ESTIMATING AEROBIC AND ANAEROBIC CAPACITIES USING THE RESPIRATORY ASSESSMENT PARADIGM: A VALIDATION USING ATLANTIC SALMON (*SALMO SALAR*) AND EUROPEAN SEA BASS (*DICENTRARCHUS LABRAX*)

by

Yangfan Zhang

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Abstract

Assessing fish performance, whether for extrapolation to natural conditions, culture conditions or toxicology, has a long history of using swim performance, metabolic rates and performance in hypoxia. However, rarely are these metrics unified in a test. Hence, thesis' objective was to test a respiratory assessment paradigm (RAP), which comprehensively evaluated aerobic and anaerobic metabolism by explicitly measuring aerobic capacity, recovery and performance in hypoxia. To provide high fish throughput with each test, RAP used multiple intermittent-flow respirometry systems. I tested RAP by examining two practical questions: do aquaculture practices compromise the respiratory robustness of domesticated Atlantic salmon (*Salmo salar*) and, if so, how might it be reversed? Second, does an acute sub-lethal exposure to chemically dispersed oil have a chronic residual respiratory effect on European sea bass (*Dicentrarchus labrax*).

RAP revealed a domesticated (*Bolaks*) strain of Atlantic salmon had a lower aerobic capacity than a wild (*Lærdal*) strain. Incremental exercise training significantly increased aerobic and recovery capacities in only the *Lærdal* strain. Thus, the *Bolaks* strain appeared to be athletically less robust than *Lærdal* strain. As a conclusion, ten generations of growth-oriented breeding program of *Bolaks* strain in commercial aquaculture have reduced its athletic robustness and cardiorespiratory plasticity as compared to their wild conspecifics. Given the success in improving athletic robustness of the wild strain, it still remains to explore whether an exercise-training protocol can be developed that will provide benefits to the salmon aquaculture industry.

RAP discovered no residual effect of oil on aerobic capacity for either hypoxia tolerant (HT) or hypoxia sensitive (HS) sea bass. Instead, RAP discovered a residual effect for only the

HT phenotype on its ability to survive in hypoxia, which was evidenced by an impaired tolerance to hypoxia and an increased oxygen deficit in hypoxia. Therefore, RAP identified a chronic impairment from acute exposure to oil to the hypoxia-tolerant segment of the sea bass population, perhaps increasing their risk of perishing in hypoxic episodes.

These successful applications illustrate that RAP is a reliable methodology that could find further application to comparative studies of thermal, hypoxic and anthropogenic effects on the ecological performances of fishes.

Preface

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List of Abbreviations

AAS	absolute aerobic scope
AMR	active metabolic rate
AMS	active metabolic scope
AOD	accumulated oxygen deficit
ATP	adenosine triphosphate
CS	citrate synthase
DO	dissolved oxygen
EPHOC	excess post-hypoxia oxygen consumption
EPOC	excess post-exercise oxygen consumption
FAO	Food and Agriculture Organization of the United Nations
FAS	factorial aerobic scope
FL s^{-1}	fork length per second
Hb	hemoglobin
HCT	hypoxia challenge test
HS	hypoxia sensitive
HT	hypoxia tolerant
ICES	International Council for the Exploration of the Sea
Ifremer	Institut Français de Recherche pour l'Exploitation de la Mer
ILOS	incipient lethal oxygen saturation
LDH	lactate dehydrogenase
$\dot{M}O_2$	rate of oxygen uptake
$\dot{M}O_{2max}$	maximum rate of oxygen uptake

MS	metabolic scope
MS-222	tricaine methanesulfonate
O_{2crit}	critical oxygen level
OCLTT	oxygen- and capacity-limited thermal tolerance
PAH	polycyclic aromatic hydrocarbons
FSOD	factorial scope for oxygen deficit
SMR	standard metabolic rate
SOD	scope for oxygen deficit
U_{burst}	burst swimming speed
U_{crit}	critical swimming speed in a prolonged swimming test
U_{max}	maximum swimming speed in an incremental acceleration swimming test

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Dedication

To my mother and father.

For showing me courage, patience and wisdom

&

Always seeing my potential at times perhaps others could not

Chapter 1: INTRODUCTION

Anthropocene is the current time period, when the rate of mass extinction of plant and animal species due to humans is moving at a pace faster than any era since Cretaceous–Paleogene extinction (Crutzen and Steffen, 2003). One major contributor to this concern is the pollution of the aquatic environment, which is often the final resting place for most toxicants. Beyond the thousands of synthetic toxicants that aquatic biota have never experienced before, rapidly expanding human population has introduced increasing levels of human waste into the aquatic environment and this has resulted in aquatic eutrophication and hypoxia. Eutrophic and hypoxic dead zones are increasing in size and number, particularly in coastal environments (Diaz and Rosenberg, 2008; Breitburg et al., 2009). The 7.4 billion humans also need food security. Because humans have over-exploited wild fish stocks and disrupted their aquatic habitats, a growing aquaculture industry has domesticated wild stocks and commercial aquaculture now supplies about half of the seafood eaten each year by humans (Bergheim et al., 2009; FAO, 2014).

My thesis considers how best to assess the impacts of oil spills and aquaculture on two important fish species Atlantic salmon (*Salmo salar*) and European sea bass (*Dicentrarchus labrax*). Atlantic salmon and European sea bass are iconic model species for both aquaculture and conservation in Northern America and Western Europe (ICES, 2000, Kousoulaki et al., 2015). World-wide commercial Atlantic salmon aquaculture has replaced wild capture and provides a year round supply of protein-rich food to a large portion of the western world (Naylor et al., 2009; Lefevre et al., 2014; FAO, 2014). Sea bass are similarly cultured in Europe, but their wild stocks are more abundant, yet under pressure (Kousoulaki et al., 2015). For example, sea

bass off Northwest France were threated when the crude oil tanker Erika sank in 1999 (Claireaux et al., 2004). A fast growth rate is a highly desirable trait for aquaculture. It ensures a rapid turnover rate of stocks and capital (Gjedrem, 2012). In contrast, fish ecotoxicology is concerned with how a fish prioritizes its survival when exposed to an anthropogenic challenge (Claireaux et al., 2004). Common to both fields is metabolic capability, which is critical for both growth and reproduction, and for dealing with toxicants (Pörtner et al., 2014). Therefore, both aquaculture professionals and toxicological conservationists are interested in how fish utilize their metabolic scope to survive and support life's activities.

The goal of my thesis is to test a unified suite of respiratory metabolic indices that will quantify both aerobic and anaerobic metabolic capacities within one experimental protocol, which I coin as respiratory assessment paradigm (RAP). Whole-animal respiratory robustness is defined by the effectiveness of oxygen transport cascade, which is a vertical integration of ventilation of respiratory surface, diffusion of oxygen across the respiratory-exchange surface, circulatory oxygen delivery, tissue oxygen diffusion and tissue oxygen utilization. RAP measures whole-animal respiratory robustness with respirometry as the aerobic capacity for sustained locomotion, the ability to tolerate hypoxia and temporarily support metabolic demands with anaerobic metabolism, as well as the ability to recover from oxygen deficit in both circulatory and muscle tissue levels. Using RAP, I investigate two practical questions. First, do aquaculture practices compromise the respiratory robustness of domesticated Atlantic salmon and, if so, how might it be improved with exercise training? Second, does an acute oil exposure have long-term residual effects on respiratory robustness of European sea bass?

1.1 The respiratory assessment paradigm (RAP)

Conservation physiology in fish is an emerging field that has been using the long-standing hypothesis that respiratory physiology and bioenergetics have a major influence on their ecological performance and biogeography (Fry, 1971). Some studies have measured aerobic scope to make ecological predictions that are embedded for fishery regulations of Pacific salmon *Oncorhynchus* spp. (see Eliason and Farrell, 2016). Other studies have linked high metabolic phenotypes to dominance, risk taking, and rapid growth rate that might be favoured in resource-abundant conditions but prone to high mortality in resource-scarce conditions (Metcalfe et al., 2016). Ultimately, a bioenergetics model that includes variation in metabolic phenotypes with environmental factors can increase the accuracy of energy budget estimation and thereby enhance the transfer of ecophysiological studies into policy making (Enders and Boisclair, 2016; Jørgensen et al., 2016). To reach this goal, a standard respirometry experimental protocol must be available to generate accurate metabolic data.

A simple philosophy underpins my thesis: develop effective methodological measures of a fish's performance on a scale and accuracy so that scientists will have proper tools to address challenges involved with the rapid rate of extinction in anthropocene. Traditional methodologies include critical swimming speed (U_{crit}) (Blazka, 1960; Brett, 1964, 1967), burst swimming speed (U_{burst}), ramp-U_{crit} (Jain et al., 1997) and maximum swimming speed (U_{max}) (Reidy et al., 1995; Farrell, 2008), which are integrative measures that consider either aerobic metabolism, anaerobic metabolism or an amalgamation of both. During aerobic swimming or routine activity, the respiratory system continuously supplies oxygen for mitochondria to produce ATP (Richards et al., 2002). Conversely, after burst-and-coast swimming or a bout of hypoxemia, the cost of

anaerobic metabolism must be repaid by consuming oxygen to replenish depleted oxygen stores, as well as to restore the biochemical balance of metabolites and organic phosphates (Gaesser and Brooks, 1984; Wood, 1991; Ejbye-Ernst et al., 2016). From this perspective, oxygen is a valuable and central currency that can now be easily and continuously measured in the aquatic environment using newly developed fiber optic oxygen probes in respirometry, which is a much easier methodology to quantify aerobic scope than a half of a century ago (Brett, 1964; Svendsen et al., 2016).

Fish's ability to survive in hypoxia is predominately relied on compensatory mechanisms and anaerobic metabolism. I explicitly quantify hypoxia tolerance using the critical oxygen level (O_{2crit}) as measured with respirometry, and the incipient lethal oxygen saturation (ILOS; the oxygen saturation when a fish loses its equilibrium) to quantify hypoxia resistance as measured with either respirometry or a hypoxia challenge test (HCT) (Farrell and Richards, 2009; Claireaux et al., 2013). Ideally, oxygen level at which a fish transits from an oxygen regulator to an oxygen conformer is O_{2crit} (Prosser, 1973). Fish, however, rarely show a complete regulation or conformity but exhibit a hyperbolic response of $\dot{M}O_2$ to decreasing dissolved oxygen (DO) (Mueller and Seymour, 2011). Thus I adapt a more recent definition that O_{2crit} pinpoints where oxygen shifts from a limiting to a lethal factor, i.e., standard $\dot{M}O_2$ can no longer be maintained and $\dot{M}O_2$ becomes dependent on a decreasing DO concentration (Claireaux and Chabot, 2016). Thus, below O_{2crit}, the cardiorespiratory system can no longer sustain SMR and anaerobic glycolysis must increasingly support energy demands, despite metabolic depression can present as the fish enter hypoxemia (Wang et al., 1994; Speers-Roesch et al., 2012). ILOS occurs below O_{2crit} when a fish can no longer maintain it righting reflex and rolls over. ILOS can be measured in a time- or a DO-dependent manner, because lactate are being accumulated and glycogen stores

depleted in time- and DO-dependent manners (Nilsson and Östlund-Nilsson 2008; Mandic et al., 2013). Although O_{2crit} and ILOS have been adapted to assess functional integrity in oil exposure and aquaculture (Claireaux et al., 2004; McKenzie et al., 2007a), I further refine 'scope for survival' to describe the physiological processes that make a fish resistant to hypoxia between O_{2crit} and ILOS (Hochachka, 1990; Claireaux and Chabot, 2016). The compensatory mechanisms include metabolic depression (Hochachka and Guppy, 1987; Nilsson and Renshaw, 2004), hypoxic bradycardia (Farrell, 2007), increases in hemoglobin (Hb)-oxygen binding affinity and Hb concentration (Farrell and Richards, 2009; Speers-Roesch et al., 2012), as well as regression of the interlamellar cell mass in the gill (Sollid et al., 2003; McBryan et al., 2016) and the capacity to use anaerobic metabolic fuels (Richards, 2009, 2011). This collectively forms an anaerobic scope.

