediately determined using a needle-type
55 oxygen micro sensor and a Microx TX3-trace transmitter (PreSens Precision Sensing GmbH,
56 www.presens.de). Haematocrit was immediately determined by centrifuging (Compur
57 Microspin, Bayer, www.bayer.com). The pCO_2 , plasma pH, ions (Na^+ , K^+ , ionized Ca (iCa
58 and HCO_3^-) and glucose were immediately analyzed using an i-STAT® (Medinor AS,
59 www.medinor.no). Blood samples were immediately centrifuged at 2000 g for 3 min and the
60 plasma collected and frozen in a $-20\text{ }^\circ\text{C}$ freezer for later analysis of cortisol using a
61 commercially available kit (Spectria® Cortisol RIA; Orion Diagnostica AS,
62 www.oriondiagnostica.no). All data were analysed using SAS (Statistical Analysis Software)
63 v. 9.1. The regression procedure (proc reg) was used for generating linear regression models
64 and the mixed procedure (proc mixed) for repeated measurements was used for all other
65 analyses. Normoxia1 and 2 were considered to be repeats of the same treatment (normoxia) in
66 further analysis. T-test with Tukey-Kramer adjustment for multiple comparisons was used
67 throughout and resulting p-values <0.05 were considered to be statistically significant.

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70 The p_aO_2 increased with increasing p_wO_2 (Fig. 1A and Table I) and pO_2 difference
71 ($p_wO_2 - p_aO_2$), which is the driving pressure for oxygen diffusion, was the same (about 6 kPa)
72 at all oxygen levels (Table I). These results are in accordance with other studies that indicate
73 that p_wO_2 affect the p_aO_2 in fish (Perry *et al.*, 1991; Perry and Reid, 1992; Takeda, 1993;
74 Kristensen *et al.*, 2010). However, Kristensen *et al.* (2010) observed that the difference
75 between p_wO_2 and p_aO_2 increased during hyperoxia in Atlantic salmon *Salmo salar* L. No
76 such increase was observed in the present study (Table I), possibly due to the mild nature of
77 the hypoxic and hyperoxic levels used. The elevated p_aO_2 level observed in *S. alpinus* in

78 hyperoxia indicates that *S. alpinus* may be exposed to increased oxidative stress (Olsvik *et al.*,
79 2005; Olsvik *et al.*, 2006; Finne *et al.*, 2008) in aquaculture with subsequent negative effects
80 on fish welfare and performance (Stefansson *et al.*, 2007). Gilmour and Perry (1994) reported
81 increased ventilation and consequently decreased arterial pCO₂ in hypoxia, while hyperoxia
82 initiated the opposite response, in rainbow trout *Oncorhynchus mykiss* Walbaum. Both pCO₂
83 and HCO₃⁻ increased with increasing p_wO₂ in the present study (Fig. 1BC and Table I),
84 suggesting increased ventilation in hypoxia and decreased ventilation in hyperoxia. Gilmour
85 and Perry (1994) also reported decreased pH in hyperoxia, likely caused by their observed
86 decrease in ventilation. No change in pH was detected in the present study (Table I), possibly
87 due to the rather small differences between the p_wO₂ levels used. The hypoxic p_wO₂ level used
88 in the present study is much higher than what is necessary to induce a stress response in *O.*
89 *mykiss* (Perry and Reid, 1992). However, plasma cortisol levels were in the high range for
90 resting, seemingly undisturbed salmonids (Table I) and lowest in hypoxia; the last treatment
91 in the experiment. The relatively high cortisol levels which decreased with time suggest that
92 the *S. alpinus* did not fully recover from the DA cannulation procedure despite a recovery
93 time of 48 hrs, which has proved to be adequate for *S. salar* (Djordjevic *et al.*, 2010). Minor
94 changes in plasma glucose and no changes in blood haematocrit or plasma Na⁺, K⁺ or iCa
95 indicate that the oxygen treatments did not initiate a stress response in *S. alpinus* (Table I).
96 We conclude that the p_aO₂ of *S. alpinus* is dependent on p_wO₂ in conditions from mild
97 hypoxia to mild hyperoxia. Thus, supersaturated levels of oxygen, commonly used in modern
98 fish farming, will cause elevated p_aO₂ and may increase oxidative stress levels in *S. alpinus*.
99 As hypoxia is also problematic in terms of the growth and welfare of salmonids, the oxygen
100 levels in salmonid aquaculture should be maintained at normoxia in order not to risk
101 compromising the performance or welfare of farmed fish.

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103

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Table I: Blood parameters in *S. alpinus* in hypoxia, normoxia and hyperoxia

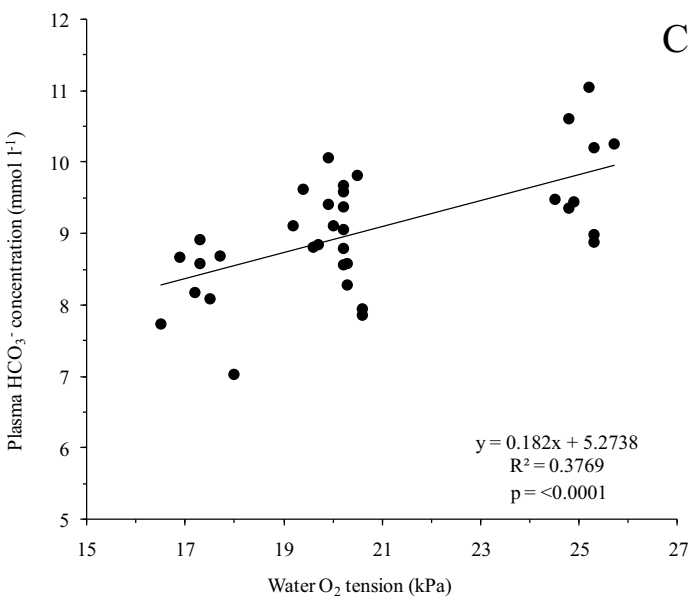
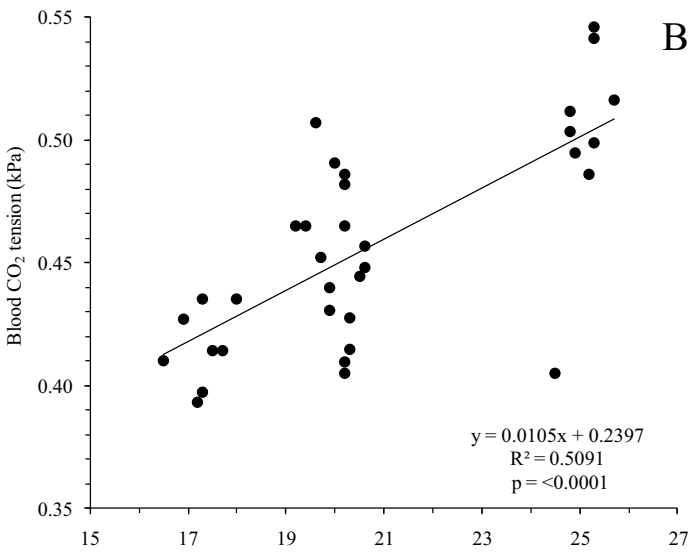
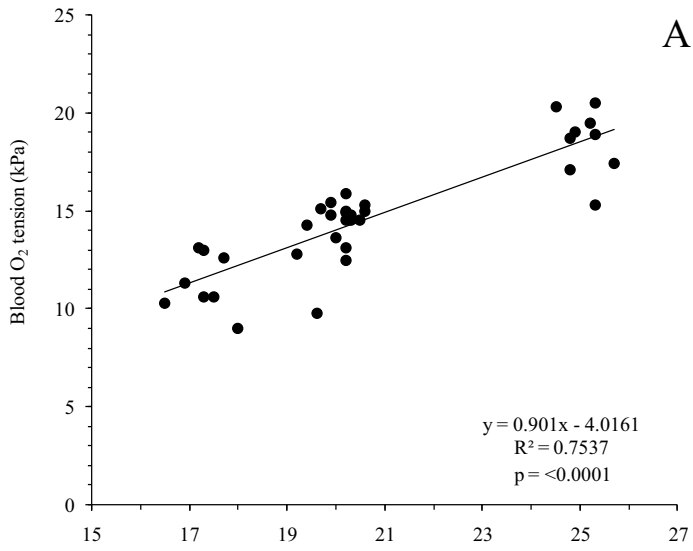
	Hypoxia		Normoxia		Hyperoxia	
p _w O ₂	17.3 ± 0.1	A	20.1 ± 0.1	B	25.1 ± 0.1	C
p _a O ₂	11.9 ± 0.7	A	14.7 ± 0.6	B	19.1 ± 0.7	C
pO ₂ difference	6.0 ± 0.5		5.9 ± 0.4		6.6 ± 0.5	
pCO ₂	0.42 ± 0.01	A	0.45 ± 0.01	B	0.50 ± 0.01	C
HCO ₃ ⁻	8.3 ± 0.2	A	9.0 ± 0.2	B	9.8 ± 0.2	C
pH	8.034 ± 0.014		8.031 ± 0.013		8.024 ± 0.014	
Cortisol	31.2 ± 9.7	A	59.8 ± 7.5	B	62.1 ± 9.3	B
Haematocrit	13.9 ± 1.7		16.1 ± 1.5		15.7 ± 1.6	
Glucose	4.13 ± 0.13	A	3.86 ± 0.11	B	3.86 ± 0.12	B
Na ⁺	152.5 ± 0.6		152.1 ± 0.5		151.8 ± 0.6	
K ⁺	2.32 ± 0.04		2.33 ± 0.03		2.33 ± 0.03	
iCa	1.51 ± 0.01		1.50 ± 0.01		1.49 ± 0.01	

Figure 1:

Arterial blood O₂ (A) and CO₂ (B) tensions and plasma HCO₃⁻ concentration (C) in Arctic charr as a function of water O₂ tension.

Table I:

Different letters indicate significant difference between treatments ($p < 0.05$). Values are LSmean \pm S.E. All parameters except p_wO_2 (measured in tank water) were measured in arterial blood of *S. alpinus*. Gas tensions are expressed as kPa. HCO₃⁻, glucose, Na⁺, K⁺ and iCa are expressed as mmol l⁻¹ plasma. Cortisol is expressed as ng ml⁻¹ plasma and haematocrit as % red blood cells (volume).



Paper II

1 Pre-anaesthetic metomidate sedation affects recovery from caudal
2 artery cannulation in Atlantic cod (*Gadus morhua*)

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17 Keywords:

18 Metomidate; metacain; stress response; fish; blood physiology; cortisol;

19

20 Abstract

21

22 Atlantic cod (*Gadus morhua*) were either metomidate sedated or not sedated prior to
23 anaesthesia with metacain, and subsequently subjected to a non invasive caudal artery
24 cannulation method never before described for Atlantic cod. Post surgery (PS) recovery was
25 assessed by comparison of plasma cortisol, glucose, electrolytes and acid-base parameters
26 with values in non cannulated cod obtained by sacrificial sampling (reference level).
27 Metomidate sedation caused a delayed stress response in cod, causing plasma cortisol to
28 return to reference levels quicker in unsedated cod (day 2 PS) than sedated cod (day 4 PS)
29 while plasma glucose was elevated in both sedated and unsedated cod up to and including 5
30 days PS. Plasma potassium (K^+) was elevated and plasma pH was lowered until 24 hours PS,
31 where after both variables were stable and significantly different from reference levels;
32 indicating a stress effect of sacrificial sampling on plasma potassium and pH.

33 We conclude that caudal artery cannulation can be a useful tool in obtaining repeated blood
34 samples from Atlantic cod. The necessary recovery time from caudal artery cannulation was
35 determined to be six days PS irrespective of pre-anaesthesia sedation status for total recovery.
36 However, depending on the variable studied, shorter recovery times may be used. Omitting
37 metomidate sedation prior to caudal artery cannulation of Atlantic cod should be considered if
38 a recovery time of less than three days is necessary.

39 Introduction

40

41 Cannulation techniques have been used in physiological experiments on fish for over 40 years
42 (Conte *et al.* 1963). The cannulation techniques have proved to be valuable research tools, and
43 several blood vessels have been targeted, e.g. the dorsal aorta (DA) (Conte *et al.* 1963; Smith
44 and Bell 1964; Soivio *et al.* 1975; Kiessling *et al.* 2003; Sunde *et al.* 2003; Djordjevic *et al.*
45 2010), the ventral aorta (Axelsson *et al.* 1994), the hepatic portal vein (McLean and Ash
46 1989; Eliason *et al.* 2009) and the caudal artery (CA) (Forgue *et al.* 1989) with duration of
47 experimental period varying from hours to several weeks. The various cannulation methods
48 allow repeated sampling of blood from the same individual, providing an alternative to
49 repeated sacrificial sampling when studying changes in blood variables. The repeated
50 sampling setup allows a reduction in the number of experimental animals by default, and
51 therefore it coincides well with the 3 R's principle of experimental animal welfare (Russell
52 and Burch 1959). It can also greatly reduce experimental variability as it allows internal
53 paired comparison as reference; test and recovery data can be measured in the same
54 individual, further decreasing the number of animals necessary in order to produce
55 statistically significant results. If proper care is taken the fish can be practically undisturbed
56 by the sampling, eliminating stress caused by the acute and brutal sacrificial sampling
57 procedure, consequently refining the experimental setup (Djordjevic *et al.* 2010). Cannulation
58 is a necessity when studying blood parameters that may be very rapidly affected by gill
59 ventilation or immediate stress responses, such as plasma pH, HCO_3^- or partial pressures of
60 respiratory gases (Djordjevic *et al.* 2010).

61 The DA cannulation has been one of the most frequently used cannulation methods, possibly
62 because of its uncomplicated and little invasive surgical procedure compared to other
63 cannulation techniques. Although the DA cannulation is suitable for many species, the
64 anatomy of some species does not allow cannulation of the DA if the fish are to be unaffected
65 by the cannula. In the case of the Atlantic cod (*Gadus morhua*), the gill-efferent blood vessels
66 merge to form the DA so far back in the roof of the mouth that the cannula would have to be
67 positioned in, or very close to, the pharyngeal sphincter (visual inspection by dissection). If
68 inserted in that position, the fish would likely be severely disturbed by the cannula. In turn, it
69 could lead to a stressed and/or suffering fish; undermining the justification for cannulation

70 and possibly producing unreliable and/or ambiguous results. Previous studies involving
71 cannulation of Atlantic cod all report occlusive cannulation of gill afferent and/or efferent
72 blood vessels; effectively removing one gill arch (Smith *et al.* 1985; Axelsson and Nilsson
73 1986; Perry *et al.* 1991). Due to the occlusive and/or invasive nature of these procedures they
74 could potentially produce ambiguous results, especially for long term studies in the manner of
75 weeks. An alternative artery in the Atlantic cod is then CA. The CA cannulation was first
76 described by Forgue *et al.* (1989) in Wels catfish (*Silurus glanis*) using open surgery, and
77 later using a non-invasive method in other teleosts (Forsman *et al.* 2005). To the authors'
78 knowledge the present study is the first time the non-invasive CA technique has been
79 described for use in gadoid fishes.

80 In-tank metomidate sedation prior to cannulation has recently been reported in studies using
81 salmonids (Djordjevic *et al.* 2010; Kristensen *et al.* 2010). The pre-surgery sedation in
82 combination with other improvements has reduced the general stress level in fish post surgery
83 (PS) to such an extent that most blood variables stabilize within 1-3 hours PS (cortisol,
84 glucose, hematokrit, pH, bicarbonate (HCO_3^-) and pCO_2) and all parameters measured were
85 stable within 24-72 hours post DA cannulation (Djordjevic *et al.* 2010). Pre-anaesthesia
86 sedated Atlantic cod was recently reported to require a lower anaesthetic concentration and
87 have a faster recovery time than unsedated congeners (Zahl *et al.* 2009).

88 The objectives of this study were to evaluate the closed CA cannulation technique in terms of
89 response magnitude and recovery time and to evaluate the use of pre-anaesthesia metomidate
90 sedation prior to CA cannulation in Atlantic cod.

91

92 Materials & Methods

93

94 *Animals and experimental procedure*

95 100 Atlantic cod, approximate weight 700 g, were purchased from a commercial cod farm
96 (Profunda AS; Bardstadvik, Norway) in early February 2009 and transported by truck to the
97 Norwegian Institute for Water Research (NIVA) – Marine Research Station at Solbergstrand
98 (NIVA-MFS), Norway. No cod died during or after transportation and the fish were of good
99 health on arrival (veterinary approval). Finally, no signs of ill health were observed later on.
100 The cod were housed in a circular 6 m diameter holding tank, fed a commercial cod diet
101 (BioMar AS, Norway) to satiation five days a week and visually inspected at least once daily.
102 The cod were given a minimum of 5 weeks to acclimate to their new surroundings before
103 being transferred to the experimental unit.

104 Cod were cannulated and recovery from cannulation followed in three trials. In all three trials
105 the cod were transferred from the holding tank to the experiment tanks (square 1 m tanks, 500
106 L water volume) and given seven days to acclimate to the new environment in groups of 4-5
107 individuals per tank. The tanks were purposely designed to hold individual, cannulated fish
108 and create a low stress environment as described in detail by Djordjevic *et al.* (2010). At least
109 24 hours prior to, and throughout acclimation and experimentation, cod were deprived of
110 food. In trial 1, (Mar-Apr 2009) all cod were pre-anaesthesia in-tank sedated with 0.5 mg/L
111 metomidate (sedated) before being transferred to a separate metacain bath prior to
112 cannulation. In trials 2 (Sep-Oct 2009) and 3 (Nov-Dec 2009), cod were either pre-anaesthesia
113 in-tank sedated with 0.5 mg/L metomidate (sedated) or not sedated prior to full anaesthesia
114 and cannulation (unsedated). In all three trials each individual was cannulated and used only
115 once, thus receiving either sedation or no sedation prior to full anaesthesia and cannulation
116 surgery.

117 To follow recovery from cannulation, 22 cod, average weight 1041 ± 288 g and length 44 ± 3
118 cm (\pm standard deviation (SD)), were sampled for blood through the CA cannula at the
119 following times: Directly PS (0h), one hour PS (1h), three hours PS (3h), 24 hours PS (24h),
120 daily after 24 hours until day 7 PS (Day 2-7) and at days 9, 11 and 14 PS.

121 To establish a comparison/reference level, 7 Atlantic cod, average weight 734 ± 75 g and
122 length 41 ± 2 cm (\pm SD), from the same population as the experimental fish were placed in
123 the tanks described above (1 cod per tank). The fish were left undisturbed for 14 days, and
124 then sacrificially sampled for blood from the caudal artery/vein using aspiration with a
125 disposable syringe and needle.

126 All cod with cannulas still attached upon finishing the experiment (20 of 22 fish, 2 fish lost
127 their cannulas during the experiment) were dissected to determine the placement of the
128 cannula. 16 cod were cannulated in the CA, 3 cod were cannulated in the caudal vein (CV)
129 and cannula placement could not be determined in 1 cod. Due to the possible differences in
130 pH, $p\text{CO}_2$ and HCO_3^- between arterial and venous blood, results from individuals cannulated
131 in the CV or with unknown cannula placement (6 cod in total) were removed from statistical
132 analysis of pH, $p\text{CO}_2$ and HCO_3^- data.

133 Throughout the experiments, full strength seawater from a depth of 60 m and with a
134 temperature of 7.2-10.6 °C and a salinity of 31.8-34.5 parts per thousand (ppt) was used. The
135 water flow to the tanks was kept at 2 L/min. The flow through system secured good water
136 quality throughout. Typical water quality in the facility: oxygen tension 18.1 kPa; pH 7.84;
137 alkalinity 2.41 mmol/L; turbidity 0.36 FNU; total nitrogen 173 $\mu\text{g/L}$; ammonium 13 $\mu\text{g/L}$;
138 dissolved carbon dioxide 0.44 mg/L.

139

140 *Surgical procedure*

141 Cannulas were made from PE50 polyethylene tubing (Intramedic®; Becton Dickinson, New
142 Jersey, USA) and trocars made from PL013 steel guitar wire. The PE50 was heated and then
143 stretched and narrowed at the penetrating end, and a bubble was made by precision heating ~5
144 cm up the cannula; so that the cannula could be attached to a suture without sliding. The
145 cannula was then flushed with heparinised physiological saline (NaCl, 9 g/L; Na-heparin, 150
146 IU/mL, injection quality) (saline) and cut at the narrowing end to fit tightly around the trocar.
147 Finally, two small holes were made on the cannula tip (one on each side) to prevent the
148 cannula from suctioning onto the vessel wall. The cannulas were stored without the trocar
149 immersed in 70 % ethanol. Immediately prior to surgery, the trocar was prepared by cutting it
150 at an angle as low as possible with a pair of wire cutters to produce a sharp point with a

151 cutting edge. Finally, the trocar was inserted into the cannula so that only the cutting edge and
152 point was protruding from the PE50 tubing, and the cannula was immersed in antiseptic fluid
153 (Chlorhexidine, 0.5 g/L; Fresenius Kabi, Uppsala, Sweden).

154 Each fish was either in-tank pre-anaesthesia sedated with metomidate (0.5 mg/L) until it
155 stopped responding to visual stimuli, or not disturbed before it was transferred from its'
156 respective tank to an anaesthetic bath containing ~30 L of aerated seawater and 0.08 g/L
157 metacain (Norwegian Medical Depot, Norway). When the fish no longer responded to touch,
158 weight and length was measured before it was placed into a purposely-designed surgical
159 cradle and surrounding bath, and covered with a wet cloth. Throughout surgery, the gills were
160 ventilated with aerated seawater maintained at equal temperature as in the tank (8-9 °C) at
161 ~15 L/min containing a maintenance dose of metacain (0.04 g/L).

162 0.3-0.7 mL of lidocaine analgesia was used at the point of incision and suture placement
163 through the skin (10 or 20 g/L, with adrenaline (5 mg/L)) using a disposable insulin syringe
164 with pre-attached needle. An #11-blade and a #3-scalpel was used to make a horizontal
165 incision (~5 mm) through the skin, vertically positioned at approximately the same distance
166 from the anal fin and lateral line, and horizontally approximately half way down the length of
167 the anal fin.

168 The “closed” cannulation was performed by inserting a pre-made cannula with trocar into the
169 incision, at approximately 45° horizontally, and pushed through muscle and membrane tissue
170 until entering the CA. The wire was then retracted, and the cannula was securely placed inside
171 the vessel by pushing it 2-3 cm further into the vessel. To prevent clotting the cannula was
172 filled with saline. The cannula was secured to the fish using a single stitch of sterile, non-
173 absorbable suture (Supramid®, 3-0 USP; AgnTho’s AB, Sweden) directly behind the
174 insertion point of the cannula. After securing the cannula to the fish, the cannula end was
175 melted shut and the surgery wound covered with Stomahesive® paste (ConvaTec Norway
176 AS, Oslo, Norway). Finally, the fish received an injection of Oxytetracycline antibiotic (100
177 g/L; Ceva Sante Animale, Libourne, France) into the abdominal cavity to prevent variation in
178 condition caused by accidental bacterial infection.

179

180 *Sample collection, preparation and analysis*

181 Prior to sampling blood from the CA cannula, previously injected saline and a few drops of
182 fresh blood were discarded to ensure a pristine sample. 0.2-0.35 mL blood samples were
183 collected by light suction using a 1 ml disposable syringe with blunted tip inserted into the
184 cannula. The amount of sample extracted was volume adjusted based on visual observation of
185 haematocrit; in order to obtain enough plasma and draw as little blood as possible. After the
186 required amount of blood was sampled, saline was injected into the cannula until blood was
187 no longer visible. To prevent clotting, another 0.1-0.2 mL of saline was injected to ensure no
188 blood was left in the tip of the cannula. The cannula ends were sealed by melting after each
189 sampling.

190 Blood samples were immediately analyzed for glucose, pCO₂, pH, HCO₃⁻ and ions (Na⁺, K⁺,
191 Cl⁻) using an i-STAT® Portable Clinical Analyzer (Medinor AS, Norway). Results for pH,
192 pCO₂ and HCO₃⁻ were temperature corrected using formulas supplied by the i-STAT®
193 manufacturer (Abbott Point of Care Inc.; Princeton, NJ, USA).

194 Blood samples were immediately centrifuged for three minutes at 2000 x g to yield plasma.
195 Plasma was immediately frozen at -20 °C and transferred to -80 °C within 3 days. Plasma
196 samples (50 µL) were mixed with five times the sample volume (250 µL) of ethyl acetate
197 using a vortex mixer. The mix was centrifuged for two minutes at 7155 x g in 4°C, and the
198 resulting supernatant stored at -80 °C until analysis. The supernatant was later analyzed for
199 cortisol using a Radioactive Immuno Assay (RIA) kit (Spectria® Cortisol RIA; Orion
200 Diagnostica AS, Asker, Norway) and a NaI-gamma counter (Wizard®; PerkinElmer Norge
201 AS, Oslo, Norway) according to instructions in the RIA kit booklet. Samples determined to be
202 below the detection limit of the RIA kit (5 ng/mL) were set to be 5 ng/mL before further
203 analysis of the data.

204

205 *Data and statistical analysis*

206 Plasma chloride was above the detection limit of the instrument (140 mmol/L) in all samples
207 and was consequently excluded from further analysis. Two samples were removed from the
208 statistical analysis for all blood variables due to extreme observations of plasma cortisol; 267
209 and 315 ng/mL while no other observations were >140 ng/mL. These samples originated from

210 the same individual at 24 and 48 hours PS respectively. All other samples from the same
211 individual were within normal range and were included in the statistical analysis.

212 The data were analysed with SAS v. 9.13 (Statistical Analysis Software), using the mixed
213 procedure for repeated measurements with a heterogeneous and autoregressive covariance
214 structure. Pre-anaesthetic sedation status, recovery time and the interaction between pre-
215 anaesthetic sedation status and recovery time were tested as class variables in the model.
216 Individual was included as a random variable (subject identification). No effect of pre-
217 anaesthetic sedation status ($p=0.6255$) or interaction between recovery time and pre-
218 anaesthetic sedation status ($p=0.2085$) was found for plasma potassium. Consequently, the
219 two pre-anaesthetic sedation statuses were considered to be the same for statistical analysis of
220 plasma potassium.

221 F-test was used for determination of statistical significance of fixed effects. T-test with
222 Tukey-Kramer adjustment for multiple comparisons was used for determination of statistical
223 differences between the different sampling times and between pre-anaesthetic sedation
224 statuses within sampling time. T-test with Dunnett adjustment for multiple comparisons was
225 used for determination of statistical difference between the reference level and the different
226 sampling times. Comparisons yielding p -values <0.05 after adjustment for multiple
227 comparisons were considered to be statistically different. Unless otherwise stated, $p<0.05$ in
228 all comparisons where differences are indicated. All values are presented as least squares
229 mean (LS mean) \pm standard error (SE) unless stated otherwise.

230

231

232 Results

233

234 *Plasma cortisol and glucose*

235 Plasma cortisol and glucose were affected by recovery time ($p < 0.0001$ for both) and
236 interaction between recovery time and pre-anaesthetic sedation status ($p = 0.0015$ for cortisol).
237 No effect was found for sedation status alone (Table 1). Both plasma cortisol and glucose
238 increased initially and later decreased with recovery time irrespective of sedation status
239 (Figure 1a and b). However, the unsedated cod displayed peak plasma cortisol levels earlier
240 (~ 90 ng/mL, 1-3 hours PS) than did the sedated cod (~ 90 ng/mL, 3-24 hours PS) (Fig. 1a).
241 Plasma glucose peaked at 1 hour PS in unsedated cod and at 24 hours PS in sedated cod (~ 9
242 mmol/L) (Fig. 1b). Plasma cortisol differed between sedated and unsedated cod at 1 hour PS
243 and 5 days PS ($p = 0.0065$) (Fig. 1a). Plasma glucose differed between sedated and unsedated
244 cod only at 24 hours PS (Fig. 1b). Plasma cortisol differed from the reference level (10.6 ± 9.3
245 ng/mL) from 1 hour PS up to and including 72 hours PS in sedated cod, while unsedated cod
246 differed from the reference level from directly PS (0 hours) up to and including 24 hours PS,
247 and at day 5 PS (Table 2a and b). Plasma glucose differed from the reference level ($2.38 \pm$
248 1.08 mmol/mL) from 1 hour PS up to and including day 5 PS in sedated cod, while unsedated
249 cod differed from the reference level from directly PS up to and including day 5 PS (Table 2a
250 and b).

251

252 *Acid-base parameters*

253 Plasma pH, $p\text{CO}_2$ and HCO_3^- were affected by recovery time ($p < 0.0001$), anaesthetic
254 treatment ($p < 0.01$) and interaction between recovery time and anaesthetic treatment
255 ($p < 0.0001$ for $p_a\text{CO}_2$, $p = 0.0008$ for HCO_3^-) (Table 1).

256 Plasma pH increased from directly PS (~ 7.6) and was stable from 24-48 hours PS onwards in
257 both sedated and unsedated cod (day 3 different from days 11 and 14 PS in sedated cod).
258 Plasma pH differed from the reference level (7.59 ± 0.05) in sedated cod from 1 hour PS
259 onwards ($p < 0.001$) and in unsedated cod from 3 hours PS onwards ($p \leq 0.001$) (Figure 2c).

260 No clear pattern could be seen for pCO₂ with recovery time. Blood pCO₂ in sedated cod
261 differed from the reference level (0.52 ± 0.05 kPa) at days 1, 2, 7 and 11 PS while unsedated
262 cod never differed from the reference level (Fig. 2a).

263 From 1 hour PS onwards plasma HCO₃⁻ was relatively stable at 6-7 mmol/L and always
264 different from the reference level (4.0 ± 0.5 mmol/L) in unsedated cod. More differences
265 between sampling times and less frequent differences from the reference level were observed
266 in sedated cod (Fig. 2b).

267 Blood pCO₂ and plasma HCO₃⁻ differed between sedated and unsedated cod at days 1, 2, 7, 11
268 and 14 PS, while plasma pH differed between sedated and unsedated cod directly PS, 3 hours
269 PS and at days 1, 2 and 11 PS (Fig. 2a, b and c).

270

271 *Blood ions*

272 Plasma sodium was affected by recovery time (p=0.0002), sedation status and interaction
273 between recovery time and sedation status (Table 1). Plasma sodium levels were elevated the
274 first 24 hours PS, but from 48 hours PS onwards plasma sodium was stable at levels not
275 different from the reference level (150 ± 1 mmol/L) in both sedated (~151 mmol/L) and
276 unsedated cod (~154 mmol/L). Plasma sodium levels differed between sedated and unsedated
277 cod at days 3-7 PS (Figure 3a).

278 Plasma potassium was only affected by recovery time (p<0.0001) (Table 1). From 24 hours
279 PS onwards plasma potassium was significantly different from the reference level (3.40 ±
280 0.13 mmol/L, p<0.0001) and from 48 hours PS onwards plasma potassium was stable at ~2.4
281 mmol/L with no more significant differences between sampling times (Fig. 3b and Table 2).

282

283

284 Discussion

285

286 In both sedated and unsedated cod there was an initial increase and a following decrease in
287 plasma cortisol after CA cannulation (Fig. 1a). However, the sedated cod displayed a delayed
288 stress response compared to the unsedated cod in that their cortisol levels increased slower
289 and returned to reference levels slower than did the unsedated cod. Recent studies on recovery
290 from DA and HPV cannulation surgeries in Atlantic salmon reported that plasma cortisol
291 levels stabilized and returned to reference or lower levels within 24 hours PS when using
292 metomidate sedation prior to surgery (Eliason *et al.* 2007; Djordjevic *et al.* 2010). In the
293 present study however, plasma cortisol in unsedated cod was no longer different from the
294 reference level 48 hours PS, while sedated cod needed 96 hours PS. This indicates that the
295 Atlantic cod stress response differ from that of Atlantic salmon, especially so when cod are
296 sedated with metomidate. Plasma cortisol levels in sedated cod seemed to stabilize at a low
297 level quicker (stable at ~20 ng/mL from day 5 onwards) than did the sedated cod (~20 ng/mL
298 at day 14) (not significant). This suggests that in experiments where a recovery time of >72
299 hours is possible, metomidate sedation should be used, and the opposite for experiments
300 requiring a shorter recovery.

301 Plasma glucose exhibited many of the same characteristics as did plasma cortisol (Fig. 1a and
302 b). However, plasma glucose was elevated up to and including day 5 PS compared to the
303 reference level irrespective of sedation status. Also, plasma glucose seemed to keep
304 decreasing during the entire experimental period irrespective of sedation status. Although not
305 continuously significant, this decrease indicates that the fourteen days of recovery described
306 in the present study may not have been enough for Atlantic cod plasma glucose to fully
307 recover from CA cannulation. Again, these findings differ from that of recent studies
308 investigating recovery from cannulation surgeries (DA and HPV) using metomidate sedation
309 in Atlantic salmon, where plasma glucose was either not affected by recovery time and not
310 different from reference levels at any time PS (Djordjevic *et al.* 2010), or stabilized within
311 day 3 PS (Eliason *et al.* 2007). With regards to plasma glucose, the results in the present study
312 indicate that the use of metomidate sedation on Atlantic cod prior to CA cannulation is not
313 necessary, regardless of possible recovery time. If measurements of plasma glucose are to be
314 used in an experiment with CA cannulated Atlantic cod and considered representative of

315 experimental treatments and not affected by time PS, recovery from surgery should be at least
316 6-7 days PS, preferably two weeks or more, irrespective of sedation status.

317 After the first 1-3 hours PS both groups displayed stable pH levels, but unsedated cod
318 consistently displayed lower pH levels than did the sedated cod (Fig. 2a). In both sedated and
319 unsedated cod all sampling times from 3 hours PS onwards differed from sacrificially
320 sampled reference level cod. This indicates a very quickly induced and strong effect of
321 handling stress during sacrificial blood sampling on plasma pH, and is concurrent with a
322 recent study reporting a large deviation between resting, post-recovery pH levels in
323 cannulated and sacrificially sampled Atlantic salmon (Djordjevic *et al.* 2010). The plasma
324 acidosis observed shortly PS and in sacrificially sampled cod is likely due to erythrocyte
325 alkalization, and consequent plasma acidification, possibly caused by hypoxia induced
326 stimulation of erythrocyte Na^+/H^+ -exchangers (Fievet *et al.* 1988). However, there was no
327 concomitant decrease in plasma sodium levels; the opposite was observed as plasma sodium
328 levels were elevated the first 24 hours PS. This rise in plasma sodium was likely caused by a
329 reduced ability to maintain osmotic balance during and directly after surgery, causing plasma
330 sodium to increase despite Na^+ uptake by erythrocytes. The recovery time of cod plasma pH
331 from CA cannulation surgery observed in the present study (24-48 hours PS) is longer than
332 DA and HPV cannulated Atlantic salmon (1 hour PS) (Eliason *et al.* 2007; Djordjevic *et al.*
333 2010). The pH in arterial blood of Atlantic cod differed between sedated and unsedated cod at
334 0, 3 and 24 hours and days 2 and 11 PS (Fig. 2c). Although the difference between sedated
335 and unsedated cod is not continuously significant, the difference persisted throughout the
336 experiment and may indicate a lasting effect of metomidate sedation on the acid-base
337 regulation of Atlantic cod.