With the establishment of low-cost and high-throughput intermittent-flow respirometry systems (Svendsen et al., 2016), I see a great opportunity to build a coherent framework using the respirometry metabolic indices that were rarely converged by the comparative physiologists. To describe a true metabolic scope, measurements of aerobic capacity will be combined with the measurements of capability to recover from exhaustion and the capacity to survive hypoxia. An automated, multi-channel fish respirometry system will simultaneously monitor $\dot{M}O_2$ in 8 fish every 3 minutes for a minimum of 3 days, during which the fish become quiescent and SMR can be accurately measured (Chabot et al., 2016). However, instead of swimming fish to exhaustion to determine the maximum rate of oxygen uptake ($\dot{M}O_{2max}$), fish are chased to exhaustion before being placed in the respirometer to measure it (Norin and Clark, 2016), a technique that can also measure excess post-exercise oxygen consumption (EPOC) as the fish recovers from exhaustion. This approach, therefore, provides reliable data to derive absolute and factorial aerobic scope

(AAS and FAS). Immediately after the period used to measure SMR, a hypoxia challenge experiment can be conducted to measure O_{2crit} and ILOS (Fry, 1971; Ultsch et al., 1978; Claireaux et al., 2013). From O_{2crit} and ILOS, I derived three new indices, the scope for oxygen deficit (SOD), the factorial scope for oxygen deficit (FSOD) and the accumulated oxygen deficit (AOD) to assess 'scope for survival' (Hochachka, 1990; Claireaux and Chabot, 2016). Altogether, I have coined this measurement framework as RAP, an estimation of the true metabolic scope by assessing capacity and performance of both aerobic and anaerobic metabolism using $\dot{M}O_2$ as the currency. RAP, in a broader sense, will help describe how the environment influences energy allocation for life-support activities (Figure 1.1; Kerr, 1990; Neill et al., 1994; Lefrançois and Claireaux, 2003; Claireaux and Lefrançois, 2007).

To understand the generality of RAP, I will validate my methodological approach using two different conditions and fish species. An aquaculture hatchery represents a condition where fish growth is optimized, whereas survival is prioritized during oil contamination. Each of these applications is now introduced.

1.2 An application to aquaculture

Commercial aquaculture, since its inception in the late 1970s, is playing an ever increasing role in food security by supplying a regular source of high quality of protein for a growing human population (Gjedrem and Baranski, 2009). Global aquaculture production has been steadily increasing because of an increased per capita consumption of fish. In contrast, the global wild fishery capture has plateaued since the late 1980s. In 2009, aquaculture reached a milestone of producing 50% of human consumed finfish and shellfish (FAO, 2014). Besides

human nutrition, wild fish populations are essential for ecological balance and are involved in recreational activities and cultural values. Thus, aquaculturists hold the notion that providing a sustainable fish source for human diet through aquaculture is an effective approach to protect wild fish populations (Gjedrem, 2012).

Commercial aquaculture, however, still strives to enhance the efficiency of its production procedure and the welfare of its domesticated Atlantic salmon. For instance, transfer of Atlantic salmon smolts to seawater is a routine operational procedure in salmon aquaculture (Specker and Schreck, 1980; Schreck et al., 1989; Gjedrem and Baranski, 2009), yet concerns remain about the practice because of the alarming 15–20% smolt mortality that occurs in the first 90 days of oceanic grow-out (Eggset et al., 1997; Kristensen et al., 2012b). If these lost smolts had survived to market size (e.g. 5 kg), the resulting 170,805 tonnes of salmon (15% of 1,138,700 tonnes annual productions in 2011; FAO, 2014) would translate to a financial gain of at least CAN$1.57 billion (priced at CAN $9.18 / kg, April 2016; Indexmundi.com). Thus, the industry has great interest in finding a solution for a better survivorship during seawater transfer, and I propose that an enhanced respiratory robustness can be the solution, as reasoned below.

The aquaculture industry has selectively bred farmed Atlantic salmon for a variety of the economically important traits, including rapid growth rate, body weight at harvest and age at sexual maturation (Gjedrem, 2012). However, these selective breeding strategies, as well as the sheltered aquaculture environment with unlimited feed and the absence of predatory threats, potentially trade off physiological integrity, developmental stability and immune responses to a stressful environment (Mercier et al., 2001; McKenzie et al., 2007a). In fact, natural selection leads to an optimum rather than the absolute maximum growth rate in order to have the functional integrity (Sundström et al., 2005; Arnott et al., 2006; Arendt, 1997). For instance, a

high-latitude strain of Atlantic silverside (*Menidia menidia*) with a fast growth rate had inferior swimming performance compared with the low-latitude strain with a slower growth rate, suggesting a trade-off between respiratory performance and growth (Billerbeck et al., 2000, 2001). Aquaculture breeding programs similarly select individuals with rapid growth rate that have a more active growth hormone-insulin-like growth factor I axis of endocrine growth regulation, but these fish show a suppressed immunity (Fleming et al., 2002; Krasnov et al., 2016).

Aquaculture settings also fail to provoke the development of superior swimming performance at the pre-smolt stages (Vincent, 1960; Anttila et al., 2014a). In contrast, long upstream migrations and negotiating rapid water currents stimulate a natural athleticism among wild salmonids (Hayashida et al., 2013; Eliason et al., 2011, 2013). In fact, impaired swimming stamina of domesticated salmonids has been documented since the 1950s (Reimers, 1956; Green, 1964; Bams, 1967; Duthie, 1987; Brauner, 1994; MacDonald et al., 1998). Cultured salmonids also characteristically have a higher incidence of cardiac aberrations. This has been associated with reduced cardiac pumping and oxygen delivery capacities that affect respiratory capacity, resulting in a compromised swimming performance (Gamperl and Farrell, 2004; Claireaux et al., 2005). Thus, I reasoned that the protected rearing environment allow the domesticated Atlantic salmon with cardiac impairments and reduced respiratory performance reach parr stage (Mercier et al., 2001), but these domesticated smolts become more vulnerable with the higher $\dot{M}O_2$ demand induced by handling and osmotic stress associated with seawater transfer (Wagner et al., 2006).

Therefore, my primary objective was to use RAP to characterize and compare the aerobic capacity and hypoxia tolerance of Norwegian domesticated (*Bolaks*) and wild (*Lædal*) strains of

Atlantic salmon held in a common garden rearing condition. I also explored the possibility of employing an exercise-training regime to enhance the respiratory robustness in an aquaculture setting with the aim of reducing the smolt mortality post-transfer. In order to stimulate swimming in the hatchery, I used an 18-day incremental exercise-training regime (water velocity of 2.0–2.8 FL s^{-1}) in an industry-scale swimming flume in which 160 parr (34 g, 14 cm each) could be trained at a time. This is because a proper exercise regime can strengthen the cardiovascular system (Farrell et al., 1990) and skeletal muscle (Hochachka, 1961), as well as enhance osmoregulation in seawater (Jørgensen and Jobling, 1993; Gallaugher et al., 2001) and swimming performance (Totland et al., 1987; Young and Cech, 1993, 1994), in addition to the improved feed conversion efficiency and somatic growth that are favorited by aquaculture industry (Davison, 1997; Kieffer, 2010).

1.3 An application to toxicology

Severe oil spills that contaminate marine coastal ecosystems have become a regular occurrence since the *Torrey Canyon* shipwreck off southwest United Kingdom in the 1960s (Petrow, 1968; Southward and Southward, 1978). Among the most catastrophic to local ecosystems include the *Exxon Valdez* in Alaska 1989 (Marty et al., 1997), *Erika* in western France 1999 (Claireaux et al., 2004), *Prestige* in Spain 2002 (González et al., 2006) and *Deepwater Horizon* in the Gulf of Mexico 2010 (US Coast Guard, 2011). Beyond the catastrophic physical effects of oil sedimentation and a surface oil slick, polycyclic aromatic hydrocarbons (PAH) are of great concern to aquatic biota because they are the water-soluble fraction of petroleum hydrocarbons and are more bioavailable to marine biota (Moles et al.,

1994; Moles et al., 1998; Claireaux et al., 2004). Moreover, they have acute and carcinogenic properties (Neff and Anderson, 1981). While chemical dispersants reduce the direct physical effects of an oil spill, bioavailability of the petroleum compounds to fishes increases by dissolving and dispersing a larger portion of crude oil into water-soluble PAH (Chapman et al., 2007).

Crude oil contamination commonly occurs in marine coastal zones and estuaries, which interact with regional aquatic hypoxia episodes and propose a large threat on the survival of residential fishes (Diaz and Rosenberg, 2008; Negreiros et al., 2011). The catastrophic effects are firstly induced by the surface oil slick that physically blocks the air-water interface, which reduces the oxygenation of the water underneath and forms a temporary hypoxic bloom (Malan 1986). Low dissolved oxygen (DO) slows oxygen diffusion across gill epithelia and produces arterial hypoxemia, which triggers a series of compensatory mechanisms to enhance fish's ability of acquiring oxygen from environment and deliver to tissues. These include increases in gill ventilation, gill perfusion, cardiac output, blood Hb concentration and tissue oxygen extraction (Farrell and Richards, 2009). However, elevated ventilation rates increase the exposure to and uptake rate of PAH, increasing the chance of contaminating the cardiorespiratory system (Whitehead, 2013). Most known cardiorespiratory effects of crude oil fall into the acute exposure category, for instance gill morphological disruptions (Claireaux et al., 2004; Kennedy and Farrell, 2005; Kochhann et al., 2015) and cardiotoxicity (Incardona et al., 2004; Incardona et al., 2009; Incardona et al., 2013; Hicken et al., 2011; Brette et al., 2014; Millemann et al., 2015). The damaged gill histology further hinders oxygen diffusion across the gill epithelia in hypoxia, which directly decreases oxygen supply for other compensatory mechanisms, eventually causing

hypoxemia (Richards, 2009). These syndromes have been associated with impaired aerobic capacity (Davoodi and Claireaux, 2007; Claireaux and Davoodi, 2010).

PAH bioaccumulate in the bile (Aas et al., 2000), liver (Cohen et al., 2001) and gills (Bols et al., 1999; Lyons et al., 2011), leading to chronic toxicity (Whitehead et al., 2012). Although PAH can be effectively excreted from fish, leaving minor residual PAH (Tuvikene 1995; Blahova et al., 2013, Dévier et al., 2013), this does not imply that residual toxicity has subsided. For instance, adult pink salmon (*Oncorhynchus gorbuscha*) survival was reduced by 36% after exposure to less than 20 μg/L PAH at the embryonic stage (Heintz et al., 1999; Heintz et al., 2000; Heintz, 2007). Chronic residual effects can be better understood when the sub-lethal toxicity can be placed in a physiological framework, an approach that dates back to the 1970s when the swimming performance tested the sub-lethal effects of pollutants on fish (Sprague, 1971; Beamish, 1992; Hammer, 1995). For example, U_{crit}, an integrative trait, has proven to be sensitive to dissolved metals (Waiwood and Beamish 1978; Wilson et al., 1994; Beaumont et al., 1995a, b; McKenzie et al., 2003), organophosphate pesticides (Petersen 1974), organochlorine fungicides (MacKinnon and Farrell 1992; Nikl and Farrell 1993; Wood et al., 1996) and ammonia (Shingles et al., 2001; Wicks et al., 2002; McKenzie et al., 2003). Likewise, oil-exposed fish have chronically compromised swimming performance (Hicken et al., 2011; Mager et al., 2014).

Underpinning swimming performance is aerobic metabolism, which uncovers the energetic costs associated with metabolizing toxicants (Rice, 1990; Blahova et al. 2013; Dévier et al. 2013), as well as anaerobic metabolism that supports hypoxic survival (Richards, 2009). $\dot{M}O_2$ is known to be sensitive to organochlorine pesticides (Holmberg and Saunders, 1979; Farrell et al., 1998), methylmercury (Rodgers and Beamish, 1981), some herbicides (Johansen

and Geen, 1990; Janz et al., 1991) and oil contamination (Claireaux et al., 2004; Davoodi and Claireaux, 2007). Therefore, I explore the possibility of applying RAP to study the chronic residual effect of chemically dispersed crude oil on European sea bass.

1.4 Thesis objectives and hypotheses

The primary objective of my thesis was to develop RAP as an experimental protocol for automatic intermittent-flow respirometry that generates 10 reliable respiratory metabolic indices in a minimum of 3 days. Specifically, RAP measures metabolic scope using $\dot{M}O_2$, which can quantify an individual's aerobic capacity (SMR, $\dot{M}O_{2max}$, FAS, AAS), recovery from exhaustion (EPOC) and the ability to survive hypoxia (O_{2crit}, ILOS). The scope for oxygen deficit (SOD), the factorial scope for oxygen deficit (FSOD) and the accumulated oxygen deficit (AOD) were derived from O_{2crit} and ILOS as new indices of scope for survival. The second objective was to validate RAP by testing an overarching hypothesis that fish respiratory physiology and bioenergetics have a major influence on functional integrity. Hence, the validation was conducted in two different anthropogenic environments, where an aquaculture hatchery represents a resource-rich circumstance and an oil exposure represents a resource-threatened circumstance.

The form of my thesis is as follows. In Chapter 2, I present work on exercise-trained *Bolaks* (domesticated) and *Lærdal* (wild) strains of Atlantic salmon that were reared under identical hatchery conditions. A range of swimming performance was assured by pre-categorizing fish using a constant acceleration screening protocol to measure maximum swimming speed (U_{max}) and segregating fish into groups of inferior and superior swimmers.

Using RAP, I predicted that exercise training increases athletic robustness, but with the domesticated strain having less cardiorespiratory plasticity and a lower athletic robustness than the wild strain (Mercier et al., 2001; Gamperl and Farrell, 2004). I also predicted that a constant acceleration screening protocol should segregate fish according to athletic robustness measured by RAP based on their swimming ability (Claireaux et al., 2005).

In Chapter 3, I conducted HCTs on yearling European sea bass both pre- and post-exposure to chemically dispersed oil to assure a good representation of the range of hypoxia tolerance of sea bass. In doing so, however, I discovered that two phenotypes for hypoxia tolerance that were further tested with a separate study of plasma lactate and glucose accumulation. To more precisely characterize a fish's capacity to handle hypoxic episodes (Hochachka, 1990; Pörtner and Knust, 2007), I incorporated new indices of scope for survival (SOD, FSOD, AOD) into my RAP, which tested fish 176 days post-exposure to quantify chronic residual effects of oil. I predicted that European sea bass, with their need for a high aerobic capacity, hypoxia tolerance and hypoxia resistance, would remain chronically impaired after a prolonged recover from an acute exposure (48 h) to chemically dispersed crude oil regardless of the hypoxia tolerance phenotypes.

In Chapter 4, I summarized the novel discoveries regarding RAP, potential limitations of RAP along with the advancements made to our understanding of scope for survival that revealed chronic residual effect of oil in ecotoxicology research. I then further discussed the refinement of exercise screening protocols and applications of exercise training in aquaculture industry.

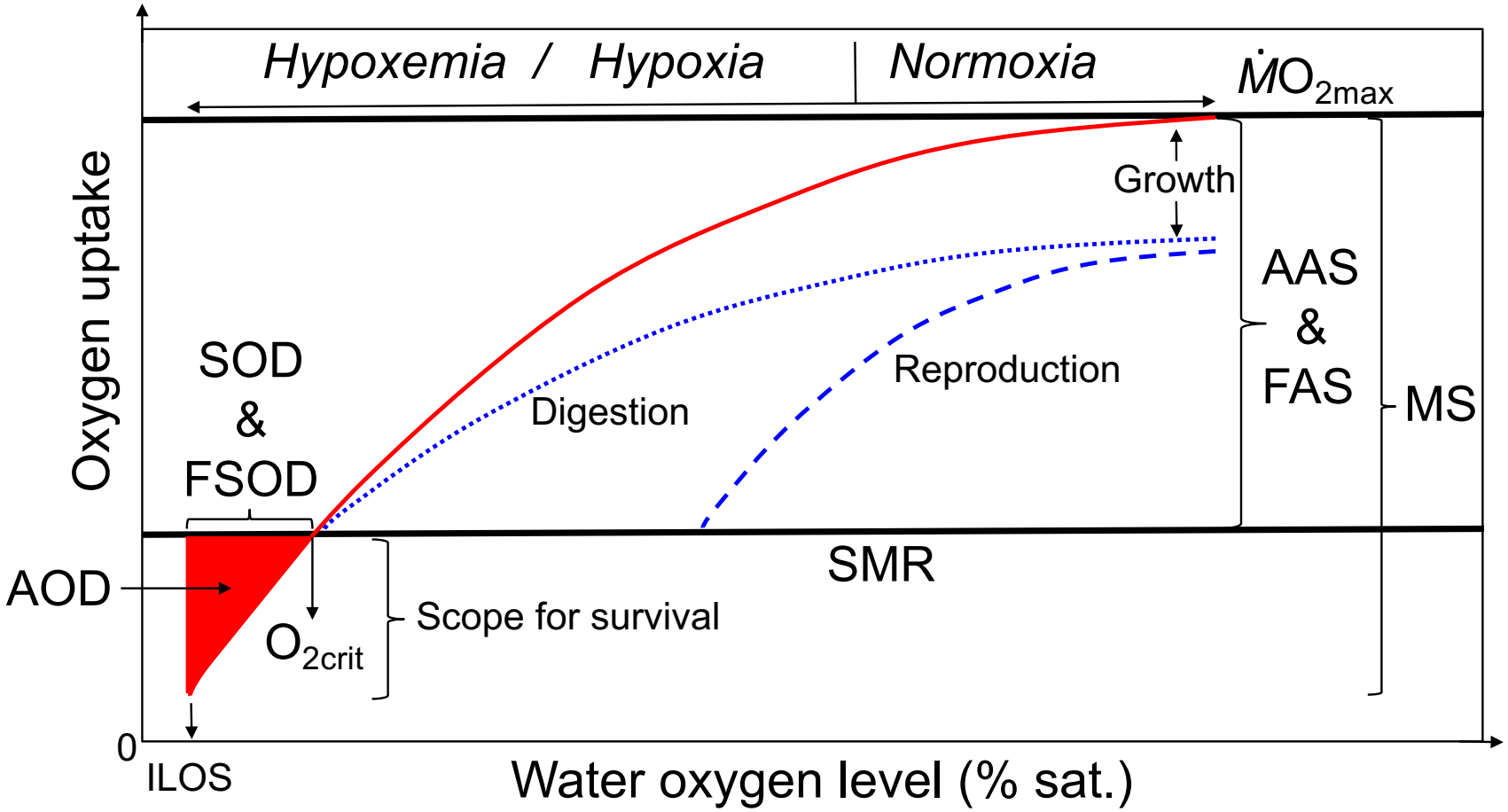

Hypoxemia / Hypoxia
Normoxia
$\dot{M}O_{2max}$
Growth
AAS
&
FAS
MS
Oxygen uptake
SOD
&
FSOD
Digestion
Reproduction
AOD
SMR
O_{2crit}
Scope for survival
0
ILOS
Water oxygen level (% sat.)

Figure 1.1. Schematic illustration of respiratory assessment paradigm (RAP). Fish have maximum rate of oxygen uptake ($\dot{M}O_{2max}$), standard metabolic rate (SMR), absolute aerobic metabolic rate (AAS) and factorial aerobic scope (FAS) in normoxia. In hypoxia, fish reach critical oxygen level (O_{2crit}) and incipient lethal oxygen saturation (ILOS), which forms a scope for survival and quantified by scope for oxygen deficit (SOD), factorial scope for oxygen deficit (FSOD) and accumulated oxygen deficit (AOD) in this thesis. These indices, collectively estimate metabolic scope. Theoretical apportioning curves of digestion (blue dot line) and reproduction (blue dash line) decide the surplus aerobic scope for growth potential. This figure is developed based on Fig.11.1 Farrell and Richards 2009 and Fig.1 Claireaux and Chabot 2015.

Chapter 2: DOMESTICATION COMPROMISES ATHLETICISM AND RESPIRATORY PLASTICITY IN RESPONSE TO AEROBIC EXERCISE TRAINING IN ATLANTIC SALMON (*SALMO SALAR*)

2.1 Introduction

Freshwater production of Atlantic salmon smolts in commercial aquaculture is characterized by high intensity production (Kristensen et al., 2009, 2012a) and several handling procedures, such as grading, vaccination and transport, which are physiologically challenging. A particularly critical production procedure is the transfer of smolts to seawater because it is associated with significant mortality (Maxime et al., 1986, 1990; Jørgensen and Jobling, 1994; Iversen et al., 2005). In Norway alone, a 15–20% mortality rate still accompanies salmon grow-out in sea at the first 90-day, which amounts to 40–50 million fish annually (Kristensen et al., 2012a). Both the osmo-respiratory compromise (McCormick and Saunders, 1987; Randall and Brauner, 1991; Gallaugher et al., 2001; Sardella and Brauner, 2007) and lower cardiorespiratory fitness (Castro et al., 2011) have been implicated as underlying mechanisms for this mortality, with secondary disease outbreaks as a diagnosed cause of death (Aunsmo et al., 2008).

A fish's activities are ultimately governed by the capacity of its cardiorespiratory system to supply oxygen to working tissues, making this system a determining factor of a fish's ability to face environmental contingencies, whether of natural or anthropogenic origin. It therefore seems reasonable to assume that within a population, those individuals with the greatest cardiorespiratory capacity will be less likely to suffer energy budgeting conflicts as they have a greater capacity to multitask (Neill et al., 1994). In farmed Atlantic salmon, this advantage could

translate into improved survival during seawater transfer of smolts and other challenging aquaculture procedures, e.g., chemical delousing procedures. Here we used a suite of established cardiorespiratory indices, ones that can be reliably measured in individual fish over a period of several days, to define the general athletic robustness of a salmon. Collectively, these indices measured the fish's aerobic capacity for sustained swimming, ability to recover from exhaustion, ability to produce ATP aerobically at muscle tissue level, and ability to tolerate hypoxia and temporarily support metabolic demands with anaerobic metabolism.

Selective breeding for rapid growth, delayed maturity and high fecundity (as well as rearing in a sheltered environment with an unlimited food source and the absence of predators) appears to have reduced athletic robustness in farmed fish. For instance, cultured salmonids characteristically have a high body condition factor, a rounded cardiac ventricle and a low plasma level of atrial natriuretic peptides (Poppe et al., 2003; Claireaux et al., 2005; Kristensen et al., 2012b). They also display a higher incidence of cardiac aberrations (e.g., a misaligned bulbus arteriosus; Mercier et al., 2000), which have been associated with reduced capacities for cardiac pumping and oxygen delivery (Poppe and Taksdal, 2000; Poppe et al., 2002, 2003; Gamperl and Farrell, 2004; Claireaux et al., 2005). Domesticated fish also have lower swimming muscle contraction capacity and lower enzymatic capacity for aerobic energy production as compared to wild fish (Anttila et al., 2008a; Anttila and Mänttäri, 2009). Poorer swimming performance associated with domestication (McDonald et al., 1998; Jonsson and Jonsson, 2006) could reflect a reduced aerobic capacity (Fry, 1971; Claireaux et al., 2005). At an extreme, the rapid growth rate of growth hormone transgenic salmonids is similarly associated with reduced aerobic capacity and hypoxia tolerance (Farrell et al., 1997; Stevens et al., 1998; Cook et al., 2000). Thus, intensive selection for beneficial traits during commercial rearing (e.g., rapid

growth) appears to have negatively impacted the physiological integrity of farmed fish (McKenzie et al., 2007), with possible consequences on smolt's ability to handle the additional energy costs and osmotic stress that accompany transfer into seawater (Usher et al., 1991).

In intensive aquaculture, exercise training can benefit fish, provided the exercise regime is not too severe, i.e., plasma stress hormones are not greatly elevated, ionic/osmotic homeostasis is not disrupted and blood flow to the digestive system is not diverted to skeletal muscle (Farrell et al., 1991; Thorarensen et al., 1993; Kiessling et al., 1994; Davison, 1997; Kieffer, 2000; McKenzie et al., 2003). Indeed, aerobic training in fish is viewed by some as an integrated approach to improve animal wellbeing (Nelson, 1989), in part because training is known to enhance oxygen extraction efficiency and supply, anaerobic metabolism, the capacity to recover from exhaustion, and reduces the osmo-respiratory compromise (Kieffer, 2000; Gallaugher et al., 2001; Kieffer, 2010). Also, aerobic training can enlarge muscle fibers in swimming muscles and mitochondria in cardiac myocytes (Davison, 1997; Castro et al., 2013), and increase oxidative enzyme activities in both red and white skeletal muscles, while reducing their anaerobic enzymes activities (Anttila et al., 2006, 2008b). Lipid utilization (McClelland et al., 2006; Anttila et al., 2010) and the capacity to deplete muscle glycogen similarly increase after aerobic training (Hochachka, 1961; Poston et al., 1969; Pearson et al., 1990; Gamperl et al., 1994; Milligan, 1996). Furthermore, aerobic training of Atlantic salmon parr before sea transfer enhanced gene expression for infectious pancreas necrosis virus resistance and seawater relevant ion-transportation pathways (Castro et al., 2011, 2013; Esbaugh et al., 2014). Consequently, the aquaculture industry has growing interests in identifying appropriate exercise regimes that would strengthen stress tolerance, growth and survival before and after sea transfer (Grisdale-Helland et al., 2013).