338 No clear trend could be seen for blood pCO_2 in either sedated or unsedated cod PS. However,
339 as for pH, intermittent differences were observed between sedated and unsedated cod (Fig.
340 2a). Almost no changes were found in unsedated cod between sampling times while sedated
341 cod displayed comparatively large variations in blood pCO_2 PS. Previous studies using
342 Atlantic salmon report elevated blood pCO_2 directly PS and stable blood pCO_2 levels from 1
343 hour PS onwards (Eliason *et al.* 2007; Djordjevic *et al.* 2010). No consistent differences were
344 observed between the reference level and cannulated cod, indicating that the effect of

345 sacrificial sampling on blood pCO₂ seen by Djordjevic *et al.* (2010) was not evident in the
346 present study.

347 In both sedated and unsedated cod plasma bicarbonate was lowered directly PS (Fig. 2b).
348 From 1 hour PS onwards, plasma bicarbonate was mostly stable and different from the
349 reference level throughout in unsedated cod. However, in the sedated cod, there was more
350 variation between sampling times PS, and less frequent and inconsistent differences from the
351 reference level. Together with similar results for pCO₂, this suggests that sedated cod
352 experienced more difficulty in maintaining their acid-base balance PS compared to unsedated
353 cod (Fig. 2a and b). Eliason *et al.* (2007), also using metomidate sedated Atlantic salmon,
354 reported infrequent and inconsistent differences in (venous) plasma bicarbonate between
355 sampling times PS.

356 Plasma sodium was elevated in both sedated and unsedated cod directly PS until 24 hours PS,
357 where after no changes were evident in either of the treatments and neither sedated nor
358 unsedated cod differed from reference levels (Fig. 3a and Table 2). The increased sodium
359 levels the first 24 hours PS occurred irrespective of sedation status and was likely caused by
360 Na⁺ influx from the water across the gills. For comparison, the average water Na⁺
361 concentration was 581 mmol/L in the experiment, or roughly 4 times that of cod arterial
362 blood. Although stable within sedation status, sodium levels differed between sedated and
363 unsedated cod at days 3-7 PS. The difference between sedated and unsedated cod was small
364 (~3 mmol/L or ~2 % difference) and likely had little practical significance. The necessary
365 plasma sodium recovery time observed in the present study (48 hours) fits well with previous
366 studies investigating recovery from cannulation in Atlantic salmon, reporting 24 to 96 hours
367 PS (Eliason *et al.* 2007; Djordjevic *et al.* 2010). Plasma potassium was elevated up to and
368 including 24 hours PS, where after it was stable and different from the reference level
369 throughout the experiment (Fig. 3b). This suggests that standard blood sampling procedures,
370 involving chasing, netting and killing fish prior to sampling, yields unrepresentative samples
371 for plasma potassium in Atlantic cod, with a near two-fold increase in plasma potassium
372 concentration. Eliason *et al.* (2007) and Djordjevic *et al.* (2010) reported very similar resting
373 and PS plasma potassium levels as in the present study for cannulated Atlantic salmon. A
374 recovery time of 48 hours after CA cannulation is necessary and sufficient for plasma sodium
375 and potassium to stabilize, irrespective of sedation status.

376 Plasma pH, bicarbonate, $p_a\text{CO}_2$ and sodium differ more or less consistently between sedated
377 and unsedated cod (Figs. 2 and 3a). The differences appear 24-72 hours PS and seem to occur
378 when the parameters have stabilized after the initial PS stress. These sedation status induced
379 differences are puzzling, and although they are mostly small, indicate a lasting physiological
380 effect of sedation status. Which sedation status is preferable is difficult to determine, but the
381 acid-base parameters seemed to be more fluctuating in sedated cod. This indicates a lowered
382 capacity to maintain acid-base homeostasis in sedated cod, suggesting no sedation is better
383 than metomidate sedation to reduce PS stress in Atlantic cod. The closed CA cannulation
384 technique was judged to be a promising substitute for more invasive cannulation procedures
385 previously used in Atlantic cod (Smith *et al.* 1985; Axelsson and Nilsson 1986; Perry *et al.*
386 1991). With the proper recovery time it will most likely prove to be a useful tool for
387 performing scientific experiments on Atlantic cod. As opposed to sacrificial sampling
388 techniques, the CA cannulation produces more reliable results, especially so for plasma
389 potassium and parameters affected by hypoxemia/arrested ventilation in sacrificially sampled
390 fish. The use of metomidate sedation prior to anaesthesia and subsequent cannulation surgery
391 was not so undecidedly positive as could be expected based on previous studies using
392 metomidate sedation prior to cannulation of salmonids (Kiessling *et al.* 2003; Sunde *et al.*
393 2003; Karlsson *et al.* 2006; Kiessling *et al.* 2006; Eliason *et al.* 2007; Djordjevic *et al.* 2010).
394 In Atlantic cod, pre-anaesthetic sedation with metomidate seemed not to suppress nor reduce
395 the stress response caused by the cannulation procedure, but rather produce a delayed
396 response of equal magnitude. Plasma cortisol levels were higher than the reference level for a
397 longer time PS in sedated compared to unsedated cod, but sedated cod seemed to reach lower
398 levels than unsedated cod from day 4 PS onwards (Fig. 1a). Due to the slow plasma glucose
399 removal observed in the present study, combined with elevated cortisol levels for a somewhat
400 shorter time, a recovery time of more than 5 days PS is necessary to produce representative
401 results for all studied variables, irrespective of sedation status. However, if a short recovery
402 time after cannulation is absolutely necessary to carry out the experiment (2 days or less),
403 Atlantic cod should not be sedated with metomidate prior to cannulation and the studied
404 variables should be limited in order to achieve the most representative data.

405

406

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412 experiment.

413

414

415 Figure and table captions/legends

416

417 Figure 1: Arterial blood plasma cortisol (a) and glucose (b) concentration after CA
418 cannulation surgery in Atlantic cod either sedated with metomidate (○) or not sedated (●)
419 prior to anaesthesia and subsequent cannulation. Solid line represents reference level
420 sacrificially sampled from the caudal vein/artery. Boxes show development the first 4 hours
421 PS. Results are presented as LSmeans with S.E. bars. * or ** indicates significant difference
422 between sedation statuses at respective sampling time at $p<0.05$ or $p<0.01$ respectively.

423

424 Figure 2: Arterial blood partial pressure of CO₂ (a), bicarbonate concentration (b) and pH (c)
425 after CA cannulation surgery in Atlantic cod either sedated with metomidate (○) or not
426 sedated (●) prior to anaesthesia and subsequent cannulation. Solid line represents reference
427 level sacrificially sampled from the caudal vein/artery. Boxes show development the first 4
428 hours PS. Results are presented as LSmeans with S.E. bars. * or ** indicates significant
429 difference between sedation statuses at respective sampling time at $p<0.05$ or $p<0.01$
430 respectively.

431

432 Figure 3: Arterial blood plasma sodium (a) and potassium (b) concentrations after CA
433 cannulation surgery in Atlantic cod. Solid line represents reference level sacrificially sampled
434 from the caudal vein/artery. Results are presented as LSmeans with S.E. bars. 3a: Atlantic cod
435 either sedated with metomidate (○) or not sedated (●) prior to anaesthesia and subsequent
436 cannulation. Box shows development the first 4 hours PS. * or ** indicates significant
437 difference between sedation statuses at respective sampling time at $p<0.05$ or $p<0.01$
438 respectively. 3b: Plasma potassium irrespective of sedation status (●), see materials &
439 methods for justification. Different letters denote significant differences between sampling
440 times at $p<0.05$. # indicates significant difference between respective sampling time and
441 reference level at $p<0.0001$.

442

443 Table 1: P-values for tests of fixed effects on blood variables

444

445 Table 2: Significant differences between sampling times post surgery and between sampling
446 times and reference level in caudal artery cannulated Atlantic cod either sedated (a) or not
447 sedated (b) with metomidate prior to anaesthesia.

448 Table 2 legend: Different letters denote significant differences between sampling times within
449 sedation status at $p < 0.05$. *, ** or *** denotes significant difference between respective
450 sampling time within sedation status and reference level at $p < 0.05$, $p < 0.01$ or $p < 0.0001$
451 respectively.

452

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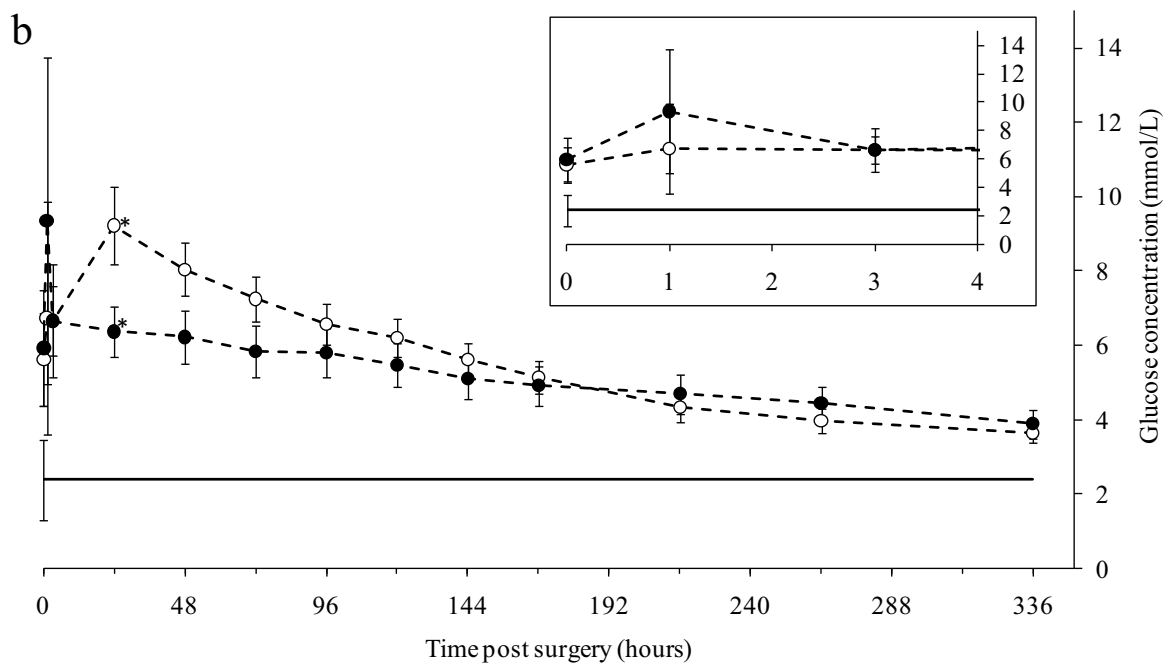
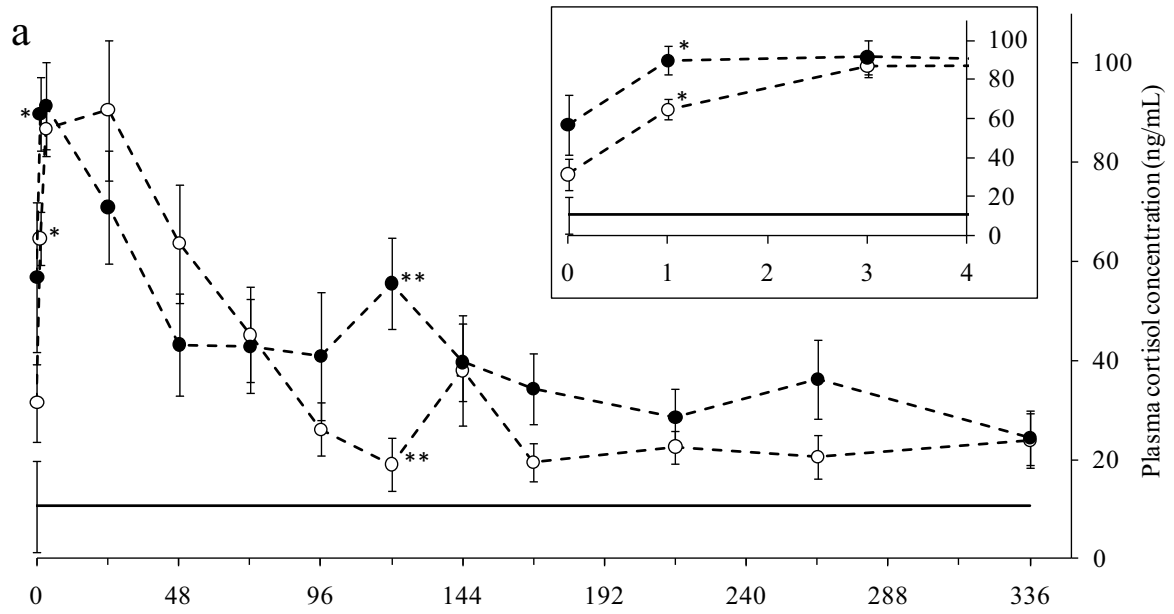
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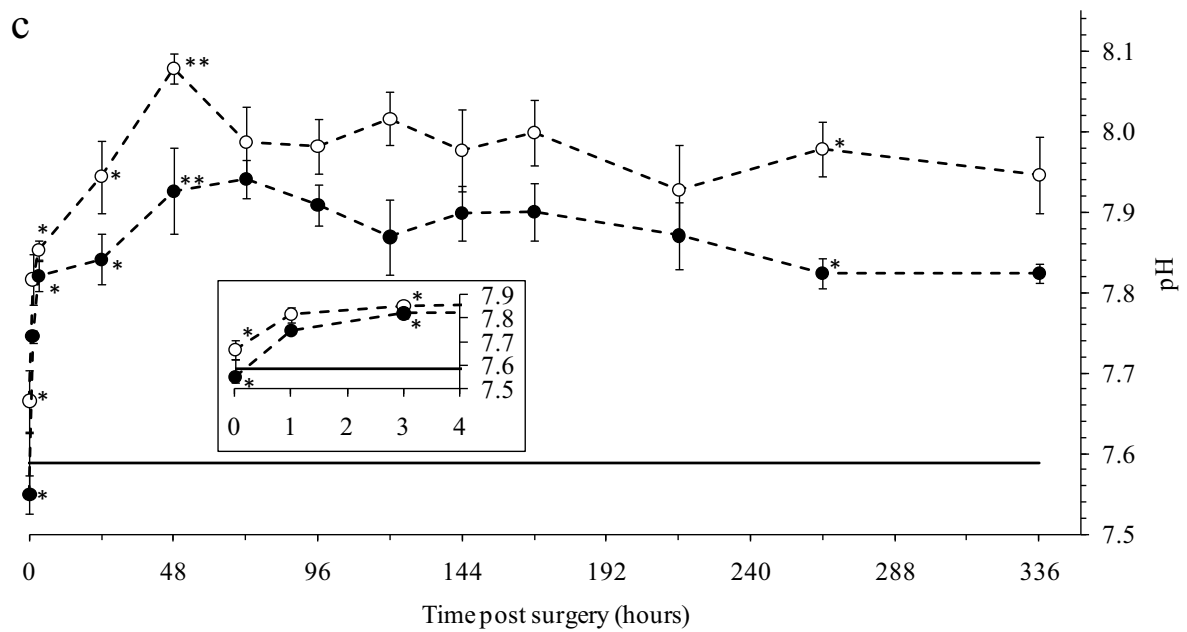
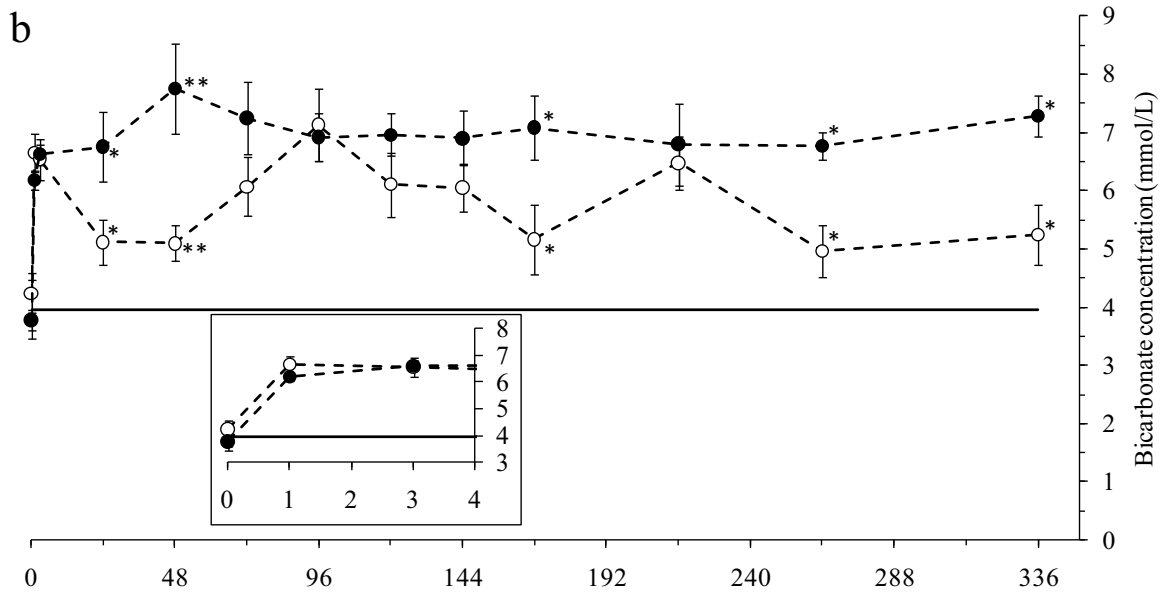
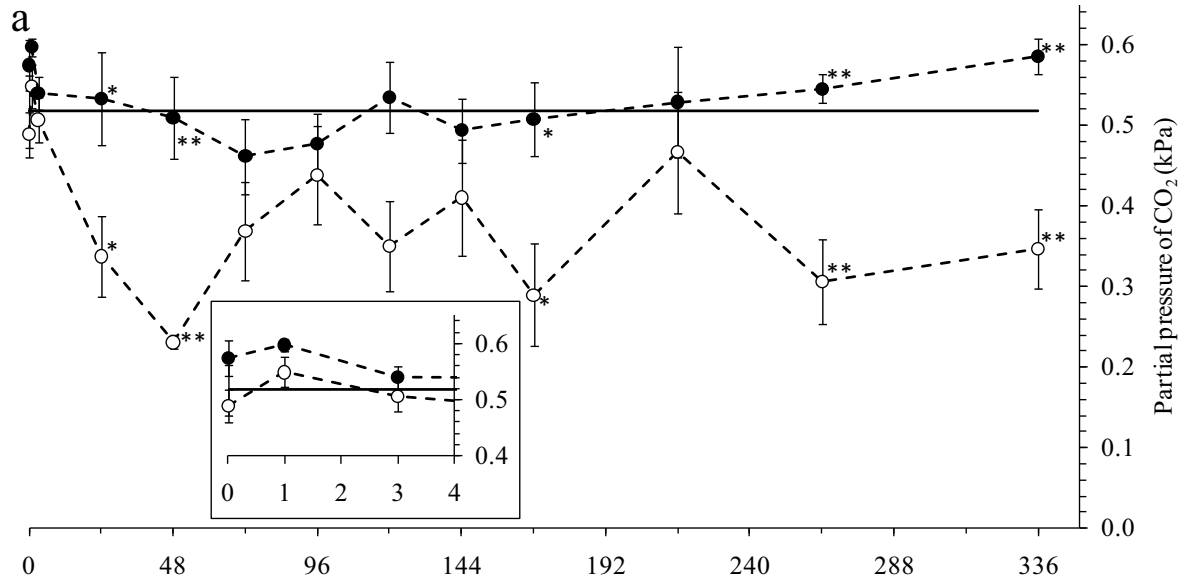
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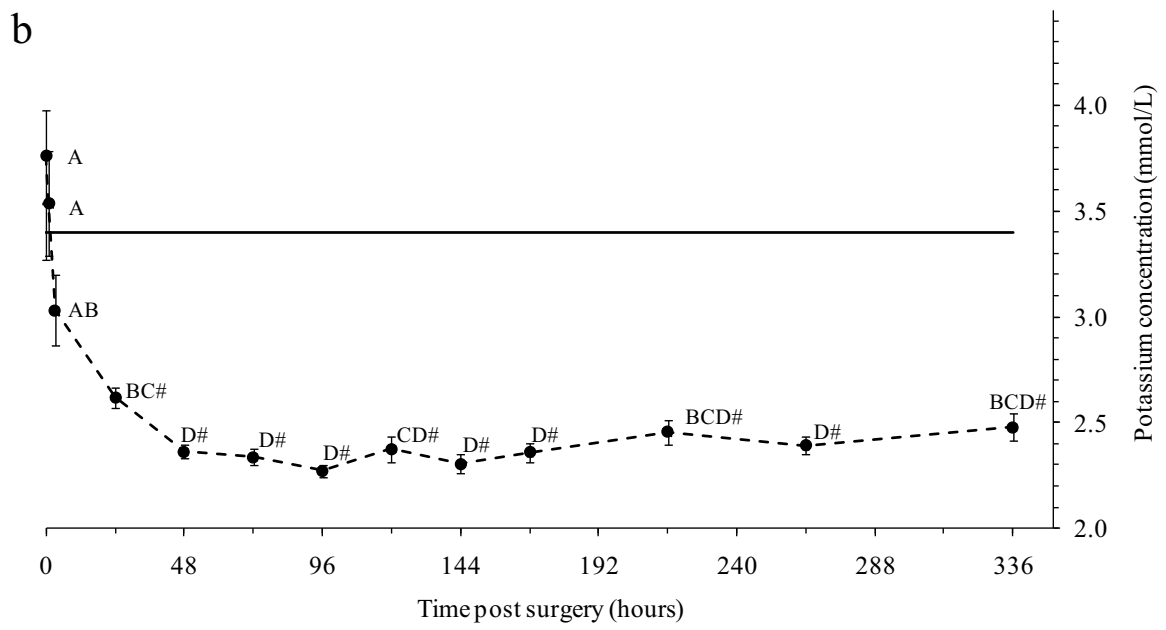
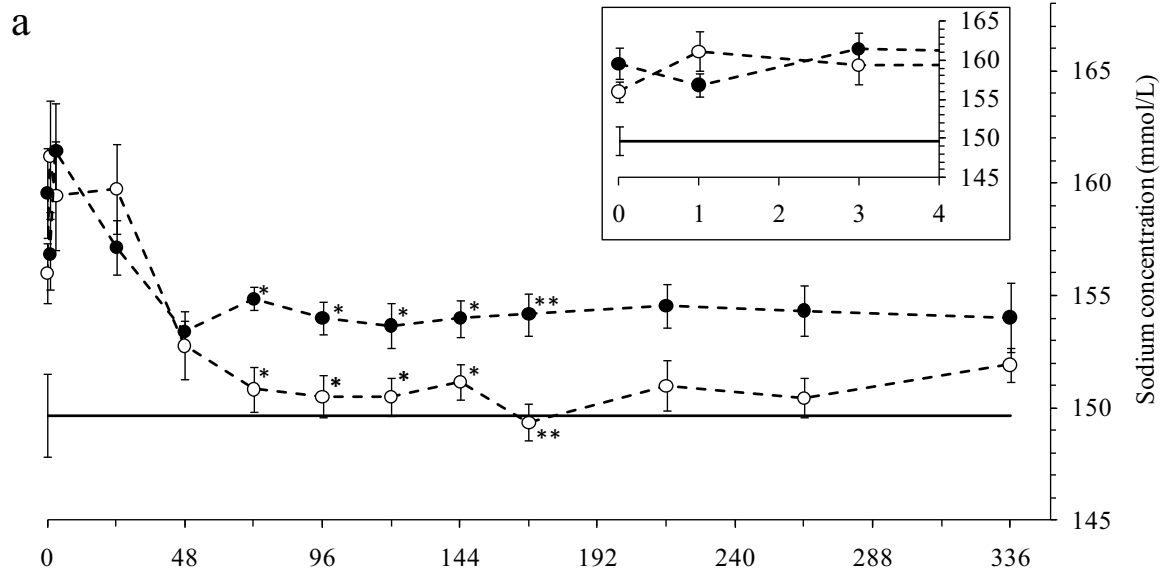
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	Cortisol	Glucose	Sodium	Potassium	pH	p _a CO ₂	Bicarbonate
Time post surgery	<0.0001	<0.0001	0.0002	<0.0001	<0.0001	<0.0001	<0.0001
Sedation status	0.2826	0.7882	0.0465	0.6255	0.0035	0.0019	0.0076
Time*sedation	0.0015	0.0424	0.0366	0.2085	0.0127	<0.0001	0.0008