For the present study, I tested the hypothesis that exercise training of domesticated and wild strains of Atlantic salmon parr would improve their athletic robustness measured at biochemical and whole animal levels. I also reasoned that by screening swimming performance ahead of training with a constant acceleration test, superior and inferior swimmers could be segregated according to their athletic robustness (Anttila et al., 2014a). Thus, after the screening protocol, Atlantic salmon parr were given an 18-day incremental aerobic exercise training regime in a hatchery setting followed by measurements of a suite of respiratory indices to evaluate athletic robustness. I further hypothesized that the domesticated *Bolaks* strain should have a lower athletic robustness and less cardiorespiratory plasticity in response to exercise training than the wild *Lærdal* strain. To ensure a broad characterization of athletic robustness, I measured each fish's standard metabolic rate (SMR, the minimal maintenance metabolic rate of an aquatic ectotherm in a post-absorptive and inactive states; Fry and Hart, 1948) and maximum rate of oxygen uptake ($\dot{M}O_{2max}$, as an estimate proxy of maximum metabolic rate; Fry, 1947; Brett and Groves, 1979), as well as the absolute aerobic scope (AAS) and factorial aerobic scope (FAS), which are indices of the aerobic capacity for activities (Pörtner and Farrell, 2008; Pörtner, 2010; Clark et al., 2013). The capacity for both aerobic and anaerobic energy production in red and white swimming muscles were assessed by measuring the citrate synthase (CS) and lactate dehydrogenase (LDH) activities, respectively. Hypoxia tolerance and anaerobic capacity were characterized by measuring, respectively, the critical oxygen level (O_{2crit}, the capability to extract dissolved oxygen; Ultsch et al., 1978) and the incipient lethal oxygen saturation (ILOS, the minimal oxygen saturation that aquatic animal maintaining equilibrium; Claireaux et al., 2013). Lastly, I measured excess post-exercise oxygen consumption (EPOC; Lee et al., 2003) to quantify the ability to recover from exhaustion.

2.2 Materials and methods

2.2.1 Experimental and rearing conditions

The *Bolaks* (domesticated) strain was generated from eggs of 7 females that were fertilized by 2 males and the fertilized eggs were incubated at 7 °C in SalmoBreed (Bergen, Norway) until 396 degree days (dd). The *Lærdal* (wild) strain were generated from eggs of 5 females that were fertilized by 2 males and the fertilized eggs were incubated at 7 °C on site until 410 dd. Eyed eggs were then transported to Nofima research station, Sunndalsøra, Norway and incubated in 5–6 °C freshwater until hatching using side-by-side incubators (463–487 dd for *Bolaks* and 513–518 dd for *Lærdal*). Emergent fry were similarly reared under identical standard conditions and fed the same diet (Skretting, Stavanger, Norway) in side-by-side 5.3 m^3 circular fiberglass tanks (approximately 25 kg m^{-3} stock density). Rearing temperature was progressively increased to 12 °C in accordance with Norwegian aquaculture industry standards and maintained at 12 °C throughout the experiment. At 3 g size (bulk weighed), fish were graded to obtain homogenous populations with respect to body mass/fork length and maintain stocking density (35 kg m^{-3}). At 25 g size and two weeks prior to swim screening, 600 fish per strain were selected to limit the variance in body mass and fork length to ± 3 g and ± 1 cm, respectively, and individually tagged with a passive integrated transponder (Jojo Automasjon ÅS, Sola, Norway). Each stock was then reared in five replicate circular tanks (0.1 m^3, n = 120 per strain, 36 kg m^{-3} stock density) until the fish were screened for their swimming performance. Throughout, water exchange and current were set and routinely adjusted to self-clean the tanks in accordance to standard procedures and to provide a nominal water current that was slightly lower [0.2–0.3 fork

lengths (FL) s^{-1}] than that used later for the control fish (see Section 2.2.2). Specific growth rates were 3.40 and 3.07 in the *Bolaks* and *Lærdal* strains, respectively, during this period. Therefore, to minimize the size dichotomy, the faster growing *Bolaks* strain were screened, trained and tested two months ahead (September 2014) of the *Lærdal* strain (November 2014). The experiments were approved by the National Animal Research Authority, according to the 'European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes' (EST 123).

The *Lærdal* and *Bolaks* strains are characteristic Norwegian wild and domesticated Atlantic salmon, respectively. The *Lærdal* strain, from the Norwegian *Lærdal* River (61°N), was selected because of its relatively large spawning population size, genetic purity, genetic stability and a limited genetic influence from aquaculture escapees (Glover et al., 2012, 2013; Finstad et al., 2013; Johnsen et al., 2013). Its freshwater habitat has a large water discharge and low water temperature, which are typical conditions for wild Atlantic salmon in Norway (Brooks et al., 2006; Urke et al., 2013). The *Lærdal* strain consists mainly of late maturing (2 and 3 sea-winter) spawners and the migration route passes through the longest (200 km) Norwegian fjord system, Sognefjorden. The domesticated *Bolaks* strain originates from and is primarily produced as broodstock in Western Norway. The two founding strains were from the Vosso (60°N) and Årøy (61°N) Rivers. For the first five generations until 2000, selective breeding with other strains focused on promoting characteristics of rapid growth and delayed sexual maturation, after which a family-based breeding program was established to enhance specific disease resistance, fillet quality and coloration as selective breeding traits in addition to growth and delayed sexual maturation.

2.2.2 Screening and training protocols

The experimental design is summarized in Figure 2.1 For each strain, 480 parr were initially screened using the following protocol. Batches of 120 parr were fasted for 1 day before being transferred to a pair of side-by-side transparent PVC tunnels (60 fish per tunnel; stocking density = 29 kg m^{-3}). The swimming section was 2 m long and 20 cm in diameter, which allowed 4 fish to easily swim abreast, if required. Fish were habituated for 4 h, while maintaining station in the water current (0.5 FL s^{-1}) without tail beats. Water velocity was incremented by 5 cm s^{-1} every 10 min until all the fish in a tunnel had reached exhaustion (typically ≤ 145 cm s^{-1}). Fatigued fish, which were too refractory to remove themselves from the net at the rear of the swimming section, were manually removed via a hatch situated above the back grid and scanned for their pit-tags. Their body mass, fork length, final water speed (U_{max}; Farrell, 2008) and swimming duration were recorded. U_{max} for each fish was calculated from the proportion of the time period spent at the final velocity increment (Brett, 1964). Both swim tunnels received rearing tank water (12 ± 0.5 °C) from a 7 m^3 reservoir via a pump (VAKI Heathro Self Priming 6″ pump, VAKI Aquaculture Systems Ltd., Kópavogur, Iceland). Water flow from each pump and to each swim tunnel was independently controlled with a Cubix remote controller (HBC-radiomatic GmbH, Crailsheim, Germany) and a Micronics Portaflow 300 ultrasonic flowmeter that monitored water flow in the swim tunnel (Micronics Ltd., Buckinghamshire, UK). The design of the apparatus was based on a Brett-type swimming respirometer, which has been extensively and critically tested since its conception over 50 years ago (Brett, 1964). I improved on Brett's original design by inserting flow straightening devices in the section situated upstream

from the swimming section to ensure lamellar flow and a proper cross-sectional water velocity profile. The maximum water velocity was approximately 145 cm s^{-1}.

For each population and test, the first 20% and last 20% of each group of 120 fish to reach fatigue were categorized as inferior and superior swimmers (Figure 2.2.). The respirometry tests (Section 2.2.3) and enzyme activity assays (Section 2.2.4) were performed only on these sub-groups to maximize our chances of segregating athletic robustness. The remaining fish were returned to their original rearing tanks.

For the exercise-training regime, each swim tunnel contained 40 inferior and 40 superior swimmers (stock density = 38 kg m^{-3}). The training lasted 18 days, followed by a 2-day recovery period (Figure 2.1). One swim tunnel (water velocity of 0.5 FL s^{-1}) was used for control fish, which would spread themselves along the length of the swim tunnel and only swim occasionally (using slow and small-amplitude tail beats to move forward). The other swim tunnel was used for aerobic exercise training, which involved maintaining the water velocity at 2 FLs^{-1} for the first 7 days, at 2.4 FL s^{-1} for next 7days and at 2.8 FL $s^{-1}$for the last 4 days. Again, fish spread themselves along the length of the swim tunnel and typically maintained station in the water current with only occasional changes in location. Fish were fed a daily ration of 2% biomass through a hatch situated above honeycomb grid at the front of the swim tunnels, which was connected to an automatic belt feeder. A technician monitored feeding twice daily to ensure the pellets were evenly spread and fish were feeding. After the 18-day period, fish were segregated into 8 groups of 40 fish, from which up to 12 fish per group were subsequently used for the respirometry testing and 15 fish per group were used to measure enzyme activities. The rest of the fish were used for other purposes that were separate from current experiments.

2.2.3 Respirometry trials

Prior to testing, the fish groups were returned to their rearing tanks for 3 days where they were held on a 24-h photoperiod with a limited diet to prevent smoltification and further growth. The control group were tested first to allow the exercised group some recovery, a practice that would likely be needed for an industry application prior to sea transfer. Each batch of respirometry trials simultaneously examined 8 fish that had been fasted for 3 days using intermittent-flow respirometers (water volume = 2.1 L each) were submerged in a water reservoir (3 m × 0.5 m and 0.3 m deep), where aerated water (12 ± 0.5 °C) flowed continuously. Water flow through the respirometers was regulated using computer-controlled flush pumps (Compact 600, EHEIM, Germany) and relays (AquaResp, University of Copenhagen, Helsingør, Denmark). Continuously mixing of the respirometry chamber was assured by a circulation loop, into which an optical oxygen probe (Robust Oxygen Probe OXROB3, Pyroscience, Germany & Oxygen Minisensor, PreSens, Germany) that continuously monitored dissolved oxygen saturation (% sat.). Prior to every respirometry trial, the oxygen probes were calibrated to 0% sat. (water saturated with sodium sulfite) and 100% air saturation (fully aerated water).

A respirometry trial began with a chasing protocol, during which each fish was individually hand chased in a 10-L bucket for 10-min and then given a 2-min air exposure. At the end of air exposure, the fish was placed immediately in a respirometer during flushing mode. An oxygen uptake rate ($\dot{M}O_2$) measurement cycle consisted of flush, stabilization and measurement periods. Only oxygen saturation values obtained during the measurement periods were used to calculate $\dot{M}O_2$. Oxygen saturation was then monitored using a measuring cycle comprised of a 30 s flush, a 45 s stabilization and a 105 s measurement at the first 2 h to capture the maximum

$\dot{M}O_2$, which was assigned $\dot{M}O_{2max}$. The computer recorded the first $\dot{M}O_2$ value about 150 s to 180 s after the fish was placed in the respirometer. As $\dot{M}O_2$ decreased toward SMR, the measurement cycle was changed to a 120 s flush, a 80 s stabilization and a 400 s measurement to guarantee that oxygen saturation was > 97% at the start of the measurement period. These measurements continued for 2 or 3 days in a dark and quiet environment, allowing the fish to fully adjust to the respirometer and reach a minimal maintenance $\dot{M}O_2$ (SMR; Section 2.2.5).

O_{2crit} and ILOS were determined at the end of a trial by introducing hypoxic water into the respirometers. Water was pumped (Compact 600, EHEIM, Germany) from the water reservoir to the top of a gas equilibration column where it trickled down through nitrogen gas that was injected into the bottom. Air-saturation of the water decreased progressively to 10% over an 8-h period, during which the $\dot{M}O_2$ measurement cycles were a 60 s flush, a 45 s stabilization and a 195 s measurement. When a fish lost its equilibrium, the air saturation was noted as the ILOS. O_{2crit} was determined with a post-experiment calculation (Section 2.2.5). Fish were immediately resuscitated with well-aerated water at 12 °C (survival rate was 94%). To eliminate microbial respiration, the entire system was disinfected with Virkon S (Lilleborg Profesjonell, Oslo, Norway) for 6 h on completion of every respirometry trial.

2.2.4 Enzyme activity assays

Fish were weighed, measured and euthanized 5 days after the 18-day training regime. Blocks of red and white muscles were removed from the mid-line at the mid-point between the adipose fin and tail fin and frozen immediately and separately in liquid nitrogen for storage at −80 °C until analyzed. For enzymatic analysis, each muscle sample was homogenized separately

in 19 vols of homogenization buffer (0.1% Triton, 50 mM Hepes, 1 mM EDTA, pH 7.4). Citrate synthase (CS, EC 2.3.3.1) and lactate dehydrogenase (LDH, EC 1.1.1.27) activities were measured according to Dalziel et al. (2012) at 21 °C. The measurements were done with EnSpire 2300 Multilabel Reader (Perkin Elmer, Turku, Finland) and the final substrate concentrations followed the optimizations were done by Dalziel et al. (2012). For CS the concentrations were 0.15 mM DTNB, 0.15 mM acetyl CoA and 0.5 mM oxaloacetic acid in 50 mM Tris (pH 8.0) and for LDH 0.27 mM NADH and 25 mM sodium pyruvate in 50 mM Tris (pH 7.4). Assays were performed in triplicate for each sample and a background reaction rate was subtracted. The activities of enzymes were calculated g^{-1} tissue. All the reagents were purchased from Sigma-Aldrich Chemie GmbH, Steinheim, Germany.

2.2.5 Data analysis

Only % air saturation data with a linear decrease and $R^2 > 0.8$ during the measurement cycle were accepted for an accurate calculation of $\dot{M}O_2$. SMR was determined from between 288 and 432 measurement cycles per fish. In the absence of a fully accepted method to estimate SMR (see Chabot et al., 2016), I used an R script (Chabot et al., 2016) to determine two estimates: a) a quantile of 0.2 (q0.2), and b) the mean of the lowest 10 $\dot{M}O_2$ values (Low10) after removing the lowest 2% of the dataset. $\dot{M}O_{2max}$ was not always the first $\dot{M}O_2$ measurement made after chasing and was as late as the 5th $\dot{M}O_2$ measurement, but averaged the 2nd $\dot{M}O_2$ measurement. AAS = $\dot{M}O_{2max}$ − SMR , while FAS = $\dot{M}O_{2max}$ / SMR. EPOC was calculated from the integral of the area bounded by the actual ṀO2 measurement and SMR. However, I only used the recovery period until $\dot{M}O_2$ remained at or below to SMR + 10% for at least 3 measurement cycles, rather than

using SMR per se. This strategy limited the obvious overestimate of EPOC and its duration due to spontaneous fish activity. I acknowledge that while our approach slightly underestimates EPOC and its duration, the effect of spontaneous activity on EPOC would be numerically much greater. Yet, even with this modification, EPOC could not be calculated reliably in 20 individuals given the extent of their spontaneous activity during the recovery period. O_{2crit} was derived using R script from the intersection of a regression line for $\dot{M}O_2$ versus oxygen saturation and the SMR (Claireaux and Chabot, 2016).

Statistical comparisons of SMR, $\dot{M}O_{2max}$, AAS, FAS, EPOC, O_{2crit}, ILOS, CS and LDH activities were made using a two-way (strain & training) multivariate analysis of variance (MANOVA) with Pillai's trace test to correct for unbalanced sample size, followed by multiple ANOVAs and Tukey HSD *post-hoc* (Zar, 1996). Log10 transformations were applied on $\dot{M}O_{2max}$ and EPOC in Low10 dataset to achieve the assumption of multivariate normality. Statistical comparisons of U_{max}, regarding strain and initial swimming capacity, were made using a two-way ANOVA with Tukey HSD *post-hoc*. Statistical analyses were conducted in R (ver. 3.2.2; R Development Core Team, 2015) and SigmaPlot (ver. 12.5). Statistical significance was assigned when $\alpha < 0.05$.

2.3 Results

2.3.1 Swimming capacity and growth

Prior to respirometry, body mass was 9.2% larger ($p = 0.002$) in the domesticated *Bolaks* strain and their fork length was 2.1% longer than in the *Lærdal* strain ($p = 0.001$) (*Bolaks*: 35.6 ± 0.8 g, 14.4 ± 0.1 cm; *Lærdal*: 32.6 ± 0.5 g, 14.1 ± 0.1 cm) (Appendices A7). Regression analysis

found no significant correlations either between respiratory indices and body mass, or between enzymatic variables and body mass within either strain or swim groupings (Appendices A1, A2).

The cumulative frequency polygons demonstrated that neither absolute nor relative U_{max} had normal distributions (see insets in Figure 2.2) and that the distribution patterns were similar for each screening trial and for both strains (Appendices A3, A4, A5, A6). Superior swimmers swam on average almost twice as fast as inferior swimmers using absolute swimming speed (entire fish population: 117.0 ± 1.9 vs. 65.1 ± 0.3 cm s^{-1}; p = 0.001; only fish used for respirometry: 121.9 ± 2.6 vs. 66.8 ± 2.7 cm s^{-1}; p = 0.001; Figure 2.2) as well as relative swimming speed (entire fish population: 8.6 ± 0.1 vs. 4.8 ± 0.1 FL s^{-1}; p = 0.001; only fish used for respirometry: 9.0 ± 0.2 vs. 4.9 ± 0.2 FL s^{-1}; p = 0.001; Figure 2.2). Unexpectedly, the wild *Lærdal* strain had a significantly lower U_{max} than the domesticated *Bolaks* strain for both superior and inferior swimmers (Figure 2.2).

2.3.2 Respirometry indices (based on Low10 SMR values)

Contrary to the initial hypothesis, screening had no main effect on the metabolic and enzymatic indices (three-way MANOVA: p = 0.7; Appendices A8). Therefore, the data of superior and inferior swimmers were pooled for subsequent analyses of effects of training and strain. Training and strain (two-way MANOVA: training p = 0.001; strain p = 0.001; Table 2.1), as well as the interaction (two-way MANOVA: p = 0.002; Table 2.1), significantly influenced several of the metabolic and enzymatic indices.

Overall SMR (n = 90) was 65.8 ± 1.6 mg O_2 h^{-1} kg^{-1}. Neither training, nor strain had independent effects on SMR ($p \geq 0.19$; Table 2.1). Nevertheless, strain significantly influenced

$\dot{M}O_{2max}$, with wild strain displaying a 24% higher $\dot{M}O_{2max}$ than domesticated strain (392.8 ± 10.7 vs. 317.8 ± 7.3 mg O_2 h^{-1} kg^{-1}, p = 0.001, Table 2.2). Moreover, there was an interaction between training and strain (p = 0.036, Table 2.2), with training increasing $\dot{M}O_{2max}$ by 16% in wild fish (p = 0.045) but not in domesticated fish (p = 0.98, Figure 2.3).

Since AAS and FAS are derived from SMR and $\dot{M}O_{2max}$, the training and strain effects on AAS and FAS were similar to those for $\dot{M}O_{2max}$. Training increased AAS by 12% (306.2 ± 12.2 vs. 272.7 ± 7.9 mg O_2 h^{-1}- kg^{-1}, p = 0.003, Table 2.1) and AAS was 30% higher for wild fish than domesticated fish (327.1 ± 10.9 vs. 251.4 ± 5.9 mg O_2 h^{-1} kg^{-1}, p = 0.001, Table 2.1). Wild fish had a 17% higher AAS after training (p = 0.009), whereas AAS was unresponsive to training in domesticated fish (p = 0.96, Figure 2.3). Wild fish had a 24% higher FAS than domesticated fish (6.2 ± 0.2 vs. 5.0 ± 0.2, p = 0.001, Table 2.1) and training increased FAS by 7% (5.8 ± 0.2 vs. 5.4 ± 0.2, p = 0.005; Table 2.1). There was an interaction between strain and training (p = 0.012), but neither the domesticated nor wild fish reached statistical significance for an effect of training on FAS (Figure 2.3).

Training significantly increased EPOC by 30% (914.0 ± 55.7 vs. 704.1 ± 25.0 mg O_2 kg^{-1}, p = 0.002, Table 2.1), but strain (p = 0.92, Table 2.1) had no independent main effect on EPOC. There was no interaction between training and strain (p = 0.32, Table 2.1) and while EPOC increased significantly (p = 0.02) by 49% as a result of training in the wild fish, there was no training effect (p = 0.3) on EPOC in the domesticated fish (Figure 2.3).

Training and strain significantly impacted O_{2crit} and ILOS. Training decreased overall O_{2crit} by 6% (16.2 ± 0.4 vs. 17.2 ± 0.3 % sat., p = 0.001, Table 2.1) and O_{2crit} was 9% lower in wild fish than domesticated fish (15.9 ± 0.5 vs. 17.5 ± 0.3 % sat., p = 0.0028, Table 2.1). Training also decreased overall ILOS by 5% (14.6 ± 0.5 vs. 15.3 ± 0.4 % sat., p = 0.001, Table

2.2) and ILOS was 12% lower in wild fish than domesticated fish (14.0 ± 0.5 vs. 15.9 ± 0.3 % sat., $p = 0.002$, Table 2.2). Although there were interactions between strain and training for both O_{2crit} and ILOS ($p \leq 0.038$), the training effect on O_{2crit} and ILOS did not reach statistical significance for either strain (Figure 2.3).

Aerobic ATP production capacity, as measured by CS activity, was 16-times higher in red than in white swimming muscle (Figure 2.4; see Appendices A9 for morphometrics), as anticipated. Training had significant effects on CS activity that were more pronounced in wild than in domesticated fish. In red muscle, training significantly increased CS activity by 22% in wild fish ($p = 0.001$) and by 11% in domesticated fish ($p = 0.001$, Table 2.2, Figure 2.4). In white muscle, CS activity was 30% higher in wild compared with domesticated fish ($p = 0.001$), but training did not influence the CS activity of white muscle in either strain (Table 2.2, Figure 2.4). LDH activity, an index of anaerobic capacity, was 7-times higher in white swimming muscle than in red swimming muscle (Figure 2.4), as expected. Training reduced LDH activity in red muscle by 35% ($p = 0.001$) in wild fish but not in the domesticated fish (Table 2.2, Figure 2.4). In white muscle, LDH activity was 6% significantly lower in wild fish compared with domesticated fish ($p = 0.0016$), and training significantly increased LDH activity by 8% ($p = 0.048$) in wild fish but not in domesticated fish after training (Table 2.2, Figure 2.4). Therefore, in terms of key metabolic enzymes, wild fish generally had higher aerobic enzyme activity levels and a higher plasticity that responded to aerobic training than domesticated fish.

2.3.3 Respiratory indices (based on q0.2 SMR values)

By definition, the q0.2 estimate of SMR will always generate numerically higher value compared with the Low10 estimate. The q0.2 estimates (data not shown) were always < 11% numerical higher than the Low10 estimates (Appendices A8). Even so, the outcomes of the comparisons for training and strain main effects were no different (Table 2.1). For clarity, our figures only present the Low10 estimate of SMR.

2.4 Discussion

The general goal of this work was to examine if generations of selective breeding for growth (on average a 14% enhancement per generation for the first six generations; Gjedrem and Baranski, 2009) have influenced the athletic robustness and cardiorespiratory plasticity of domesticated *Bolaks* Atlantic salmon strain. This goal was pursued using a suite of indices for respiratory performance and hypoxia tolerance, along with measurements of muscle enzymatic activities that collectively characterized aerobic and anaerobic capacities and which I collectively term athletic robustness. Plasticity in response to aerobic exercise training was examined and the comparison between the domesticated *Bolaks* strain and the Norwegian *Lærdal* wild strain provided additional insights into the effects of domestication. Lastly, by experimentally separating each strain into inferior and superior swimmers, I hoped to establish an association between athletic robustness and swim performance during a simple screening test. The most important potential of this work for aquaculture is the insight into how selective breeding for growth has produced negative and possibly unforeseen effects on athletic robustness and cardiorespiratory plasticity. The domesticated strain certainly had a reduced athletic robustness and also less plasticity in response to a short exercise-training regime compared with a wild

strain. Further, the potential of domesticated fish benefiting from exercise training was not completely lost but remained below that for the wild strain, providing further insight into the compromised athletic robustness resulting from commercial aquaculture practices when compared with cardiorespiratory performance of a wild strain. Thus, the potential of using exercise protocols to train fish to reduce seawater transfer mortality in aquaculture and providing physiological basis for selective breeding to regain athletic robustness and cardiorespiratory plasticity warrant further investigation.

2.4.1 Training effects

The potential benefits of exercise training in fish are well established (Davison, 1997; Kieffer, 2010). Therefore, I anticipated that an 18-day regime would be sufficiently long and intense enough to improve athletic robustness (defined generally here as a high aerobic capacity, rapid recovery from exhaustion and superior hypoxia tolerance). This proved to be true for the wild *Lærdal* strain to a much greater extent than for the domesticated *Bolaks* strain. Training induced increases in $\dot{M}O_{2max}$, AAS, EPOC, CS activity in red muscle and LDH activity in white muscle in the wild *Lærdal* strain, effects that were absent in the domesticated *Bolaks* strain. Training only enhanced CS activity in red muscle in the *Bolaks* strain.