a) Sedated cod

Time PS (hrs)	Plasma cortisol	Glucose	Sodium	pH	p _a CO ₂	HCO ₃ ⁻
0	AD	ACEF	ACE *	A	AC	A
1	B ***	ABEF *	AE ***	B **	A	BC **
3	C ***	ABEF *	ADE **	BD **	AD	BC **
24	BC ***	B ***	E **	BE ***	BCE *	AD
48	ABC **	BC **	AF	CF ***	B **	AD
72	ABD *	BC **	CF	BF ***	ABE	ACD *
96	AD	BCD *	BF	CEF ***	AE	BDE **
120	DE	BCD *	BF	CEF ***	ABE	ACE
144	ABE	DE	CDF	BF ***	ABE	ACE
168	DE	DE	BF	CDEF ***	BCDE **	ACE
216	AE	F	CDF	BF ***	ABE	BDE *
264	DE	AF	BF	CEF ***	BCDE *	ACE
336	AE	AF	CDF	BF ***	BCDE	ACE

b) Unsedated cod

Time PS (hrs)	Plasma cortisol	Glucose	Sodium	pH	p _a CO ₂	HCO ₃ ⁻
0	AB **	ABC *	AB ***	A	AB	A
1	A ***	ABC ***	AB **	B	A	B *
3	A ***	AB **	A ***	C **	B	B **
24	AC **	A **	AB **	BC ***	AB	B **
48	B	AB **	B	CD ***	AB	B ***
72	BC	AB *	AB	D ***	AB	B **
96	BC	AB *	AB	CD ***	AB	B **
120	AB *	AB *	AB	BCD ***	AB	B **
144	BC	AB	AB	CD ***	AB	B **
168	BC	ABC	AB	CD ***	AB	B **
216	B	ABC	AB	BCD ***	AB	B **
264	BC	B	AB	C **	AB	B **
336	B	C	AB	C **	AB	B **

Paper III

Changes in arterial PO_2 , physiological blood parameters and intracellular antioxidants in free-swimming Atlantic cod (*Gadus morhua*) exposed to varying levels of hyperoxia

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Abstract Free-swimming Atlantic cod (*Gadus morhua*) were exposed to water oxygen pressures (P_wO_2) ranging from 18.1 to 41.5 kPa and sampled for blood using an indwelling caudal artery cannula. Arterial blood oxygen pressure (P_aO_2) increased with increasing P_wO_2 , from 12.0 kPa in normoxia (18.1 kPa) to 34.2 kPa in the highest hyperoxic level tested (41.5 kPa). Blood CO_2 pressure and plasma bicarbonate concentration increased with P_wO_2 , indicating reduced ventilation with increased P_wO_2 . Plasma glucose, sodium and potassium were not affected by water oxygen level. Blood oxidative stress biomarkers, reduced glutathione, oxidized glutathione and the oxidative stress index (ratio between oxidized and total glutathione) differed intermittently between normoxia and hyperoxia. The oxidative stress index was higher in the blood of exposed compared to

unexposed control cod. Together with elevated P_aO_2 , these findings suggest increased production of reactive oxygen species and increased oxidative stress in Atlantic cod exposed to hyperoxia.

Keywords Oxygen supersaturation · Normoxia · Cannulation · Oxidative stress · Glutathione · Oxidation

Introduction

Teleost fish live and thrive in a variety of different aquatic environments with regard to temperature, salinity, light conditions and oxygen pressure (P_wO_2). As teleosts inhabit such a wide range of conditions, one would expect to find an array of environmentally induced inter-species differences in physiology and morphology, which is indeed the case. It appears to be true also for arterial oxygen pressure (P_aO_2) levels between species. While salmonid P_aO_2 levels are highly dependent on P_wO_2 (14–15 kPa in normoxic water; Smith and Jones 1982; Thomas et al. 1988; Perry and Reid 1992; Gilmour and Perry 1994; Brauner et al. 2000; Kristensen et al. 2010), other teleosts such as the American eel (*Anguilla rostrata*; Perry and Reid 1992), Wels catfish (*Silurus glanis*; Fogue et al. 1989) or common carp (*Cyprinus carpio*; Takeda 1993) have lower normoxic P_aO_2 levels. In the case of the Wels catfish, the P_aO_2 is maintained at a very low level (~ 2 kPa) in a wide

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range of oxygen conditions (P_wO_2 3–39 kPa; Fougue et al. 1989). The Atlantic cod (*Gadus morhua*) seem to have resting, normoxic and hypoxic P_aO_2 levels in the same range as do the salmonids, possibly with a larger variation (Kinkead et al. 1991; Sartoris et al. 2003). However, the P_aO_2 of Atlantic cod exposed to hyperoxic conditions has, to the authors' knowledge, not been previously reported.

Water-breathing aquatic animals such as molluscs and crustaceans, and the Wels catfish, have been shown to actively reduce P_aO_2 levels during rest both in normoxia and in hyperoxia (see Massabuau (2001) for review). The active reduction in P_aO_2 during rest was proposed by Massabuau (2001) to be a mechanism to protect tissues from oxidative stress. The reduced P_aO_2 levels coincide well with intracellular PO_2 levels observed in most animals and likely also with extracellular PO_2 levels present in the environment of the first obligate aerobic eukaryotes (Massabuau 2001). The actively reduced P_aO_2 strategy is likely more advantageous for animals that are less capable of avoiding a changing and frequently hypoxic environment, than for mobile animals. Increased P_aO_2 levels may increase the risk of damage caused by oxidative stress (Finne et al. 2008), especially if the oxidative stress is not countered by an increased ability to detoxify reactive oxygen species (ROS). During recent years, a series of biomarkers have been utilized to identify oxidative stress induced in fish exposed to unfavorable environmental conditions such as hyperoxia (van der Oost et al. 2003; Olsvik et al. 2005; Olsvik et al. 2006; Finne et al. 2008).

In intensive aquaculture practice, oxygenation to increase biomass production per volume of available water has become the norm. The use of oxygenation is especially widespread in on-land facilities where available water volume (natural oxygen supply) often limits production. Water oxygenation in intensive aquaculture may lead to prolonged or intermittent hyperoxic conditions and can create a situation where the animal is unable to move to another environment. Atlantic salmon (*Salmo salar*), normally inhabiting waters with relatively stable and well-saturated oxygen levels, show a concurrent increase in P_aO_2 with that of hyperoxic water; they have no ability to counteract hyperoxic conditions (Kristensen et al. 2010). In Atlantic cod aquaculture, on-land facilities produce mostly juveniles for growing in sea cages.

The early life stages of fish are usually considered the most sensitive to environmental influences. Therefore, cod in intensive aquaculture are being exposed to hyperoxic conditions in what is likely their most sensitive phase, possibly causing detrimental effects on health and welfare of the animals. A report published by the Norwegian Research Council (NFR) on the status of the Atlantic salmon farming industry in Norway supports the hypothesis that supersaturated levels of oxygen in the water during early life stages lead to impaired health and welfare, and concurrent lowered performance, of farmed salmon later in life (Stefansson et al. 2007).

Therefore, the objectives of the present study were to investigate the influence of varying levels of hyperoxia on P_aO_2 and key physiological blood variables, and oxidative stress levels in blood, kidney and liver tissues in free-swimming Atlantic cod.

Materials and methods

Animals and experimental conditions

A total of 100 Atlantic cod (*Gadus morhua*), roughly 700 grams each, were acquired from Profunda AS in early February 2009. The cod were housed in a six-meter-diameter circular holding tank at the Norwegian Institute for Water Research (NIVA) Marine Research Station (NIVA–MFS) at Solbergstrand, Norway. The cod were fed 5 days a week with a commercial cod feed (BioMar AS) for approximately 4 months prior to the experiment (June 2009). The cod were starved for 48 h prior to cannulation until the end of experimentation.

Fourteen Atlantic cod were in turn transported from the holding tank in 20-L buckets containing 0.5 mg/L metomidate sedation to an anesthetic bath containing 0.08 g/L metacain (Norwegian Medical Depot, Norway). When ventilation ceased and the cod no longer responded to touch, the cod were cannulated in the caudal artery (CA) following the procedure described by Karlsson et al. (2010). After cannulation, each cod was placed in one of the eight individual tanks and left to recover. Cannulation was unsuccessful in four fish and two fish stopped yielding blood within the first 24 h post surgery (PS). These cod were immediately euthanized by percussive stunning. The cod that stopped yielding

blood were replaced, yielding eight cod, average weight $1,268 \pm 115$ g and length 46.5 ± 1.4 cm (\pm standard deviation), to be used in the experiment. Because of this replacement, six cod had a recovery period of 96 h, while two cod recovered for 72 h prior to the experiment. The experimental tank setup was identical to the one described by Djordjevic et al. (2010). In brief, the cod were free-swimming in individual 1-m² tanks with cannulas trailing freely behind them and were provided with water current to swim against and shelter to hide under. Prior to sampling, cannulas were carefully retrieved using a hook-shaped steel wire in order to minimize any disturbance to the fish.

The cod were exposed to four hyperoxic treatments and a normoxic control, with P_wO_2 levels of 18.1 ± 0.2 (Normoxia), 24.7 ± 0.3 (Hyperoxia 1), 32.6 ± 0.3 (Hyperoxia 2), 38.6 ± 0.3 (Hyperoxia 3) and 41.5 ± 0.3 kPa (Hyperoxia 4). The experiment lasted for 8 days: At day 1, the fish were exposed to normoxic control water (18.1 kPa); on days 2–3, they were exposed to hyperoxic water (24.7 kPa); at days 4–5, they were exposed to normoxic control (18.1 kPa); and at days 6–8, they were exposed to hyperoxic water (day 6 = 32.6 kPa, day 7 = 38.6 kPa and day 8 = 41.5 kPa). Blood samples (250 μ L) were drawn from the cannulas daily, following the blood sampling procedure described by Karlsson et al. (2010). Hyperoxic conditions were created by diffusing pure oxygen gas into a header tank at ambient air pressure. The different hyperoxic levels were created by varying oxygen gas diffusion and afferent water flow rates to the header tank. Efferent water flow from the header tank was kept constant throughout the experiment to allow a constant water flow to the tanks.