These observations support our hypothesis that the domesticated *Bolaks* strain had less cardiorespiratory plasticity than the wild *Lærdal* strain in response to exercise training. This important discovery means that commercial aquaculture practices might need to adopt a longer duration of training than used here to sufficiently exploit the full plasticity of the cardiorespiratory system in domesticated strains (Davison, 1997; Gamperl and Farrell, 2004),

because previous long-term, but less intensive (1.0–1.5 FL s^{-1}) exercise regimes have induced training effects, i.e., improved growth, greater ventricle mass and enhanced disease resistance in both domesticated and wild Atlantic salmon (Castro et al., 2011, 2013; Grisdale-Helland et al., 2013; Anttila et al., 2014a). It is also possible that our trained fish underwent some detraining prior to the start of the respirometry measurements and potentially the domesticated strain may have detrained faster. A recovery period post-training is inevitable given the need to properly measure the respirometry indices and the industry requirement for transferring smolts to seawater. The diverse training effects observed for the wild strain after 17 days clearly illustrates the retention of training effects despite this recovery period, as well as the practical potential for the aquaculture industry. Indeed, previous studies report retention of training effects well beyond 17 days. For example, following a 48-day aerobic training regime (up to 1.0 FL s^{-1}), 119 days were needed before juvenile Atlantic salmon lost the growth benefit and 48 days to lose the gene expression for the acquired resistance to infectious pancreas necrosis virus (Castro et al., 2011). Coho salmon smolts exercised for 40 days (at 1.5 FL s^{-1}) retained enhanced swimming endurance for 60 days (Besner and Smith, 1983), while striped bass fry exercised for 60 days (at either 1.5–2.4 FL s^{-1} or 2.4–3.6 FL s^{-1}) retained enhanced swimming performance for 56 days (Young and Cech, 1993). Nevertheless, the current training regime was shorter than previous studies, which might lead to faster detraining. The relatively short training duration used here was aimed to discover a minimum training period (and associated economic cost) to enhance physiological robustness. Atlantic salmon aquaculture may benefit from water velocities in the range of 1– 2 FL s^{-1} and a longer training duration than used here. Of course, once suitable protocols are established for training intensity, duration and timing for different life-stages, the ultimate goal is to measure marine survival of trained smolts during commercial grow out.

Alternatively, a different genetic selection program may be needed to prevent the trade off of cardiorespiratory robustness.

Exercise training enhanced anaerobic glycolytic capacity (LDH activity) and utilization in white muscle of wild strain, an effect not reflected in either O_{2crit} or ILOS of these two strains. Instead, it was reflected in EPOC, which is an index of a) the rapid recharging oxygen storage on hemoglobin and myoglobin (~20% of total; Burnett et al., 2014), b) the rapid resynthesis of creatine phosphate and adenosine triphosphate, and c) the slower lactate clearance and glycogen resynthesis (Gaesser and Brooks, 1984; Scarabello et al., 1991; Wood, 1991) and possibly restoring ionic imbalances due to the osmo-respiratory compromise (Gallaugher et al., 2001). A higher CS activity in red skeletal muscle of trained fish, as observed previously (Davison, 1997; Kieffer, 2000; Gallaugher et al., 2001; Anttila et al., 2006, 2008a, 2008b), would not only aid aerobic ATP production for swimming, but would benefit aerobic ATP production during recovery. A higher LDH activity in white skeletal muscle could enhance lactate clearance (Milligan et al., 2000), something of less important in red skeletal muscle, as indicated by a reduction of LDH activity in trained wild fish here and previously (Anttila et al., 2006, 2008b).

2.4.2 Strain differences

The present comparison between a domesticated strain and a wild strain was intended to provide insight into the effects of domestication. Indeed, I found support for our hypothesis that the domesticated *Bolaks* strain would have less cardiorespiratory plasticity and a lower athletic robustness than the wild *Lærdal* strain. However, precisely separating out the effect of domestication (i.e., genetic selection for growth, plentiful food, lack of predators, habituation to

aquaria and people, limited environmental extremes etc.) by simply comparing a wild and a domesticated strain is impossible, especially given the mixed stock origins of the *Bolaks* strain following ten generations selective breeding for growth. Also, the genetic divergence between the two strains remains undetermined, even if single-nucleotide polymorphism analysis shows a 0.30–0.32 genetic dissimilarity when compared with themselves and only a 0.31–0.38 genetic dissimilarity when compared against each other (Nick Robinson, pers. comm.). Thus, the differences in performance between strains observed here could be due to: a) genetic selection of desirable traits, such as a faster growth rate for the *Bolaks* strain, b) selective pressures encountered only by the wild fish, c) random genetic drift (possibly exacerbated by population bottlenecks), or d) pre-existing genetic differences between the *Lærdal* strain and the base populations that founded the *Bolaks* strain. I propose that the observed differences between *Bolaks* and *Lærdal* strains are a combination of strain and domestication since the two strains of experimental fish were grown from eyed eggs in the identical hatchery conditions, and that northern and southern strains of European wild juvenile Atlantic salmon had an almost indistinguishable cardiac physiological response to warming after common garden rearing from eggs (Anttila et al., 2014b).

Support for domestication reducing the aerobic capacity of *Bolaks* strain comes from the lower $\dot{M}O_{2max}$, AAS, FAS and CS activity in white muscle when compared with the wild strain. Importantly, enhancement of AAS and FAS in the wild strain was solely through $\dot{M}O_{2max}$ with no change in SMR. Superior cardiorespiratory and muscular capacity of wild fish is likely the result of natural selection and adaptation to habitat, as observed in sockeye salmon (*Oncorhynchus nerka*; Eliason et al., 2011) and tropical killifishes (*Aphyosemion* sp.; McKenzie et al., 2013). Conversely, compared with wild or semi-wild hybrid strains, domesticated fish can have up to 9-

times lower aerobic enzyme activities in their swimming muscle (Anttila et al., 2008a; Anttila and Mänttäri, 2009), a lower swimming speed (McDonald et al., 1998; Reinbold et al., 2009) and a 25–84% lower holding velocity than wild yearlings (Rimmer et al., 1985), but a higher lipid concentration in swimming muscles (McDonald et al., 1998). Underpinning the differences in swimming capacity might be the cardiac malformations common to domesticated fish (Poppe and Taksdal, 2000; Mercier et al., 2000; Poppe et al., 2003; McKenzie et al., 2007; Kristensen et al., 2012b), but hearts were not examined here simply because it is unlikely that a routine aquaculture practice prior to smolt transfer will be cardiac sampling.

2.4.3 Segregation of superior and inferior swimmers with an exercise screening protocol

Despite good evidence that a more robust cardiorespiratory system positively correlates with a superior swimming capacity in salmonids (e.g., Keen and Farrell, 1994; Claireaux et al., 2005; Farrell, 2007; McKenzie et al., 2007), I found no support for our hypothesis that superior swimmers (top 20% of each strain and despite an almost 2-fold difference in their U_{max} compared with bottom 20% of each strain) benefitted from a higher aerobic capacity at the biochemical and whole animal levels, a more rapid recovery from exhaustion and a better hypoxia tolerance. Even an exploratory three-way MANOVA model restricted to just the fastest 10% swimmers ($140 \leq U_{max} \leq 145$ cm s^{-1}) and slowest 10% swimmers ($60 \leq U_{max} \leq 67.5$ cm s^{-1}) yielded no significant differences for any respiratory index. Previous screening protocols for Atlantic salmon smolts have similarly failed to segregate inferior and superior swimmers according to their routine metabolic rate, $\dot{M}O_{2max}$ and aerobic scope, despite a 55% difference in their swimming capacity (Anttila et al., 2014a). Theoretically, fish cannot swim faster or longer without a metabolic cost,

unless swimming is mechanically more efficient, a possibility not tested here. Interestingly, the numerical difference in EPOC between top and bottom 10% of both strains almost reached statistical significance (898.5 ± 63.8 vs. 729.8 ± 57.4 mg O_2 kg^{-1}, ANOVA: $F = 3.9$, $p = 0.06$), without any interaction between strain and swimming capacity. Therefore, perhaps the constant acceleration screening protocol used here does a better job of distinguishing anaerobic rather than aerobic capacity by segregating aerobic swimmers and burst-and-coast swimmers, two swimming phenotypes previously observed in wild European sea bass (*Dicentrarchus labrax*) (Marras et al., 2010). Fish use different gaits (burst and sprint) and muscle groups (glycolytic white skeletal muscle) to swim faster and this is why a prolonged swimming test uses incremental water velocities lasting 10 min (Jones, 1982; Rome, 1992; Hammer, 1995; Burgetz et al., 1998; Martínez et al., 2003). Our screening protocol was adapted from constant acceleration test to measure U_{max}, which is approximately 33% higher than the prolonged swimming speed (Farrell, 2008). Swimming behaviours and willingness to swim also play a role in determining U_{max} (Anttila et al., 2014a), and these factors could help explain the observed results. For example, weaker swimmers in a group of 60 fish in a 2-m long swimming tunnel could position themselves behind better swimmers to use slipstream and reduce the energetics of swimming (Bell and Terhune, 1970; Anttila et al., 2014a) and therefore mask differences in cardiorespiratory indices for the truly superior swimmers. Future studies could focus, therefore, on alternative screening protocols that do a better job of assessing aerobic performance and using a commercial scale screening apparatus that can accommodate larger numbers of fish. With regard to the former, transitions in swimming gait (Peake and Farrell, 2004, 2005, 2006) could be used to highlight different swimming modes.

2.4.4 SMR estimation method

SMR is at the foundation of many of the indices reported in the current dataset (AAS, FAS, EPOC and O_{2crit} all depend on SMR). According to the definition of SMR, which is the metabolism that supports basic homeostasis without any input into locomotion, digestion, growth and reproduction, SMR should locate at the left side of the distribution curve for a range of $\dot{M}O_2$ values measured over several days in quiescent, post-absorptive fish (Steffensen, 1989; Steffensen et al., 1994; Chabot et al., 2016). However, effectively isolating true SMR values from active $\dot{M}O_2$ due to minor locomotory activity is not a trivial task. Therefore, to be objective, I provided two estimates of SMR (q0.2 and the Low10) and their derivatives (Table 2.1). The Low10 estimate will always be lower than the q0.2 estimate of SMR because it averages the lowest 10 values (here we also eliminated the lowest 2% of $\dot{M}O_2$ measurements as a compensation for any measurement errors that would underestimate SMR). Given the size of the $\dot{M}O_2$ dataset used here, these lowest 10 values would approximate a q0.12.

In conclusion, this study demonstrated that cultivation of Atlantic salmon has likely compromised athletic robustness and plasticity. The wild *Lærdal* strain had a significantly higher $\dot{M}O_{2max}$, AAS, FAS and CS activity in white muscle when compared with domesticated *Bolaks* strain. The wild *Lærdal* strain showed plasticity in response to aerobic exercise training through improvements to $\dot{M}O_{2max}$, AAS, EPOC and CS activity in red muscle, as well as LDH activity in white muscle, whereas training only improved CS activity in red muscle of domesticated *Bolaks* strain. These results suggest that the salmon breeding companies should further investigate the implications of their strong focus on growth performance because it appears to have negative consequences on athletic robustness.

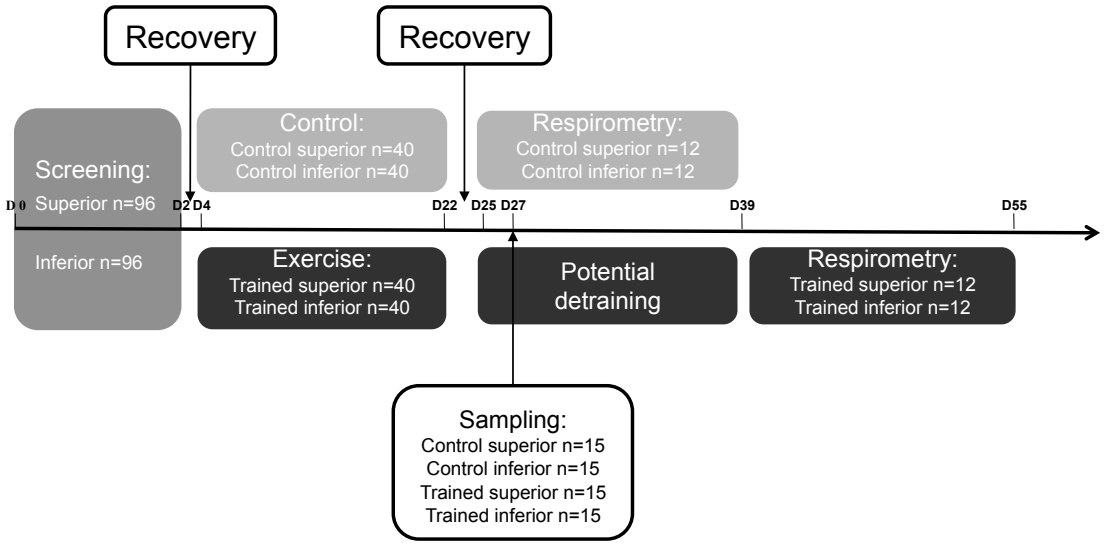
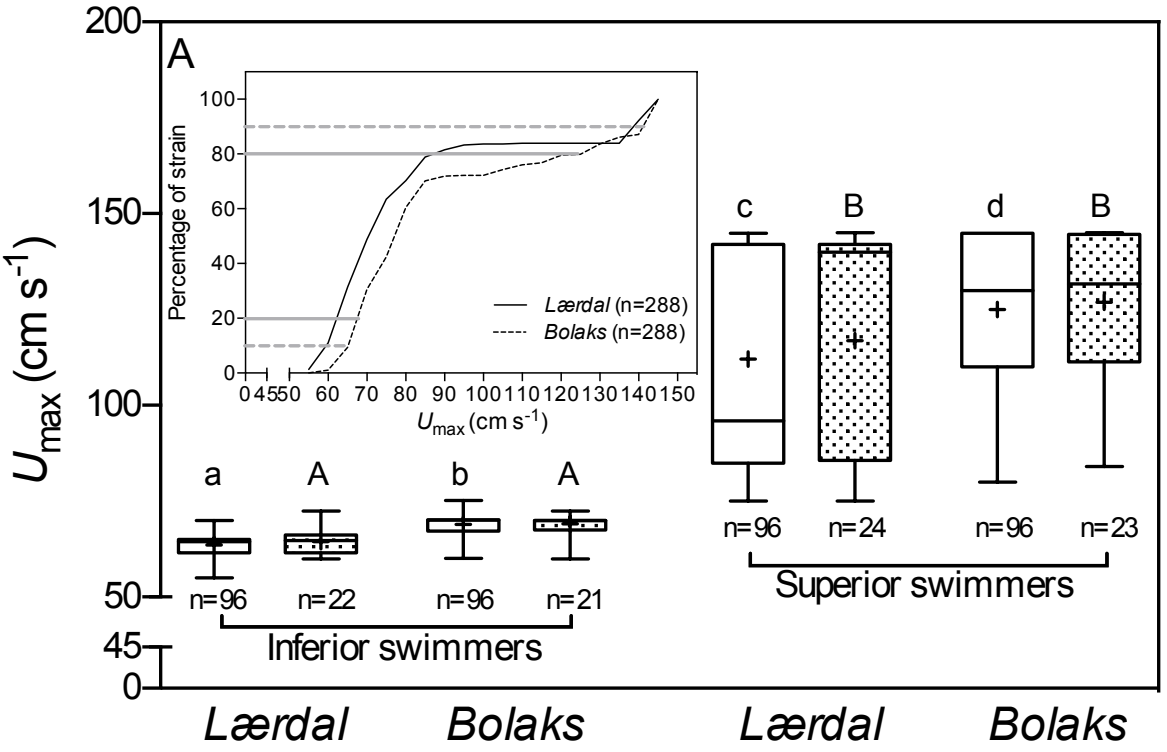


Figure 2.1. Experimental design and timeline of screening, training, tissue sampling for enzyme activity assays, and respirometry conducted on *Lærdal* (wild) and *Bolaks* (domesticated) Atlantic salmon (*Salmo salar*) parr. An identical experimental design was practiced on the two strains.


A
Percentage of strain
U_{max} (cm s^{-1})
Lærdal (n=288)
Bolaks (n=288)
a
A
b
A
c
B
d
B
n=96
n=22
n=96
n=21
n=96
n=24
n=96
n=23
Inferior swimmers
Superior swimmers
Lærdal
Bolaks
Lærdal
Bolaks

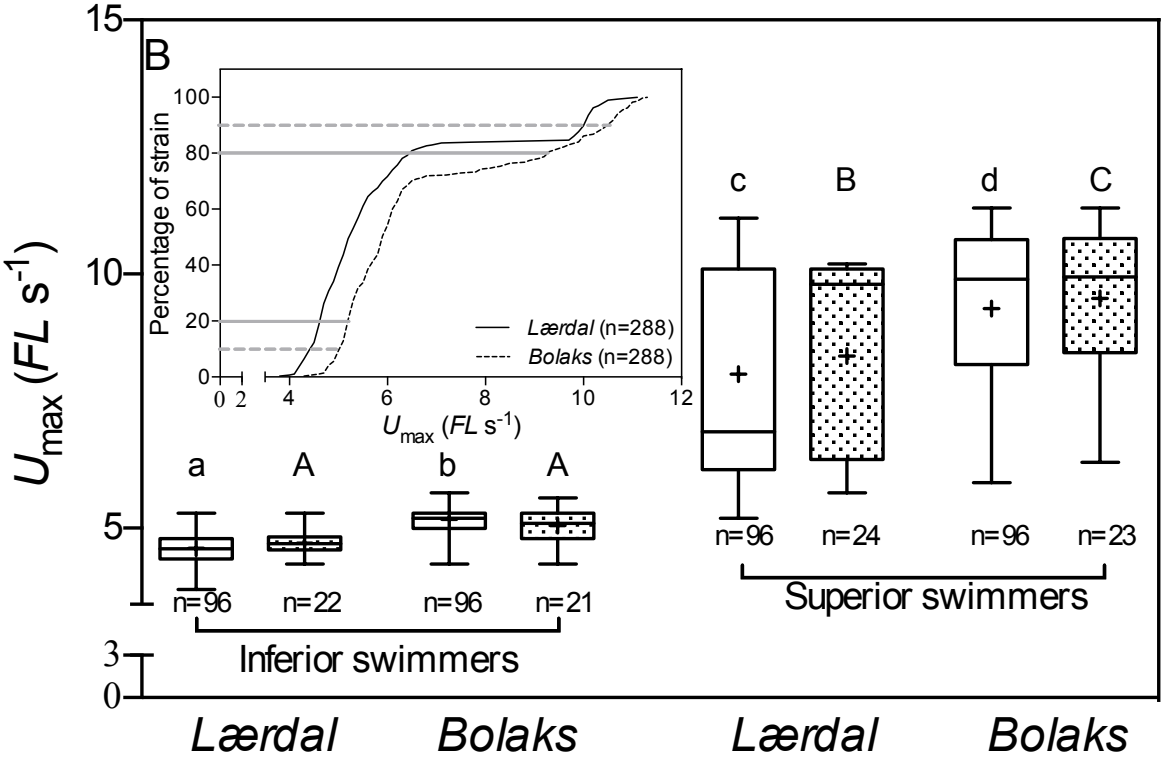


Figure 2.2. A) Absolute and B) relative maximum swimming capacity (U_{max}) of inferior and superior swimmers of the *Lærdal* (wild) and *Bolaks* (domesticated) Atlantic salmon (*Salmo salar*) parr. The results for the screening test (n=96) and the sub-sample used for respirometry (n=21-24) are presented separately. Each boxplot shows the interquartile range and 95% coefficient variation with the mean is shown as '+'. Different lowercase letters indicate significant differences ($P < 0.05$) in screening test and different uppercase letters indicate significant differences in the fish sampled by respirometry, detected by two-way ANOVA Tukey HSD *post-hoc*. There were no significant differences ($P > 0.05$) between fish tested by screening protocol and the fish sampled by respirometry in respective swimming groups, tested by Student's t-test. The insets show the cumulative frequency polygons for U_{max} of both strains. The inferior or superior 10% and 20% of swimmers are indicated as horizontal dash and solid lines respectively.

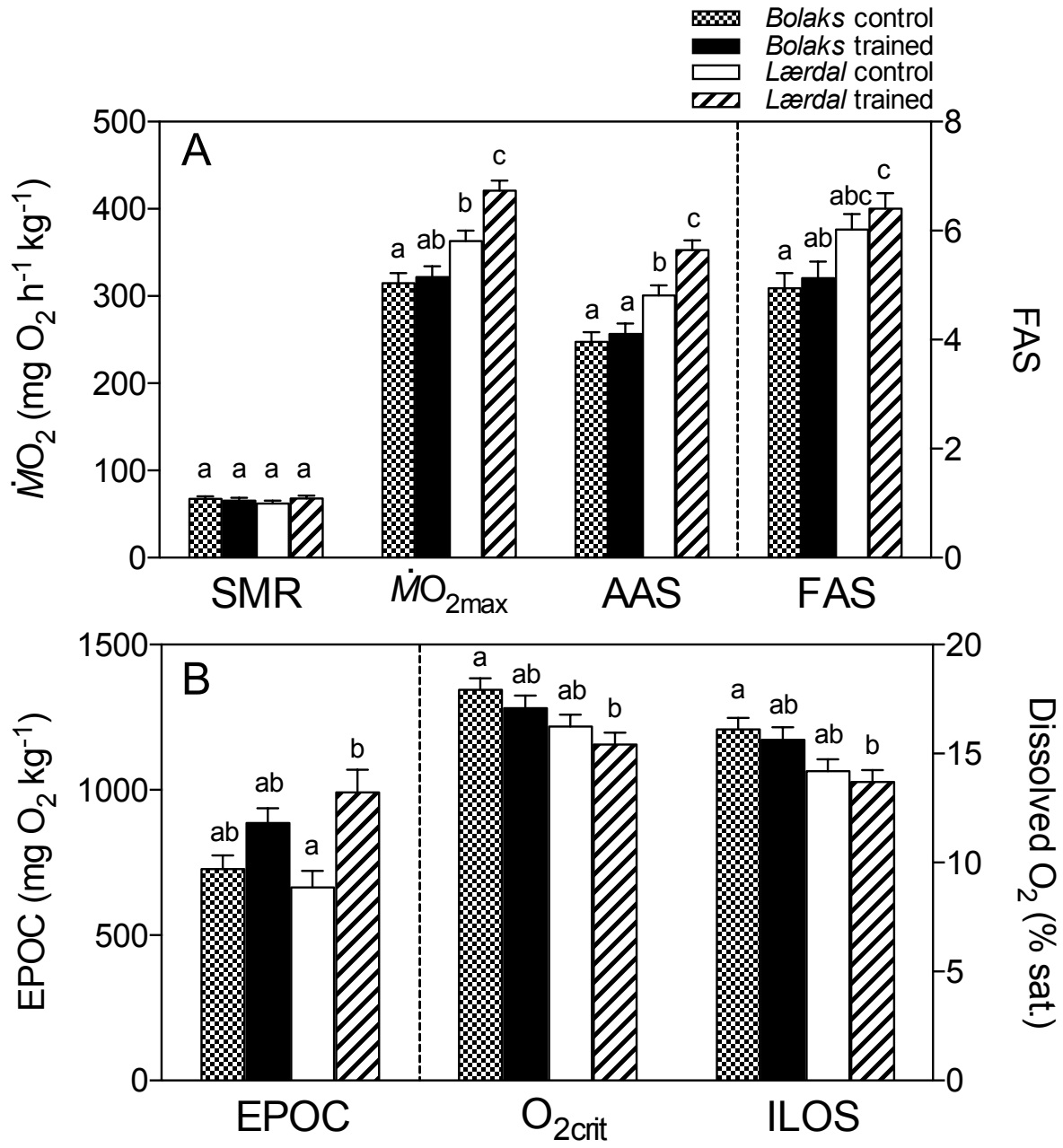

Bolaks control
Bolaks trained
Lærdal control
Lærdal trained
A
$\dot{M}O_2$ (mg O_2 h^{-1} kg^{-1})
FAS
SMR
$\dot{M}O_{2max}$
AAS
FAS
B
EPOC (mg O_2 kg^{-1})
Dissolved O_2 (% sat.)
EPOC
O_{2crit}
ILOS

Figure 2.3. Interactive effects of domestication and exercise training on A) standard metabolic rate (SMR), maximum rate of oxygen uptake ($\dot{M}O_{2max}$), absolute aerobic scope (AAS) and factorial aerobic scope (FAS); B) excess post-exercise oxygen consumption (EPOC), critical oxygen level (O_{2crit}) and incipient lethal oxygen saturation (ILOS) of Atlantic salmon (*Salmo salar*) parr. Different letters indicate significant differences between groups within a respiratory index ($P < 0.05$) by two-way ANOVAs and Tukey HSD *post-hoc*. Values are mean±s.e.m., n=24 in *Bolaks* untrained, 21 in *Bolaks* trained, 23 in *Lærdal* untrained, 22 in *Lærdal* trained.