The water source was the same throughout the experiment, full strength seawater from 60 m depth, supplied to the fish in a flow-through manner. Water salinity and temperature were recorded daily using a header tank-indwelling farming grade probe (OxyGuard[®]): salinity ranged from 32.0 to 34.6 parts per thousand (ppt) and water temperature ranged from 7.3 to 7.5°C. Additionally, water was sampled from the outlet of one of the eight experimental tanks at days 3 (Hyperoxia 1), 5 (Normoxia) and 7 (Hyperoxia 3). These water samples were analyzed at an accredited chemical laboratory (NIVA, Oslo, Norway) using standard procedures. The water quality was judged to be well suited to house Atlantic cod

(range): pH 7.83–7.86; alkalinity 2.40–2.41 mmol/L; turbidity 0.24–0.51 FNU; total nitrogen 165–180 μ g/L; ammonium <5–16 μ g/L; dissolved carbon dioxide 0.42–0.47 mg/L.

Sample preparation and analysis

Sampled arterial blood was immediately analyzed to determine P_aO_2 with a Microx TX3-trace oxygen transmitter and water temperature equalized needle-type oxygen micro sensor (PreSens Precision Sensing GmbH, Germany). Arterial blood ions (Na^+ , K^+ , Cl^-), CO_2 pressure (PCO_2), HCO_3^- , pH, glucose and urea were immediately analyzed using an i-STAT[®] Portable Clinical Analyzer (Medinor AS, Oslo, Norway). Results for PCO_2 , HCO_3^- and pH were temperature-corrected using formulas supplied by the manufacturer (Abbott point of care Inc.; Princeton, NJ, USA). Blood samples (100 μ L) were mixed with an equal volume of 5% sulfosalicylic acid (SSA) and immediately flash-frozen in liquid nitrogen. Within hours, the samples were transferred to a freezer maintaining $-80^\circ C$ for the later analysis of total glutathione (tGSH) and oxidized glutathione (GSSG) as biomarkers for oxidative stress. Tank water was sampled and analyzed to determine P_wO_2 as described above for P_aO_2 immediately after analysis of P_aO_2 .

At the start of the experiment, a second batch of cod, average weight 816 ± 188 g and length 41.2 ± 2.9 cm (\pm standard deviation), were removed from the holding tank and sacrificially sampled for blood from the caudal vein/artery. Tissue samples were taken from head kidney and liver and immediately flash-frozen in liquid nitrogen for the later analysis of tGSH, GSSG (liver) and MT (liver and kidney). The blood was treated the same way and analyzed for the same variables as in the experiment fish, except for PO_2 that was not included in the analysis. The same sampling procedure was applied to the experiment fish after the last experimental sampling (Hyperoxia 4) in comparison with the unexposed control.

Head kidney and liver samples for MT analysis were homogenized in 5 mM Tris-HCl pH 7.4 1:5 (w/v) on ice with a Potter-Elvehjelm homogenizer. Tissue homogenates were centrifuged at 10,000g for 10 min (4°C) and the supernatants frozen at $-80^\circ C$ until analysis. The tissue levels of Cd/Zn-MT were analyzed based on the method described by Bartsch et al. (1990). In brief, 100 μ L of homogenate was

added to 100 μL acetonitrile for the denaturation of high molecular mass proteins, followed by the addition of 1 mL of buffer (10 mM Tris-HCl, 1 M NaCl, pH 7.4). Samples were incubated for 5 min with 40 μL of Cd solution (^{109}Cd + stable Cd) before 100 μL of Chelex-100 (60% solution in buffer) was added to remove the excessive amounts of Cd. After 15-min rotation, samples were centrifuged at 10,000g for 5 min. A volume of 0.9 mL of the supernatant was transferred to a scintillation vial, and the activity of ^{109}Cd in the samples was measured using a NaI-detector (Wallac, Perkin Elmer, Wizard 3, 1,480 automatic gamma counter). Measured ^{109}Cd in the supernatant represented Cd bound to MT. The concentration of MT could be determined assuming the molecular mass of MT to be 7,000 dalton and a molar ration of 7 gram atoms of Cd per mole of protein.

Liver samples for the analysis of tGSH and GSSG were homogenized (approx. 1:4 w/v) in 5% (w/v) sulfosalicylic acid (SSA, Sigma-Aldrich, South-Korea), centrifuged at 10,000g for 10 min and stored at -80°C until analysis. Blood was thawed, ultrasonicated on ice and centrifuged (10,000g, 15 min, 4°C). The supernatants were subjected to the analysis of GSSG and tGSH. Determination of GSSG and tGSH was based on the method described by Vandeputte et al. (1994). In brief, samples for tGSH analysis were diluted 1:5 (v/v) in 5% SSA. Twenty microlitres of the diluted sample was added to 200 μL of reaction buffer (143 mM Na-phosphate buffer, pH 7.4, 6.3 mM EDTA, 2 mM NADPH and 8 mM DTNB) in a microtiterplate, and 40 μL of GR (6.5 U/ml in assay buffer) was added to start the reaction. Samples were measured spectrophotometrically in a plate reader at 405 nm using kinetic readings. The concentration of tGSH was calculated based on a standard curve for reduced glutathione (GSH). GSSG analysis was carried out by adding 5 μL 2-vinylpyridine (2-VP) and 14 μL triethanolamine (TEA, diluted 1:3 (v/v)) to 100 μL of tissue homogenate, blanks and standards. Following 1-h incubation at room temperature, 20 μL of blanks, standards and samples were added to 200 μL reaction buffer and 40 μL of GR solution, and the rate of GSSG conversion to GSH was analyzed spectrophotometrically at 405 nm using kinetic readings. The concentration of GSSG was calculated based on a standard curve for GSSG. Results were normalized to liver wet weight

and blood volume and expressed as $\mu\text{mol/g}$ liver and $\mu\text{mol/mL}$ blood. The oxidative stress index (OSI) was determined based on the ratio between GSSG and tGSH:

$$\text{OSI}(\%) = \left(\frac{2 * \text{GSSG}}{\text{tGSH}} \right) * 100\%$$

GSH was calculated from the concentrations of GSSG and tGSH:

$$\text{GSH} = \text{tGSH} - \text{GSSG}$$

Statistical analysis

The data were analyzed using SAS v. 9.1 (Statistical Analysis Software). The regression procedure (proc reg) was used for generating linear regression models for the analysis of the relationship between P_{aO_2} , PCO_2 and HCO_3^- (variables) and P_{wO_2} (dependent).

The mixed model procedure (proc mixed) for repeated measurements was used for generating least squares means (LS means), standard errors (SE) and tests for the determination of significant difference between oxygen treatments or days. Either oxygen treatment or experiment day was used as the only class variable. Individual was included as subject identification, and compound symmetry was used as covariance structure.

The general linear model procedure (proc glm) was used for generating LS means, SE and tests for the determination of significant difference between the control fish and experiment fish at the end of hyperoxia exposure for blood and liver GSH, GSSG and OSI, and liver and kidney MT.

Except for two observations in hyperoxia 4 (139 and 140 mmol/L), blood chloride was always above the analytical range of the instrument (140 mmol/L) and was therefore excluded from further analysis. Blood urea levels were only infrequently observed above the detection limit of the instrument (1.0 mmol/L) and were therefore excluded from further analysis.

P-values <0.05 after Tukey-Kramer adjustment for multiple comparisons were considered to be statistically significant. Where differences between treatments are indicated, the differences were significant at $P < 0.05$ unless stated otherwise. Values are presented as LS means \pm SE throughout unless stated otherwise.

Results

Respiratory and acid–base parameters

P_{aO_2} , PCO_2 and HCO_3^- increased with P_{wO_2} (Fig. 1a–c). P_{aO_2} and HCO_3^- differed between all

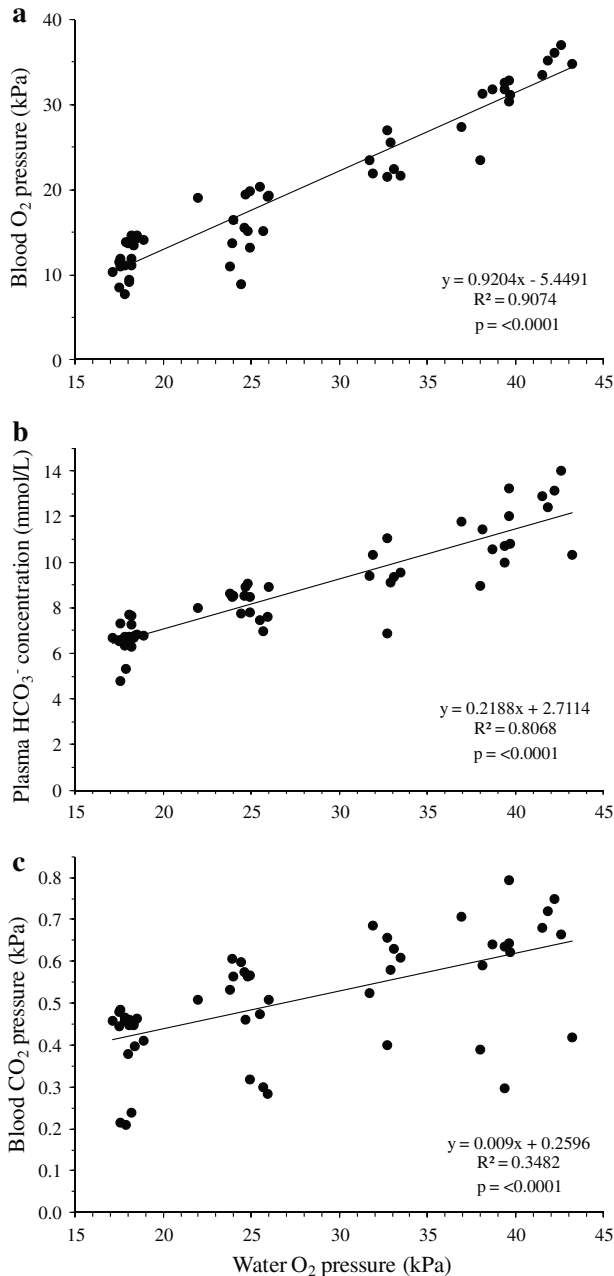


Fig. 1 O_2 pressure (a), plasma HCO_3^- concentration (b) and CO_2 pressure (c) in the arterial blood of Atlantic cod as functions of the O_2 pressure in water (P_{wO_2}). Each data point represents an individual observation

oxygen treatments (Table 1). PCO_2 differed between all treatments except between Hyperoxia 1 (0.49 ± 0.04 kPa) and 3 (0.55 ± 0.04 kPa), and between Hyperoxia 2 (0.58 ± 0.04 kPa) and Hyperoxia 3 and 4 (0.67 ± 0.04 kPa; Table 1).

PO_2 difference ($P_{wO_2} - P_{aO_2}$) was lower during days 4–5 (Normoxia) than in days 2 (day 1 of 2 in Hyperoxia 1), 6 (Hyperoxia 2) and 7 (Hyperoxia 3; Fig. 2a).

Plasma pH differed intermittently between experimental days and treatments and tended to increase with P_{wO_2} . However, the increase in pH with P_{wO_2} was not consistent throughout treatments (Fig. 2b).

Ions and glucose

Plasma sodium was stable at 145–148 mmol/L in days 1–3 and then increased between samplings at days 3 and 4 and was stable until the end of the experiment at 156–158 mmol/L (Fig. 3a). Plasma potassium was not affected by P_{wO_2} or experiment duration (Fig. 3b). Plasma glucose decreased steadily throughout the experiment from ca 9 to ca 6 mmol/L. Plasma glucose in days 1–3 differed from that in days 6–8, and days 4–6 differed from day 8 (Fig. 3c).

Oxidative stress parameters during experiment

Blood GSH was stable at ca 5 $\mu\text{mol/mL}$ in initial normoxia and the first hyperoxic treatment (days 1–3), then dropped in the following normoxia (days 4–5) and was stable at ca 4 $\mu\text{mol/mL}$ until the end of the experiment (days 6–8) during increasing P_{wO_2} (Fig. 4a). Blood GSSG was more variable than GSH with less consistent differences between days. However, the approximately opposite response to GSH observed in GSSG: blood GSSG was stable at ca 0.6 $\mu\text{mol/mL}$ during initial normoxia and hyperoxia (days 1–3), increased in the following normoxia (days 4–5) and was then stable at ca 0.9 $\mu\text{mol/mL}$ throughout the last three hyperoxic treatments (Fig. 4b). Blood OSI was stable at ca 20% at the beginning of the experiment (days 1–3), then increased through day 4 and was stable at ca 35–40% after that (Fig. 4c). Blood OSI differed between days 1–4 and days 5–8 (Fig. 4c).

Table 1 Water and blood PO_2 and blood CO_2 parameters in Atlantic cod exposed to hyperoxia

Treatment	P_wO_2 (kPa)		P_aO_2 (kPa)		PCO_2 (kPa)		HCO_3^- (mmol/L)	
	LS mean	SE	LS mean	SE	LS mean	SE	LS mean	SE
Normoxia	18.1	0.2 A	12.0	0.8 A	0.41	0.04 A	6.7	0.3 A
Hyperoxia 1	24.7	0.3 B	16.2	0.9 B	0.49	0.04 B	8.2	0.3 B
Hyperoxia 2	32.6	0.3 C	23.4	1.1 C	0.58	0.04 CD	9.4	0.3 C
Hyperoxia 3	38.6	0.3 D	29.9	1.1 D	0.55	0.04 BC	10.6	0.3 D
Hyperoxia 4	41.5	0.3 E	34.2	1.1 E	0.67	0.04 D	12.6	0.3 E

Different letters denote significant difference between treatments at $P < 0.05$. Water and arterial blood PO_2 were different in all treatments ($P \leq 0.0002$)

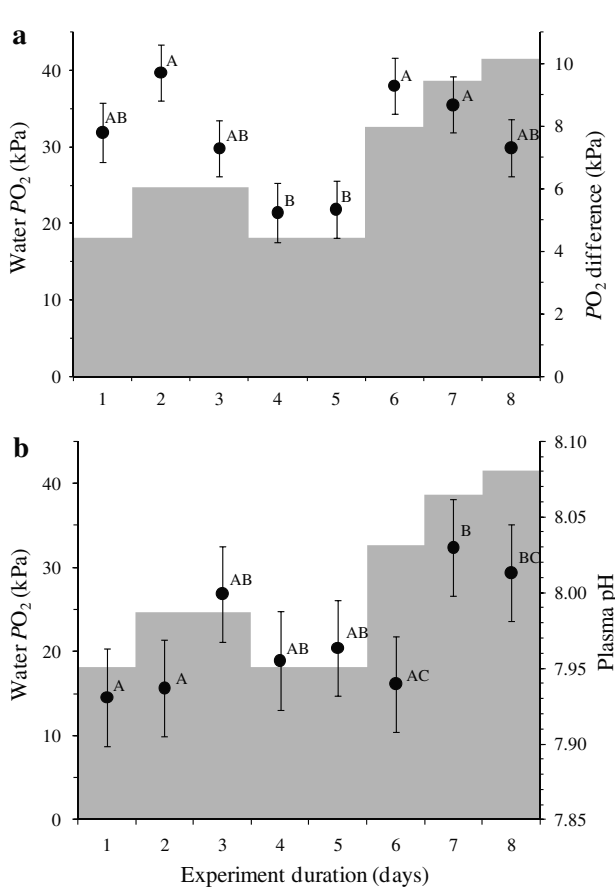


Fig. 2 Observed PO_2 difference (difference between oxygen pressure in water and arterial blood) (a) and arterial blood plasma pH (b) in Atlantic cod by duration of hyperoxia experiment. Shaded gray area shows P_wO_2 level (kPa). Values presented as LS means \pm SE error bars. Different letters denote significant difference between days within parameter at $P < 0.05$

Oxidative stress parameters in hyperoxia exposed compared to unexposed cod

Blood OSI was the only oxidative stress parameter that differed between hyperoxia-exposed cod at the end of the experiment and unexposed control cod

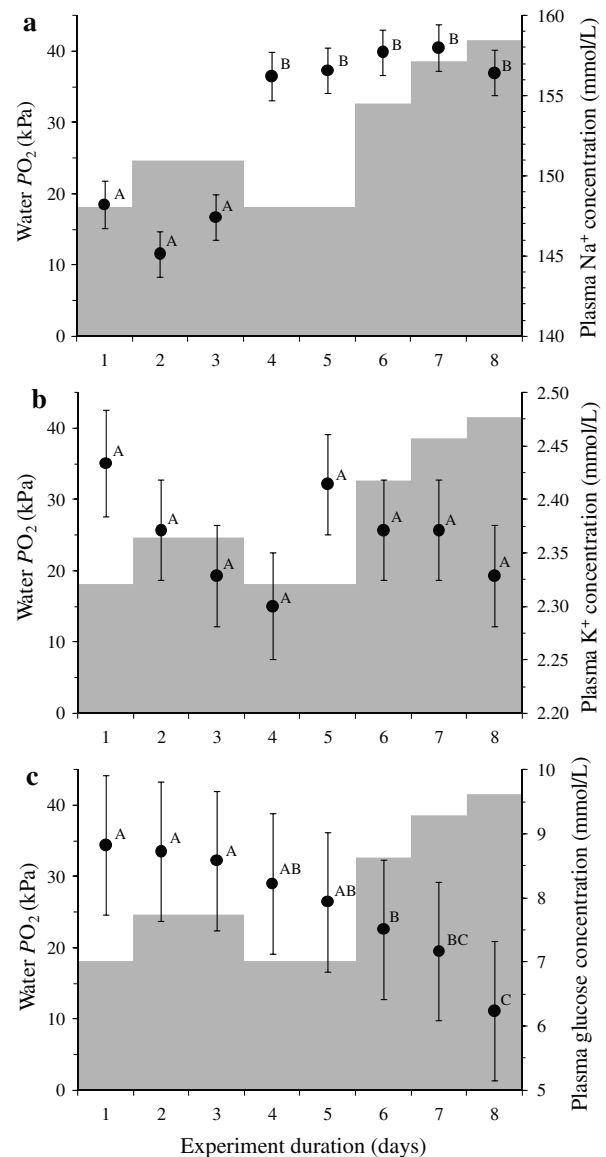


Fig. 3 Arterial blood plasma sodium (Na^+) (a), potassium (K^+) (b) and glucose (c) in Atlantic cod by hyperoxia experiment duration. Shaded gray area shows P_wO_2 level (kPa). Values presented as LS means \pm SE error bars. Different letters denote significant difference between days at $P < 0.05$

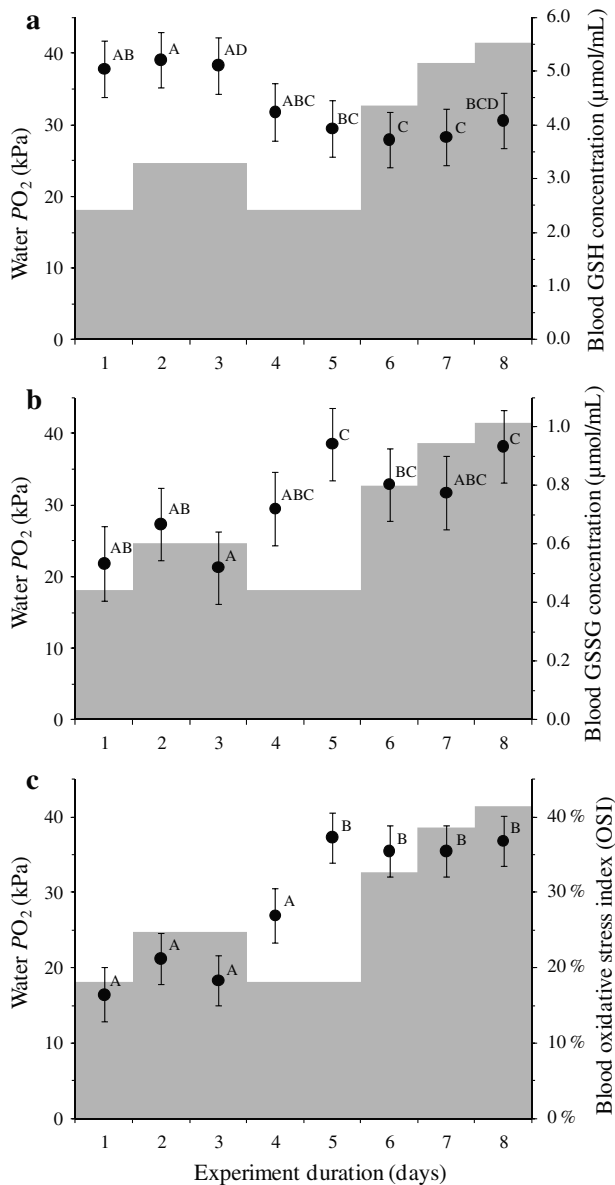


Fig. 4 Blood GSH concentration (a), GSSG concentration (b) and OSI (oxidative stress index) (c) in Atlantic cod by duration of hyperoxia experiment. *Shaded gray area* shows P_wO_2 level (kPa). Values presented as LS means \pm SE error bars. Different letters denote significant difference between days within parameter at $P < 0.05$

(Fig. 5b); all other comparisons were not significant (Fig. 5a, c).

Discussion

Atlantic cod P_aO_2 levels were highly dependent on P_wO_2 in hyperoxia (Fig. 1a); Atlantic cod do not actively regulate P_aO_2 to a predetermined level

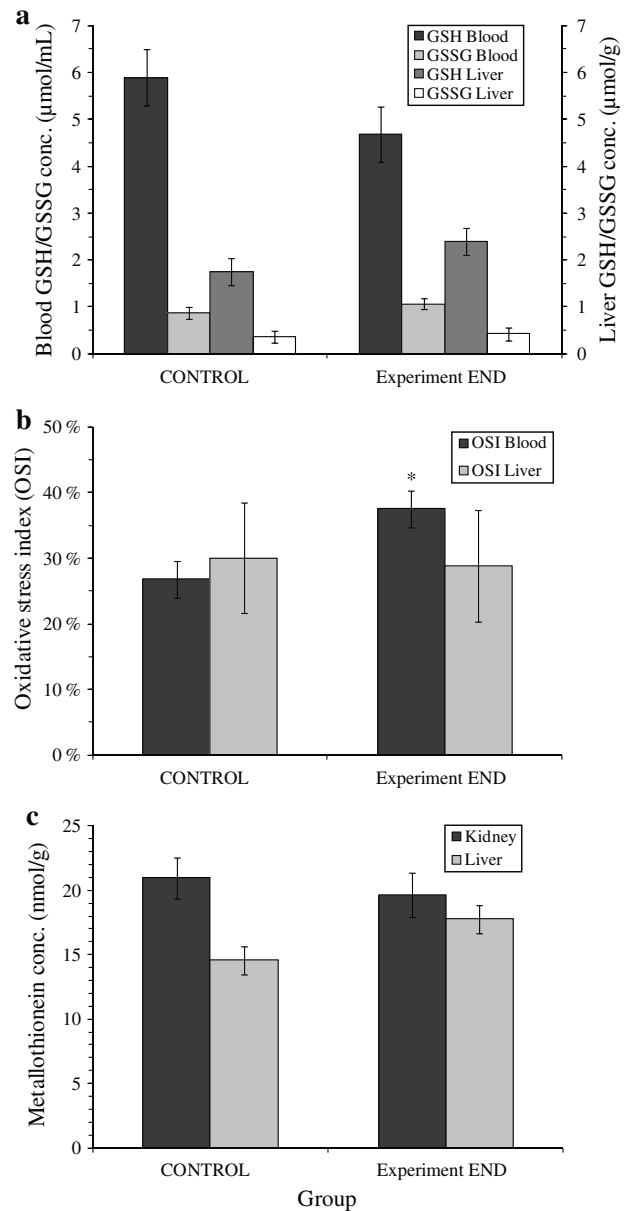


Fig. 5 GSH and GSSG concentrations in blood and liver (a), oxidative stress index (OSI) in blood and liver (b) and metallothionein concentrations in kidney and liver (c) of Atlantic cod unexposed to (CONTROL) and posterior to (Experiment END) 7 days of intermittent hyperoxia exposure. Values presented as LS means \pm SE error bars. Asterisk denotes significant difference between unexposed and exposed groups at $P < 0.05$

irrespective of P_wO_2 . This finding is in accordance with previous studies on Atlantic cod in hypoxia and normoxia (Kinkead et al. 1991; Sartoris et al. 2003) and previous studies using salmonids (Smith and Jones 1982; Thomas et al. 1988; Perry and Reid 1992; Gilmour and Perry 1994; Brauner et al. 2000;

Kristensen et al. 2010). However, indirect evidence of reduced respiration with increased P_{wO_2} was observed; HCO_3^- and PCO_2 increased with increasing P_{wO_2} (Fig. 1b, c). The reduced respiration may have been caused by reduced gill ventilation (increased ventilation reported in hypoxia exposed cod by Kinkead et al. (1991)), increased gas diffusion distance (Saroglia et al. 2000) or reduced respiratory surface area (Saroglia et al. 2002). Increased PCO_2 and HCO_3^- in hyperoxia were also reported by Kristensen et al. (2010). The reduced respiration with increasing P_{wO_2} was not sufficient to influence the PO_2 difference consistently, although day 1 of Hyperoxia 1 and Hyperoxia 2 and 3 differed from Normoxia during days 4–5 (Fig. 2a). The lack of a consistent response in PO_2 difference to increasing P_{wO_2} levels may be due to the fish ceasing to respond to the hyperoxic environment over time; as hyperoxia exposure was prolonged, the PO_2 difference dropped to a level not significantly different from normoxia (Fig. 2a). This indicates that the cod did not maintain the initial PO_2 difference during prolonged hyperoxia, a difference which in turn could have given the cod better protection against oxidative stress. Thus, the negative effects of hyperoxic P_{wO_2} levels in Atlantic cod farming are likely to be exaggerated if the hyperoxia is sustained. Plasma pH increased somewhat with P_{wO_2} (Fig. 2b), likely a secondary effect of increased HCO_3^- with increasing P_{wO_2} . Similar to the observation in the present study, Kristensen et al. (2010) reported increased blood pH in hyperoxia compared to normoxia in Atlantic salmon.

The rapid increase in plasma sodium levels observed between days 3 and 4 occurred at a time when the oxygen level was stable and simultaneously with an increase of 0.7 ppt in water salinity, possibly affecting the osmotic balance of the cod. However, larger fluctuations in salinity were observed during the experiment (2.0 ppt decrease and 1.5 ppt increase between days) without any detectable effect on plasma sodium levels. Thus, the observed shift in plasma sodium was considered unexplainable by the water quality or experimental variables measured and likely unimportant for the well-being of the cod. This finding is contrasted by what was reported in a recent study; sodium levels were lower in hyperoxia than in normoxia (Kristensen et al. 2010). Plasma potassium was stable throughout the experiment. Therefore,

hyperoxia was considered to be of no importance for plasma potassium levels in Atlantic cod. This finding is in accordance with the study by Kristensen et al. (2010) in Atlantic salmon, which reported no difference in blood potassium level in hyperoxia compared to normoxia. Plasma glucose slowly decreased from start to end of the experiment, despite varying degrees of hyperoxia and an intermittent normoxia treatment (Fig. 3c). A recent study by Karlsson et al. (2010) suggested that a recovery time of 5–7 days or more after CA cannulation surgery is necessary to reach the basal levels of blood glucose in Atlantic cod. Thus, the recovery times used in the present study before starting the experiment (3 or 4 days, depending on the individual) were likely insufficient for plasma glucose to reach basal levels. Hence, the differences observed in plasma glucose between treatments were attributed to the cannulation surgery rather than the P_{wO_2} levels. This assumption is supported by a recent study reporting no difference in blood glucose levels between normoxia and hyperoxia in Atlantic salmon (Kristensen et al. 2010).

Blood GSH levels decreased while GSSG levels increased during the second normoxic treatment, causing a near doubling of OSI during this period. Then, throughout the following 3 days of increasing hyperoxia, all three parameters remained stable (Fig. 4a–c). Glutathione is one of the major antioxidants in the cell (Halliwell and Gutteridge 2007), and the increase in GSSG, and consequently in OSI, suggests increased ROS production and induction of the antioxidant system during normoxia. However, as increased oxidative stress and consequent increased antioxidant system induction in normoxia compared to the preceding hyperoxia seem unlikely, it may be a delayed response to the preceding hyperoxia.

Blood OSI differed between the control and the experimental cod after 7 days of exposure to varying degrees of hyperoxia, indicating that the experimental oxygen levels induced ROS production and free radical detoxification responses. Olsvik et al. (2005) reported decreased blood OSI in Atlantic salmon smolts in a study with a much longer hyperoxia exposure time than in the present study (126 days). No response was observed in head kidney MT or liver MT, GSH, GSSG or OSI, indicating that the antioxidant system induction observed in blood did not affect the liver or kidney tissues. This is in accordance to a previous study by Olsvik et al. (2006), who

reported no effects of hyperoxia on liver GSH, GSSG, OSI or transcriptional level of MT. However, the same study identified an increase in the transcriptional level of GSH-Px in Atlantic cod exposed to hyperoxia, indicating increased ROS production (Olsvik et al. 2006). In the present study, GSH/GSSG and MT were the only biomarkers for oxidative stress evaluated. In future studies, measurements of additional biomarkers for oxidative stress, such as GSH-Px, superoxide dismutase or catalase, should be included to further elucidate the effects of hyperoxia on oxidative stress responses.

The hyperoxic conditions in the present study were created in a header tank at ambient air pressure in order to avoid the supersaturation of total gas pressure (TGP). However, TGP and the possible negative effects of supersaturated TGP were not monitored. Due to this, the possibility of supersaturation of TGP in the highest hyperoxic levels cannot be excluded.

In conclusion, Atlantic cod P_{aO_2} is largely dependent on P_{wO_2} in hyperoxia; an increase in P_{wO_2} is followed by an increase in P_{aO_2} . Hyperoxia also increased physiological parameters linked to respiration such as arterial PCO_2 and plasma pH and bicarbonate, while the other measured physiological parameters plasma sodium, potassium and glucose were unaffected by hyperoxia. Blood biomarkers for oxidative stress GSH, GSSG and OSI changed with varying hyperoxia exposure. Blood OSI was also different between hyperoxia-exposed and control cod after 7 days of varying hyperoxia exposure while kidney and liver MT and liver GSH, GSSG and OSI remained stable. The observed elevated P_{aO_2} and changes in blood GSH, GSSG and OSI suggest an increased oxidative stress load in hyperoxia, indicating that cultured Atlantic cod should not be exposed to supersaturated oxygen levels.

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Paper IV

8 Keywords: caudal artery cannulation; smart tag; hypoxia; hyperoxia; carbon dioxide;
9 respiratory physiology
10

11 Summary

12

13 Atlantic cod (*Gadus morhua*) were fitted with caudal artery cannulas and ventilation
14 registration tags and exposed to separate and combined hypercapnia (0.43 and 0.70 kPa) and
15 hypoxia (15.6 kPa) or hyperoxia (24.7 kPa). Ventilation rate, ventilatory stroke volume,
16 ventilatory minute volume, oxygen consumption, blood gases and relevant blood
17 physiological parameters were measured in all treatments. When exposed separately,
18 ventilation increased in hypercapnia and hypoxia, and decreased in hyperoxia. However, no
19 effects were observed on ventilation with changes in oxygen level under hypercapnic
20 conditions. Oxygen consumption was maintained in hypoxia, and increased in hyperoxia,
21 compared to the control. Oxygen consumption was not affected by hypercapnia.

22 Arterial blood oxygen tension increased with water oxygen tension, and the difference
23 between water and arterial oxygen tensions was larger in the control treatment than in
24 separate or combined hypercapnia and hypoxia. Arterial CO₂ tension increased, and plasma
25 pH and bicarbonate decreased, in hypercapnia. Plasma glucose was higher in the control
26 treatment than in separate or combined hypercapnia and hypoxia, but not in hyperoxia.
27 Plasma sodium was higher in hyperoxia than in hypoxia and plasma potassium was higher in
28 the 0.70 kPa hypercapnic level than in the control treatment.

29 We conclude that, at moderate levels, water CO₂ tension is a more potent ventilatory
30 stimulant than water O₂ tension and that the Atlantic cod pO₂ difference always decreased
31 from the control treatment into separate or combined hypercapnia and hypoxia.

32

33 Introduction

34

35 Atlantic cod (*Gadus morhua* Linnaeus) in their natural habitat live in an environment which is
36 fairly stable with regards to tensions of oxygen (p_wO_2) and carbon dioxide (p_wCO_2). In the
37 event that cod experience diversions from normal conditions it is usually in the direction of
38 low oxygen levels (hypoxia) or small increases in carbon dioxide levels (hypercapnia). Water
39 O_2 tension has long been regarded as the primary cause of ventilatory drive in fish in order to
40 maintain oxygen consumption (see Randall, 1982), but more recent reviews indicate that
41 water CO_2 (p_wCO_2) also plays an important role in controlling teleost ventilation (Gilmour,
42 2001; Perry and Gilmour, 2002). Both hypoxia and hypercapnia tend to cause
43 hyperventilation (Gilmour, 2001; Perry and Gilmour, 2002) while hyperoxia causes
44 hypoventilation (Gilmour and Perry, 1994; Soncini and Glass, 2000) and increased arterial
45 partial pressure of oxygen (p_aO_2) in teleosts (Gilmour and Perry, 1994; Kristensen et al.,
46 2010). The Atlantic cod is known to respond similarly to other teleosts, with hyperventilation
47 and decreased p_aO_2 in hypoxia (Kinkead et al., 1991; Perry et al., 1991) and increased p_aO_2 in
48 hyperoxia (Karlsson et al., 2010b).

49 Due to the hyperventilatory effect of increased water p_wCO_2 (Gilmour, 2001) and opposite
50 effect of increases in p_wO_2 (Gilmour and Perry, 1994), the effects of combined hyperoxia and
51 hypercapnia are not easily predicted. To further add to the complexity, two previous studies
52 examining responses to combined hyperoxia and hypercapnia in teleosts reported opposite
53 results: rainbow trout (*Oncorhynchus mykiss*) ventilation increased from hyperoxia into
54 combined hyperoxia and hypercapnia (Thomas et al., 1983), while common carp (*Cyprinus*
55 *carpio*) ventilation was not affected by combined hyperoxia and hypercapnia compared to
56 hyperoxia alone (Soncini and Glass, 2000).

57 Aquaculture has increased significantly over the last decade and production is expected to
58 increase as harvest from wild stocks further decreases. The production of Atlantic cod
59 juveniles is dominated by land based farming in tanks. This production system allows a large
60 degree of control with water quality parameters such as O_2 and CO_2 levels, important to
61 ensure good health, welfare and growth. However, this production system relies on pumping
62 seawater into the facilities which in turn causes technical and economical limitations to water
63 volume throughput. Therefore, this production system relies heavily on artificial oxygenation
64 to maintain sufficient O_2 levels in the tanks due to a high biomass per volume water. Hence,
65 the water is often supersaturated with O_2 . Such systems will by default accumulate CO_2
66 resulting in hypercapnic conditions. Furthermore, with high biomasses and low water flow

67 rates there may also be rapid and unpredictable fluctuations between separate or combined
68 hypoxic, hyperoxic and hypercapnic conditions.

69

70 The objective of this study was to assess the effects of moderate levels of separate and
71 combined hypercapnia and hypoxia or hyperoxia on the ventilation, oxygen consumption and
72 blood physiology of Atlantic cod.

73

74 Materials & Methods

75

76 *Animals and experimental protocol*

77 The experiment was conducted in February-March 2009 at the Tromsø Aquaculture Research
78 Station, Norway. The Atlantic cod used were hatched in March 2007 and bred by the National
79 Cod Breeding Programme of Nofima Marin, Tromsø, Norway. The fish were second
80 generation captive cod, from 50 different family groups varying in genetic background. From
81 July 2007 the fish were reared under continuous light conditions (LD 24:0) and at ambient sea
82 water temperatures. The cod were fed a commercial diet (Skretting, Norway). When the fish
83 were about 12 g, the juveniles were transferred to the research station and reared there until
84 used in the experiment.

85 A total of nine Atlantic cod of either sex, average weight 1024 ± 249 g and length 43 ± 3 cm
86 (\pm standard deviation (SD)), were cannulated in the caudal artery (CA) and left to recover in
87 individual tanks. Prior to each experimental day, a single cod was fitted with a differential
88 pressure acoustical tag termed SmartTag (developed by Nofima Marin and Thelma Biotel,
89 Trondheim, Norway). The SmartTag provides online measurements of pressure changes
90 inside the buccal cavity of the fish, thus allowing for continuous measurements of ventilatory
91 parameters. The cod was then placed in a swim tunnel respirometer (Loligo Systems ApS,
92 Tjele, Denmark) and left overnight to acclimate before experimentation. During the
93 acclimation period, the swim tunnel water source and water flushing cycles were identical to
94 those in the experiment. To protect the cod from visual disturbances, a rubber curtain
95 separated the swim tunnel from the rest of the experimental room. The CA cannula was
96 protruding through a small hole in the roof of the swim tunnel and further through a small slit
97 in the rubber curtain to allow sampling of arterial blood.

98 The cod were exposed to all possible combinations of three O₂ levels and three CO₂ levels,
99 yielding a total of nine treatments including the control level. The O₂ levels were: low (15.6
100 kPa), control (untreated water, 20.7 kPa) and high (24.7 kPa). The CO₂ levels were: control
101 (untreated water, 0.15 kPa), medium (0.43 kPa) and high (0.70 kPa). The combination control
102 O₂ and control CO₂ was used as control treatment.

103 Each treatment and control cycle lasted for a total of 18 minutes, and consisted of three
104 distinctive parts in order to generate oxygen consumption (MO₂) data from each treatment and
105 control: flush (8 min), wait and mix (1 min) and measure (9 min). Control treatments
106 preceded and followed all other treatments, yielding a series of treatments and controls
107 consisting of seventeen cycles (eight treatments and nine controls) being run back to back for

108 each fish. The order of the treatments was changed between each individual and never
109 repeated in order to eliminate carry-over effects of previous treatments. Control treatment
110 water was pumped into the swim tunnel using a circulation pump submerged in a bath
111 surrounding the swim tunnel, while exposure water was flushed into the swim tunnel from a
112 header tank. Control and exposure waters were continuously replaced (from the same source),
113 yielding practically equal water temperature in the swim tunnel, control water bath and
114 exposure water header tank throughout the experiment. Due to water flow limitations (pump)
115 from the control water bath, the entire eight minute flush period was used to flush out
116 exposure water and replace it with control water. Only three minutes of flushing was needed
117 to flush out the control water and replace it with exposure water in the swim tunnel. The
118 difference in flushing time meant the cod had five minutes longer to adjust to exposure
119 treatments than control treatments (14 vs. 9 min). Swimming speed in the swim tunnel was
120 maintained at approximately 0.3 body lengths per second for all individuals throughout
121 acclimation and experimentation.

122 Due to the possibility of cannulating the caudal vein rather than the desired CA, all
123 individuals were dissected posterior to experimentation to determine the location of the
124 cannula. All nine individuals were cannulated in the CA.

125

126 *Surgical protocols*

127 The cannulation procedure was performed according to Karlsson *et al.* (2010a) . Briefly, each
128 cod was sedated with 0.5 mg l⁻¹ metomidate (metomidate hydrochloride; Syndel Ltd, Victoria,
129 B.C., Canada), then anaesthetized with 70 mg l⁻¹ metacain (Tricaine methane sulphonate;
130 Norwegian Medical Depot; Norway) prior to cannulation. Finally, a polyethylene catheter was
131 inserted into the CA and sutured to the skin of the fish. Throughout cannulation surgery the
132 gills were continuously ventilated with aerated seawater containing a maintenance dose of
133 metacain (15 mg l⁻¹). The cod were then left to recover for an average of seven days (range 3-
134 15 days) after cannulation before being used in the experiment.

135 The day prior to experimentation a cod was sedated in the holding tank with 0.5 mg l⁻¹
136 metomidate and left until it was unresponsive to visual stimuli (moving a net repeatedly over
137 the tank). The cod was then removed from the tank with a net and placed in a bath containing
138 70 mg l⁻¹ metacain anaesthetic. When the cod had lost equilibrium and ventilation rate had
139 slowed substantially, it was placed in the same surgery bath used for CA cannulation and the
140 gills were continuously ventilated with aerated water containing a maintenance dose of
141 metacain (15 mg l⁻¹). In the surgery bath, the cod were fitted with a SmartTag (16 mm

142 diameter, 46 mm length, 6 g in water and 15 g in air) on the right side, below the dorsal fin.
143 The tag was fastened to the cod using two steel wires that were first connected to the each end
144 of the tag and then guided through the flesh of the cod using two syringes protruding from the
145 left side. To prevent pressure and friction lesions on the left side, a small rubber sheet was
146 placed on the protruding end of the steel wires before they were fastened together by pliers. A
147 water-filled pressure sensor tube (Tygon[®] T3602-13, Saint-Gobain) was guided from the
148 buccal cavity (directly anterior to the operculum base) to the outside of the fish using a
149 syringe, and then further to a pressure sensor port on the SmartTag. A sleeve had been
150 constructed by heating the buccal end of the pressure sensor tube, which made it fit snugly
151 inside the hole left by the protruding syringe, eliminating the need for sutures. A second
152 water-filled pressure sensor tube was connected to the reference sensor port of the SmartTag
153 and bent backwards so that the open end was not facing the current during swimming. The
154 fish was then transferred to the swim tunnel to recover overnight prior to experimentation.

155

156 *Sample collection, analysis and on-site measurements*

157 Blood samples were collected from each cod through the CA cannula at eleven times during
158 the experiment: at the end of the control treatment directly prior to exposure treatment 1, at
159 the end of exposure treatments 1-4, at the end of the control treatment between exposure
160 treatments 4 and 5, at the end of exposure treatments 5-8 and finally at the control treatment
161 following exposure treatment 8.

162 Sampled arterial blood was immediately analysed for pO₂ with a Microx TX3-trace oxygen
163 transmitter and water temperature equalized needle-type oxygen micro sensor (PreSens
164 Precision Sensing GmbH, Germany). Blood pCO₂ and plasma ions (HCO₃⁻, Na⁺, Cl⁻, K⁺), pH,
165 glucose and urea was immediately analyzed using an i-STAT[®] Portable Clinical Analyzer
166 (Medinor AS, Oslo, Norway). In control treatments, additional blood was immediately
167 centrifuged to determine haematocrit (Compur Microspin; Bayer, Leverkusen, Germany) and
168 at 2000 * g to yield blood plasma for later analysis of plasma cortisol. Plasma samples for
169 analysis of cortisol were immediately frozen either in liquid nitrogen or in a -20 °C freezer
170 and within hours transferred to a -80 °C freezer. Values for pCO₂ and plasma HCO₃⁻ and pH
171 were temperature corrected using formulas supplied by the manufacturer (Abbott Point of
172 Care Inc.; Princeton, NJ, USA).

173 50 µl plasma samples for analysis of cortisol were mixed with five times the sample volume
174 (250 µl) of ethyl acetate using a vortex mixer. The mix was centrifuged for two minutes at
175 7155 * g in 4 °C and the resulting supernatant was stored at -80 °C until analysis. The

176 supernatant was later analyzed for cortisol using a Radioactive Immuno Assay (RIA) kit
177 (Spectria® Cortisol RIA; Orion Diagnostica AS, Asker, Norway) and a NaI-gamma counter
178 (Wizard®; PerkinElmer Norge AS, Oslo, Norway) following the supplied instructions.

179 Ventilation data were derived from the continuous measurements of the differential pressure
180 between the inside and the outside the buccal cavity. The differential pressure was transmitted
181 on-line as frequency-modulated ultrasound, picked up by a hydrophone-receiver system
182 (Thelma Biotel, Trondheim, Norway) and displayed and stored on a computer. These data
183 were later analysed to identify ventilation rate (breaths min^{-1}), and relative measurements of
184 ventilatory stroke volume (integral area breath^{-1}) and ventilatory minute volume (integral area
185 min^{-1}). All ventilation results are calculated as the mean of the last three minutes of each
186 treatment for each fish.

187 Swim tunnel water pH (pH_w) was monitored manually using an indwelling pH meter (WTW
188 340i) and recorded at the time of each blood sampling. The pH_w measurements were used to
189 calculate free (gaseous) CO_2 concentrations in the water at each blood sampling, based on
190 results from water samples sent for free CO_2 analysis. The calculated free CO_2 concentrations
191 (mg l^{-1}) were later used to calculate p_wCO_2 (Calculation 1).

192 Swim tunnel p_wO_2 and temperature was monitored and logged continuously by the swim
193 tunnel control software using indwelling oxygen (Mini-DO galvanic oxygen probe, Loligo
194 Systems ApS, Tjele, Denmark) and temperature sensors (Loliterm Pt100, Loligo Systems
195 ApS, Tjele, Denmark). Determination of oxygen consumption was performed by the swim
196 tunnel software based on the decrease in p_wO_2 measured every second throughout each
197 measure period and taking into account blocking effect (i.e. height and width of each fish and
198 standard teleost formula, see e.g. Steffensen (1984)).

199 Additional water samples were extracted from the swim tunnel using a siphon at the end of
200 three treatments for each individual (one high, medium and control CO_2 treatment per
201 individual). The water samples were analysed for free CO_2 at the accredited laboratory of the
202 Norwegian Institute for Water Research (NIVA) following their standard procedures.

203

204 *Calculations*

205 Calculation 1: Calculation of $p_w\text{CO}_2$ from free CO_2 concentration was performed using the
206 ideal gas law:

207

208 $pV = nRT$

209 $n = \frac{nRT}{V}$

210 Where:

211 p = the absolute pressure of the gas

212 n = the number of moles of the gas

213 R = the gas constant

214 T = the temperature in degrees Kelvin

215 V = the volume of the gas

216

217 Calculation of ventilatory parameters:

218 Ventilation rate (VR) was calculated as the number of ventilation cycles per minute.

219 Ventilatory minute volume (VMV) represents the area between the pressure curve and zero
220 pressure line, and was calculated as the sum of the pressure differences recorded at all single
221 pressure measurements (sampling rate = 20 Hz) in a one minute period. For each individual
222 cod, the mean minute sum integral (3 samples) of each treatment was then divided with the
223 mean minute sum integral from the three control treatments (3 samples from each control
224 treatment) where blood samples were extracted to make it comparable between individuals.
225 VMV is therefore always expressed relative to the mean of the controls.

226 Ventilatory stroke volume (VSV) was calculated and treated as described for VMV above,
227 except the integral sum was divided by VR to give a relative measure of volume per
228 ventilation.

229

230 *Statistical analysis*

231 The data were analysed with SAS v. 9.13 (Statistical Analysis Software), using the mixed
232 model procedure for repeated measures. Oxygen and carbon dioxide levels, and interaction

233 between the two, were used as fixed effects. Individual was included as a random variable
234 (subject identification). Compound symmetry was used as covariance structure for all
235 parameters. Tukey-Kramer adjustment for multiple comparisons were used throughout where
236 applicable and p-values <0.05 considered to be statistically significant. All values are
237 presented as least squares mean (LSmean) \pm standard error (SE) unless stated otherwise. MO_2
238 and plasma Na^+ were only affected by O_2 level (Table 1). Thus, CO_2 level and interaction
239 between O_2 and CO_2 were excluded in the statistical analysis for these parameters. Plasma
240 HCO_3^- and K^+ were only affected by water CO_2 level (Table 1). Thus, O_2 level and interaction
241 between O_2 and CO_2 were excluded in the statistical analysis for these parameters.

242

243

244 Results

245

246 *Effects of varying water oxygen levels*

247 All the ventilation parameters and \dot{V}_E , p_aO_2 , pO_2 difference and plasma Na^+ were affected
248 by p_wO_2 (Table 1). VR decreased with increasing p_wO_2 while no differences were observed
249 with varying p_wO_2 in VSV. VMV increased in hypoxia compared to normoxia and hyperoxia
250 (Fig. 1ABC and Table 2). \dot{V}_E was higher in hyperoxia than in normoxia and hypoxia. The
251 p_aO_2 increased with increasing p_wO_2 levels and pO_2 difference was lower in hypoxia than
252 normoxia. Plasma Na^+ was higher in hypoxia than hyperoxia (Table 3).

253

254 *Effects of varying water carbon dioxide levels*

255 All measured parameters except \dot{V}_E and plasma Na^+ were affected by p_wCO_2 level (Table 1).
256 The ventilation parameters, VR, VSV and VMV, were higher in the two hypercapnic levels
257 than in normocapnia and the VR was also higher in the high compared to the medium
258 hypercapnic level (Fig. 1ABC and Table 2). The p_aO_2 and pO_2 difference differed between
259 normocapnia and both hypercapnic levels which were not different; p_aO_2 was higher and pO_2
260 difference was lower in hypercapnia compared to normocapnia (Table 3). The p_aCO_2 was
261 higher at the high hypercapnic level than in normocapnia and plasma HCO_3^- was higher in
262 both hypercapnic levels than in normocapnia (Table 3). Plasma pH decreased with each
263 hypercapnic level, from 7.912 in normocapnia to 7.621 in the high hypercapnic level (Table
264 3). Plasma glucose was lower in both hypercapnic levels than in normocapnia while plasma
265 K^+ was lower in the high hypercapnic level than in normocapnia (Table 3).

266

267 *Effects of combined hypercapnia and hypoxia or hyperoxia*

268 VR, p_aCO_2 and plasma pH and glucose were affected by interactions between p_wO_2 and
269 p_wCO_2 (Table 1). None of the ventilation parameters (VR included) changed significantly
270 between p_wO_2 levels within p_wCO_2 level in hypercapnia. VR and VMV increased from
271 hypoxia into combined hypoxia and high hypercapnia, and from hyperoxia into combined
272 hyperoxia and high hypercapnia (Table 2). Plasma glucose was higher in the control treatment
273 (3.8 mmol l^{-1}) than any other treatment except hyperoxia (3.5 mmol l^{-1}), which was also
274 higher than medium hypercapnia and combined hyperoxia and medium hypercapnia (both 3.1
275 mmol l^{-1}) (Table 3).

276

277 *Effect of experimentation on ventilation and blood parameters*

278 Plasma cortisol, glucose and Na⁺ were all affected by sampling time. While plasma glucose
279 did not display a clear pattern, plasma Na⁺ and cortisol increased from the start to the end of
280 the experiment. VR, haematocrit and K⁺ did not change with sampling time (Table 4).
281

282 Discussion

283

284 Hypercapnia has been shown to increase both ventilation rate and ventilation amplitude/stroke
285 volume in teleosts (Gilmour, 2001). In line with the review by Gilmour (2001), the Atlantic
286 cod in the present study responded to hypercapnia with increased VR and VSV, yielding a
287 more than doubled VMV from the control into high hypercapnia. Increased ventilation has
288 also been reported in hypoxia (Kinkead et al., 1991), and in the present study ventilation
289 increased in hypoxia (VR and VMV) while it was lowered in hyperoxia (VR) compared to
290 normoxia. While the observed changes in ventilation with p_wO_2 were only evident in
291 normocapnic conditions, VR and VMV were always higher in the high hypercapnic level than
292 in normocapnia when p_wO_2 level was maintained. Thus, at moderate levels, CO_2 seems to be a
293 more potent ventilatory stimulant than O_2 in the Atlantic cod. The ventilatory responses to
294 combined levels of hypercapnia and hyperoxia has been reported to increase ventilation in
295 rainbow trout (Thomas et al., 1983), while the common carp did not display changes in
296 ventilatory responses from hyperoxia into combined hyperoxia and hypercapnia (Soncini and
297 Glass, 2000). The hypoxic and hyperoxic levels used in the present study are less extreme
298 than what is common in previous studies (Gilmour, 2001). The rationale behind this was to
299 investigate how Atlantic cod respond to environmental conditions they are likely to
300 experience in the wild, and in well managed aquaculture situations, where oxygen levels
301 rarely stray outside a range of 70-130% saturation. The less extreme hypoxic and hyperoxic
302 conditions may explain the lack of influence of p_wO_2 on ventilation in hypercapnia; if the
303 p_wO_2 levels had been more extreme, differences likely would have appeared between
304 treatments.

305 The p_aO_2 of Atlantic cod has recently been reported to be heavily dependent on p_wO_2
306 (Karlsson et al., 2010b), corresponding well with the results in the present study, where p_aO_2
307 always increased with p_wO_2 within hypercapnic level. In the present study, a higher p_aO_2 was
308 observed in hypoxia than in combined hypoxia and hypercapnia, and in hypercapnia alone
309 p_aO_2 was higher and pO_2 difference was lower than in the control. These changes were most
310 likely caused by the increased ventilation observed in hypercapnia, and further support the
311 conclusion that, at moderate levels, CO_2 is a stronger ventilatory stimulant in the Atlantic cod
312 than O_2 .

313 Maintenance of oxygen consumption through increased ventilation is a common response in
314 teleosts exposed to hypoxia (Randall, 1982) above their critical oxygen level (Plante et al.,
315 1998) and was also observed in the present study. The effect of hyperoxia on oxygen

316 consumption of rainbow trout was investigated by Thomas et al. (1983), and like in the
317 present study on Atlantic cod, they reported increased oxygen consumption in hyperoxia
318 compared to normoxia. The increased MO_2 observed in hyperoxia in the present study may
319 have been an effect of increased metabolic load in detoxification of free radicals induced by
320 hyperoxia, common in exposure to other inducers of oxidative stress (Walker et al., 2001).
321 Thomas et al. (1983) also reported on the effect of hypercapnia on oxygen consumption, and
322 again their findings were similar the ones in the present study; there is no effect of
323 hypercapnia on MO_2 .

324 Atlantic cod ventilation has been reported to increase in hypoxia (Kinkead et al., 1991). In
325 turn, increased ventilation is known to increase removal of CO_2 from blood, and consequently
326 decrease p_aCO_2 , in rainbow trout (Gilmour and Perry, 1994). Removing CO_2 from blood by
327 increased ventilation causes net loss of H^+ ions in Atlantic cod, and consequently causes pH
328 to increase (Kinkead et al., 1991). The opposite effects were found when rainbow trout were
329 exposed to hyperoxia (Gilmour and Perry, 1994). In the present study, none of the known
330 effects of hypoxia or hyperoxia on acid-base parameters occurred, possibly due to the less
331 extreme hypoxic and hyperoxic levels used in the present study compared to previous studies
332 (see the review by Gilmour (2001) for examples of common hypoxic and hyperoxic levels).
333 However, a recent study using Atlantic cod reported increased levels of p_aCO_2 and plasma
334 HCO_3^- even at a hyperoxic level identical to the one used in the present study (Karlsson et al.,
335 2010b), albeit with much longer exposure time (24-48 hrs) than what was used in the present
336 study (14 min). In the study by Karlsson et al. (2010b), the longer exposure time likely
337 allowed p_aCO_2 to build up over time due to reduced ventilation in hyperoxia. In the present
338 study, the selected hypoxic and hyperoxic levels were likely not extreme enough with the
339 present time frame to elicit changes in blood acid base status. Hypercapnia at a similar level
340 (0.67 kPa) as used in the present study (0.70 kPa) is known to cause increased p_aCO_2 and
341 decreased pH in rainbow trout (Gilmour and Perry, 1994). The same responses were observed
342 in the present study in both hypercapnic levels for pH and at the high hypercapnic level for
343 p_aCO_2 . Hypercapnia at a similar level (0.67 kPa) as used in the present study (0.70 kPa) was
344 reported to initially increase (5-10 min after start of exposure), then decrease (15 min) and
345 finally increase (20 min to 30 hrs) blood $HCO_3^- + CO_3^{2-}$ in rainbow trout (Thomas and Leruz,
346 1982). The small decrease in blood $HCO_3^- + CO_3^{2-}$ reported by Thomas and Leruz (1982)
347 occurred at a time (15 min) which was practically identical to our exposure time (14 min), and
348 at the same time as their lowest observed blood pH value. The 15 min time point was
349 suggested by Thomas and Leruz (1982) to be the onset of blood pH compensatory

350 mechanisms in response to hypercapnia, and is followed by a large increase (more than
351 doubling) in $\text{HCO}_3^- + \text{CO}_3^{2-}$ and a rise in pH of roughly 0.3 units over the course of the next
352 30 hours. In the present study, both hypercapnic levels lowered plasma HCO_3^- slightly (from
353 7.1 mmol l^{-1} in normocapnia to 5.9 mmol l^{-1} at medium hypercapnia and 5.6 mmol l^{-1} at high
354 hypercapnia). Thus, according to the observations of Thomas and Leruz (1982), the exposure
355 time used in the present study was likely to short to initiate pH compensatory mechanisms and
356 explains why no increase in plasma HCO_3^- was observed in hypercapnia.

357 Plasma glucose levels were higher at the control level than all other treatments except
358 hyperoxia. Although the magnitude of this difference was small ($<1 \text{ mmol l}^{-1}$ change), the
359 effect is opposite of what could be expected if the cod had been stressed as a result of the
360 hypercapnic/hyperoxic treatments; in that case an increase in blood glucose would be more in
361 line with previous studies (Eliason et al., 2007; Olsen et al., 2008; Djordjevic et al., 2010;
362 Karlsson et al., 2010a).

363 Plasma potassium decreased by ca 25% from control to the high CO_2 levels, indicating a
364 hypercapnia-activated K^+ uptake from plasma to tissues or erythrocytes. Hypoxia has
365 previously been shown to induce stimulation of Na^+/H^+ exchangers in the erythrocytes (Fievet
366 et al., 1988), causing plasma Na^+ and pH to drop. This response to hypoxia was not observed
367 in the present study. Although small (1% change), the opposite response was observed;
368 plasma Na^+ was higher in hypoxia than in hyperoxia.

369 Plasma cortisol and Na^+ increased, while plasma glucose fluctuated, throughout the
370 experiment, indicating an increase in the stress level of the cod. The differences in plasma
371 glucose and Na^+ between control treatments were small, and no clear pattern was observed for
372 glucose; these differences likely had no practical importance. The increase in plasma cortisol
373 was also rather small compared to the initial level, which must be said to be in the high range
374 for resting Atlantic cod. The initial high plasma cortisol value was likely an unintended effect
375 of the metomidate sedation prior to the SmartTag attachment procedure. The sedation
376 procedure was incorporated based on experiences with Atlantic salmon in order to reduce post
377 surgery stress (Djordjevic et al., 2010). However, a recent study reported elevated cortisol
378 levels for a longer time post surgery in sedated cod compared to unsedated cod (Karlsson et
379 al., 2010a), with peak cortisol levels 24 hours post surgery. Since experimentation was always
380 initiated ca 16 hours after attaching the tags in the present study, the experiment initiation and
381 peak cortisol levels post surgery likely coincided reasonably well, explaining the elevated
382 cortisol levels. Therefore, the initially elevated cortisol level was considered to be a delayed

383 effect of the pre-experiment sedation, anaesthesia and surgical procedure rather than an effect
384 of the experimental conditions such as water quality or the swim tunnel itself.
385 In conclusion, hypercapnia is a more potent stimulant for ventilatory drive than hypoxia or
386 hyperoxia in the Atlantic cod, and Atlantic cod do not maintain the pO_2 difference observed in
387 the control treatment in either separate or combined hypercapnia or hypoxia, effectively
388 increasing p_aO_2 above normocapnic levels.
389

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391

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393 project PROCOD (NFR project number 172263/S40).

394

395

396

397 List of abbreviations
398
399 CA, caudal artery
400 CV, caudal vein
401 DA, dorsal aorta
402 p_wO_2 , partial pressure of O_2 in water
403 p_aO_2 , partial pressure of O_2 in arterial blood
404 p_wCO_2 , partial pressure of CO_2 in water
405 p_aCO_2 , partial pressure of CO_2 in arterial blood
406 pH_w , pH in water
407 pH_a , pH in arterial blood
408 MO_2 , O_2 consumption, expressed as $mg\ O_2\ kg^{-1}\ hr^{-1}$
409 VR, Ventilation Rate
410 SV, Ventilatory Stroke Volume, area of integral below ventilation curve relative to mean of
411 control treatments (n=3) per ventilation, expressed as % of control
412 MV, Ventilatory Minute Volume, area of integral below ventilation curve relative to mean of
413 control treatments (n=3) per minute, expressed as % of control
414 SD, standard deviation
415 SE, standard error
416 LSmean, least squares mean
417
418

- 419
420
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478

479 Figure and table captions

480

481 Figure 1: Ventilation rate (A), ventilatory stroke volume (B) and ventilatory minute volume
482 (C) of Atlantic cod exposed to nine combinations of CO₂ and O₂ levels in water. The
483 combination p_wCO₂ = 0.15 and p_wO₂ = 20.7 kPa acted as control. Ventilation rate is presented
484 as LSmeans. Ventilatory stroke volume and minute volume are expressed as LSmeans relative
485 to the mean of control treatments for each individual (% of control). Concomitant SE and
486 difference between treatments are presented in table 2.

487

488

489 Table 1: p-values from tests of effects of water O₂ and CO₂ on parameters

490

491

492 Table 2: Atlantic cod ventilation by CO₂ and O₂ exposure levels

493

494 Table 2 legend:

495 * indicates control treatment. Numbers are SE, concomitant LSmeans are presented in Fig. 1.
496 Different letters denote significant difference between treatments within parameter at p<0.05.
497 The p_wCO₂ and p_wO₂ are expressed as kPa. Ventilation rate is expressed as ventilations min⁻¹.
498 Ventilatory stroke volume is expressed as volume ventilation⁻¹ (% of control). Ventilatory
499 minute volume is expressed as ventilated volume min⁻¹ (% of control).

500

501

502 Table 3: Atlantic cod oxygen consumption and blood parameters by CO₂ and O₂ exposure
503 levels

504

505 Table 3 legend:

506 * indicates control treatment. All gas tensions and pO₂ difference are displayed as kPa.
507 Glucose and ions are expressed as mmol l⁻¹. MO₂ is expressed as mg O₂ kg⁻¹ hr⁻¹. Missing
508 values in hypercapnia indicate that hypercapnia did not affect parameter. Missing values in
509 hypoxia and hyperoxia indicate that water oxygen level did not affect parameter; see Table 1.
510 Different letters denote significant difference between treatments within parameter at p<0.05.

511

512

513 Table 4: Stress parameters in Atlantic cod pre, mid and post experimentation

514

515 Table 4 legend:

516 *denotes significant effect of sampling time on parameter. Different letters denote significant
517 difference between sampling times. Plasma cortisol is expressed as ng ml^{-1} . Plasma glucose,

518 K^+ and Na^+ are expressed as mmol l^{-1} . Blood haematocrit is expressed as volume %.

519 Ventilation rate is expressed as ventilations min^{-1} .

520

Figure 1

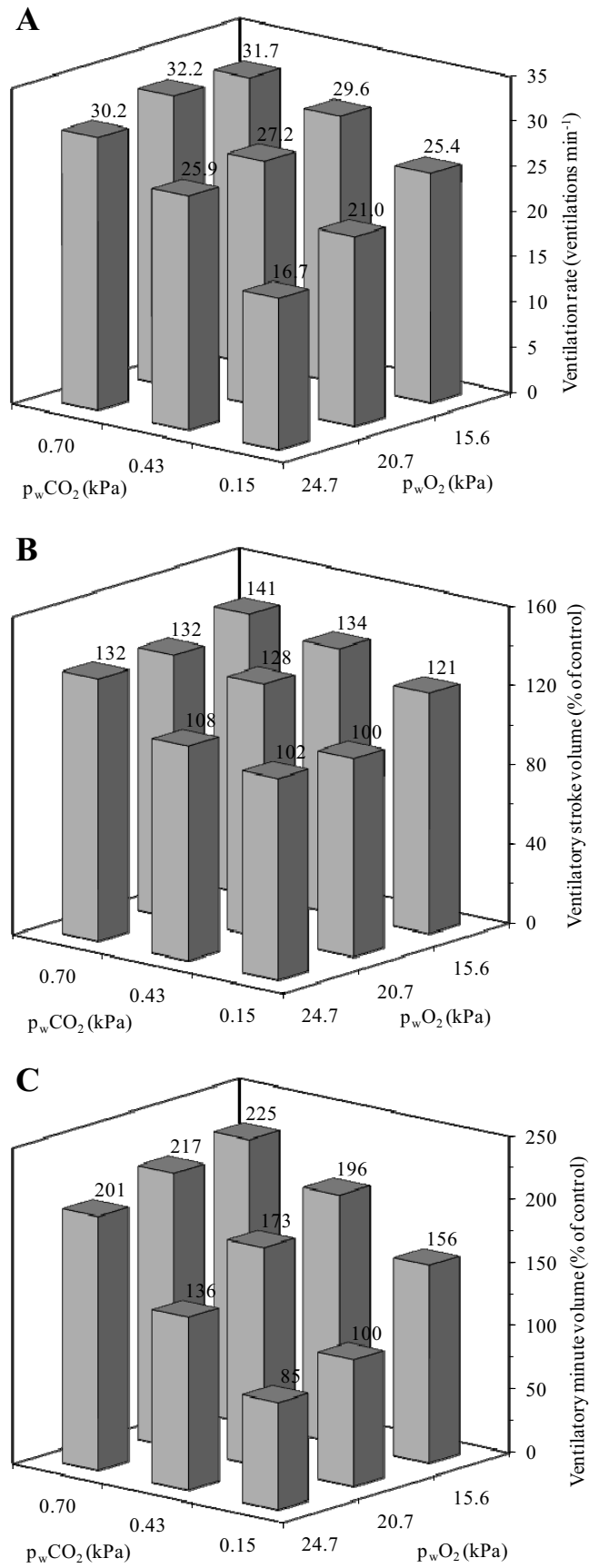


Table 1

	O ₂	CO ₂	O ₂ -CO ₂ interaction
Ventilation rate	0.0001	<0.0001	0.0112
Ventilation volume	0.0249	0.0006	0.4322
Ventilated volume	0.0011	<0.0001	0.2617
MO ₂	0.0075	0.3694	0.3920
p _a O ₂	<0.0001	<0.0001	0.1492
pO ₂ difference	0.0057	<0.0001	0.2166
p _a CO ₂	0.7619	<0.0001	0.0020
Plasma HCO ₃ ⁻	0.4372	<0.0001	0.4354
Plasma pH	0.4719	<0.0001	0.0086
Plasma glucose	0.0695	0.0003	0.0004
Plasma Na ⁺	0.0332	0.4208	0.8860
Plasma K ⁺	0.5577	0.0183	0.5287

Table 2

$p_w\text{CO}_2$	$p_w\text{O}_2$	Ventilation rate	Ventilatory stroke volume	Ventilatory minute volume
0.15	15.6	2 C	8 AB	16 CD
0.15*	20.7*	1 B*	6 A*	12 A*
0.15	24.7	2 A	8 AC	16 A
0.43	15.6	2 CD	8 BC	16 BCD
0.43	20.7	2 C	8 BC	16 BCD
0.43	24.7	2 C	8 AB	16 AD
0.70	15.6	2 D	8 B	16 B
0.70	20.7	2 D	8 BC	16 BC
0.70	24.7	2 D	8 BC	16 BC

Table 3

p _w CO ₂	p _w O ₂	MO ₂	p _a O ₂	pO ₂ difference	p _a CO ₂	HCO ₃ ⁻	pH	K ⁺	Na ⁺	Glucose
0.15	15.6	35.6 ± 3.7 A	11.8 ± 0.5 A	3.7 ± 0.5 BC	0.43 ± 0.02 A		7.937 ± 0.017 A		157.2 ± 0.7 A	3.2 ± 0.4 BC
0.15*	20.7*	36.1 ± 3.6 A*	15.1 ± 0.4 C*	5.6 ± 0.4 A*	0.49 ± 0.01 AB*	7.1 ± 0.2 A*	7.912 ± 0.012 A*	2.8 ± 0.1 A*	156.6 ± 0.7 AB*	3.8 ± 0.4 A*
0.15	24.7	46.0 ± 3.7 B	20.6 ± 0.5 E	4.4 ± 0.5 AB	0.50 ± 0.02 AB		7.879 ± 0.017 A		155.7 ± 0.7 B	3.5 ± 0.4 AB
0.43	15.6		13.5 ± 0.5 AB	2.2 ± 0.5 C	0.54 ± 0.02 B		7.768 ± 0.017 B			3.2 ± 0.4 BC
0.43	20.7		17.8 ± 0.5 D	2.9 ± 0.5 BC	0.53 ± 0.02 B	5.9 ± 0.2 B	7.788 ± 0.017 B	2.3 ± 0.2 AB		3.1 ± 0.4 C
0.43	24.7		21.3 ± 0.5 E	3.4 ± 0.5 BC	0.54 ± 0.02 B		7.769 ± 0.017 B			3.1 ± 0.4 C
0.70	15.6		13.8 ± 0.5 BC	1.8 ± 0.5 C	0.79 ± 0.02 C		7.579 ± 0.017 C			3.3 ± 0.4 BC
0.70	20.7		17.8 ± 0.5 D	2.9 ± 0.5 BC	0.71 ± 0.02 C	5.6 ± 0.2 B	7.621 ± 0.017 C	2.1 ± 0.2 B		3.3 ± 0.4 BC
0.70	24.7		22.2 ± 0.5 E	2.1 ± 0.5 C	0.72 ± 0.02 C		7.637 ± 0.017 C			3.2 ± 0.4 BC

Table 4

	Pre experiment	Mid experiment	Post experiment
Plasma cortisol*	86.6 ± 15.8 A	92.8 ± 15.8 AB	117.3 ± 15.8 B
Plasma glucose*	3.9 ± 0.5 AB	3.7 ± 0.5 A	4.0 ± 0.5 B
Plasma Na ⁺ *	156 ± 1 A	157 ± 1 AB	158 ± 1 B
Plasma K ⁺	2.48 ± 0.10 A	2.74 ± 0.10 A	2.77 ± 0.10 A
Blood haematocrit	15.1 ± 0.6 A	15.4 ± 0.6 A	14.9 ± 0.6 A
Ventilation rate	21.7 ± 2.1 A	19.7 ± 2.1 A	21.5 ± 2.1 A