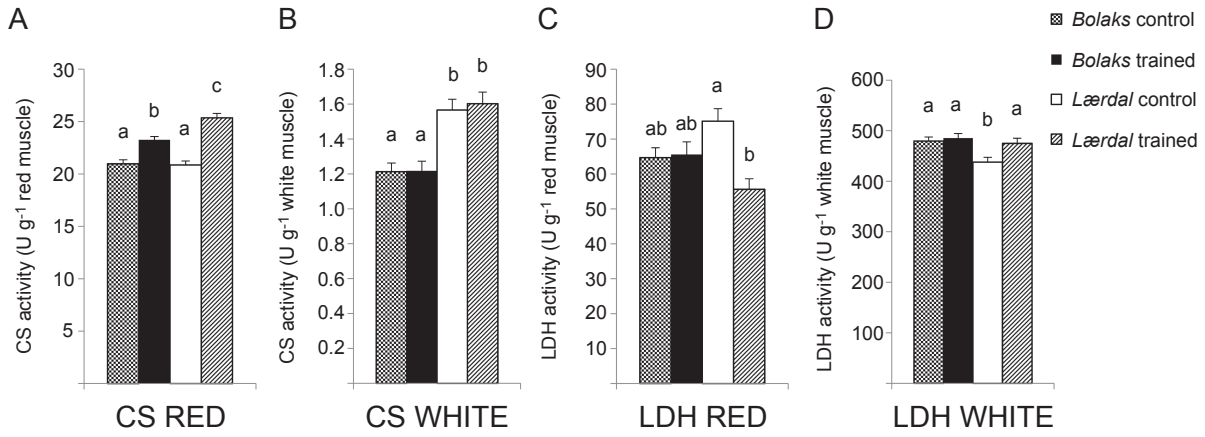


Figure 2.4. The activity of A) citrate synthase (CS) in red muscle; B) CS in white muscle; C) lactate dehydrogenase (LDH) in red muscle and; and D) LDH in white muscle of Atlantic salmon (*Salmo salar*) parr. Different letters indicate significant differences (*P* < 0.05) between groups within muscle types (two-way ANOVAs and Tukey HSD *post-hoc*). Values are means±s.e.m., n=15 per group.

Table 2.1. A summary of the main factor effects and significant interactions (F-values and *p*-values) of strain and training on respiratory variables of Atlantic salmon (*Salmo salar*). Standard metabolic rate (SMR) was calculated using two different SMR methods (Low10 and q0.2). Absolute aerobic scope (AAS), factorial aerobic scope (FAS), excess post-exercise oxygen consumption (EPOC) and critical oxygen level (O_{2crit}) also had two values corresponding with the two SMR calculation methods.

	Low10		q0.2	
	F-value	*p*-value	F-value	*p*-value
MANOVA				
Strain	**9.5**	**<0.001**	**9.7**	**<0.001**
Training	**6.8**	**<0.001**	**6.1**	**<0.001**
Strain × training	**3.2**	**0.002**	**3.2**	**0.002**
SMR				
Strain	0.77	0.38	0.45	0.51
Training	1.8	0.19	0.85	0.36
AAS				
Strain	**37.5**	**<0.001**	**36.8**	**<0.001**
Training	**9.7**	**0.003**	**8.9**	**0.0039**
Strain × training	**9.3**	**0.003**	**9.9**	**0.0025**
FAS				
Strain	**15.4**	**<0.001**	**12.6**	**<0.001**
Training	**8.4**	**0.005**	3.8	0.056
Strain × training	**6.0**	**0.012**	2.7	0.10
EPOC				
Strain	0.01	0.92	0.015	0.90
Training	**9.1**	**0.002**	**7.5**	**0.008**
Strain × training	1.0	0.32	0.18	0.67
O_{2crit}				
Strain	**9.7**	**0.0028**	**9.7**	**0.003**
Training	**16.0**	**<0.001**	**14.8**	**<0.001**
Strain × training	**4.5**	**0.038**	**5.6**	**0.028**

Table 2.2. A summary of the main factor effects and significant interactions (F-values and *p*-values) of strain and training on aerobic and anaerobic capacities of Atlantic salmon, illustrated by maximum rate of oxygen uptake ($\dot{M}O_{2max}$), lethal oxygen saturation (ILOS), citrate synthase (CS) and lactate dehydrogenase (LDH) in red and white muscles

Factor	F-value	*p*-value
$\dot{M}O_{2max}$		
Strain	**19.1**	**<0.001**
Training	2.7	0.1
Strain × training	**4.6**	**0.036**
ILOS		
Strain	**10.8**	**0.002**
Training	**11.2**	**0.001**
Strain × training	**5.4**	**0.024**
CS red muscle		
Strain	0.23	0.64
Training	**31.7**	**<0.001**
Strain × Training	**8.4**	**0.005**
CS white muscle		
Strain	**17.8**	**<0.001**
Training	1.2	0.29
LDH red muscle		
Strain	0.83	0.36
Training	3.3	0.073
Strain × Training	**5.3**	**0.025**
LDH white muscle		
Strain	**10.9**	**0.0016**
Training	1.6	0.22

Chapter 3: CHEMICALLY DISPERSED OIL HAS A CHRONIC RESIDUAL EFFECT ON HYPOXIA TOLERANCE IN EUROPEAN SEA BASS (*DICENTRARCHUS LABRAX*)

3.1 Introduction

European sea bass (*Dicentrarchus labrax*) inhabit coastal ecosystems after a period of larval and juvenile development in shallow salt marshes and lagoons that are common along Western and Mediterranean Europe. While these highly productive ecosystems supply the nutritional resources required by the fast growing juveniles (Pickett and Pawson, 1994), they are prone to aquatic hypoxia. Hence, juvenile sea bass have evolved an ability to cope with the challenge of periodic hypoxia. This ability of sea bass to cope with reduced oxygen availability is indicated, for instance, by a capacity to accumulate an oxygen deficit that is comparable to the bottom dwelling and hypoxia resistant common sole (*Solea solea*; Claireaux and Chabot 2016). European sea bass is also known to have a high maximum rate of oxygen uptake ($\dot{M}O_{2max}$) required for a piscivorous adult life that involves capture fish in open water and digest while swimming (Dupont-Prinet et al., 2009; Jourdan-Pineau, et al., 2015). Shallow marine ecosystems (e.g. *Erika* crude oil spill in the Bay of Biscay) are particularly exposed to the influence of the anthropogenic activities concentrated along coastlines and in river estuaries. Among these, exposure to accidental oil spill has a high probability. The documented negative impacts of oil exposure upon fish physiology and performance are many (Mauduit et al., 2016) and include compromised oxygen extraction (Claireaux et al., 2004), aerobic capacity (Claireaux and Davoodi, 2010) and swimming performance (Hicken et al., 2011; Mager et al., 2014). Because

very little information is available concerning residual effects remaining after an acute oil exposure, I investigated whether or not sea bass can recover their aerobic and anaerobic capacities and in hypoxic episodes after an acute exposure to chemically dispersed oil.

An acute crude oil exposure causes physical changes in gills such as aneurysms and lifting of gill secondary lamellae epithelium (Claireaux et al., 2004; Kochhann et al., 2015). If oxygen diffusion into arterial blood is impaired and the hypoxemia is uncompensated, fish must increase their reliance on glycolytic metabolism (Farrell and Richards, 2009). Fish can compensate for hypoxemia by increasing gill ventilation, gill perfusion and cardiac output (Perry, 1998; Farrell and Richards, 2009; Gamperl and Driedzic, 2009), but these compensatory responses also increase the gill exposure to the water-soluble polycyclic aromatic hydrocarbons (PAH) component of oil (Whitehead, 2013), accelerating PAH uptake and potentially increasing tissues perfusion, especially the heart, with PAH contaminated blood (Cohen et al., 2001; Heideman et al., 2005). Indeed, syndromes of PAH-induced cardiotoxicity include cardiac particulate accumulation (Millemann et al., 2015), pericardial edema, disruptions to cardiac looping during embryonic development (Incardona et al., 2013), alteration to excitation-contraction coupling in cardiomyocytes (Brette et al., 2014) and impaired cardiac contraction (Tissier et al., 2015). These symdrones result in the chronic cardiorespiratory aberrations (Hicken et al., 2011) that would potentially limit oxygen extraction and delivery under hypoxia. Indeed, both oil and hypoxia acutely reduce aerobic capacity (Davoodi and Claireaux 2007; Gamperl and Driedzic, 2009; Claireaux and Davoodi, 2010) and swimming performance (Kennedy and Farrell, 2006; McKenzie et al., 2007; Mager et al., 2014). In the present study, I combined and developed some of the current respiratory indices to comprehensively study the

chronic residual effects of an acute exposure to chemically dispersed crude oil on whole-animal respiratory robustness.

Sub-lethal acute impacts of toxicants on the aerobic metabolism in fishes have been previously diagnosed using the rate of oxygen uptake ($\dot{M}O_2$) and aerobic scope (Sprague, 1971; Rice, 1990; MacKinnon and Farrell, 1992). While whole-animal aerobic performance is well studied since Brett (1964), its ability to survive in hypoxia is not. In terms of performance in hypoxia, oxygen uptake can be measured while progressively decreasing dissolved oxygen (DO) (Neill and Bryan, 1991; Neill et al., 1994; Claireaux and Chabot, 2016) to reveal the systemic impact of hypoxia on the metabolic scope. $\dot{M}O_{2max}$ progressively decreases with DO until it reaches a critical oxygen level (O_{2crit}), when $\dot{M}O_{2max}$ equals standard metabolic rate (SMR). Below O_{2crit}, SMR can no longer be supported aerobically and anaerobic glycolysis is switched on (Wang et al., 1994; Speers-Roesch et al., 2012). With further decreases in DO, fish ultimately reach their incipient lethal oxygen saturation (ILOS; the DO when a fish loses its ability to maintain equilibrium) as anaerobic metabolic wastes accumulate and glycogen is depleted in a time- and DO-dependent manner (Nilsson and Östlund-Nilsson 2008; Mandic et al., 2013). Although O_{2crit} and ILOS are the two most established indices of performance in hypoxia (Ultsch et al., 1978; Mandic et al., 2009; Claireaux et al., 2013; Claireaux and Chabot, 2016), it is important to emphasize that O_{2crit} is a measure of hypoxia tolerance while ILOS actually measures hypoxia resistance. Between O_{2crit} and ILOS, a variety of compensatory mechanisms, including the glycolytic metabolism, govern fishes' scope for survival in hypoxia (Hochachka, 1990). To elucidate fish response to oxygen level less than O_{2crit}, I developed three new indices, scope for oxygen deficit (SOD), factorial scope for oxygen deficit (FSOD) and accumulated oxygen deficit (AOD). These indices aimed at quantifying scope for survival (Hochachka, 1990)

by integrating the severity and the effective time of hypoxia (Claireaux and Chabot, 2016). Thus, I define the trait of hypoxic survival as the ability to maintain oxygen extraction from water (hypoxia tolerance) and the ability to temporarily support metabolic needs with glycolytic metabolism (hypoxia resistance).

For the current study, I tested the hypothesis that fish with a higher ILOS switched on glycolytic metabolism at a higher DO, and therefore accumulated plasma glucose and lactate sooner during a hypoxic event, than fish with a lower ILOS. To test this hypothesis, I pre-screened two-year-old and yearling populations of European sea bass in an established hypoxia challenge test (HCT; Claireaux et al., 2013) and precautionarily assigned the two extremes of the wide breadth of ILOS distribution as hypoxia tolerant (HT) and hypoxia sensitive (HS) phenotypes. I then haphazardly sampled plasma glucose and lactate from the two-year-old population during the progress of hypoxia. Meanwhile, yearling population went through a 7-month longitudinal study to reveal the chronic residual effects of chemically dispersed crude oil on the repeatability of hypoxic survival traits under seasonal water temperature. Within this, a cross-sectional respirometry study allowed us to further test the hypothesis that aerobic capacity and the ability to survive in hypoxia of HT and HS phenotypes would be chronically impaired despite a prolonged recovery from an acute oil exposure, as seen by a reduced aerobic scope, as well as elevated O_{2crit} and ILOS, without affecting scope for survival in the oil-treated fish.

3.2 Materials and methods

3.2.1 Experimental design

All sea bass were obtained from the same commercial farm (Aquastream, Lorient, France). Experiments were conducted in two phases (Figure 3.1). The first phase used a population of two-year-old European sea bass to specifically compare the time course of plasma glucose and lactate accumulation in hypoxia tolerant (HT) and hypoxia sensitive (HS) phenotypes during a hypoxic exposure. To achieve this objective, fish with RFID tags were first submitted to a hypoxia challenge test (HCT) to measure the individual incipient lethal oxygen saturation (ILOS). After recovery, the challenge test was repeated while fish were haphazardly sampled to measure plasma glucose and lactate. The second phase of the experiment consisted in investigating the influence of oil exposure on cardiorespiratory performance of yearling European sea bass. The effects of oil exposure on the individual repeatability of hypoxia resistance were assessed with pre- and post-exposure HCT to chemically dispersed crude oil or a control exposure. June–October (3–7 months post-exposure), the yearling fish were maintained in the laboratory under identical control conditions prior to a 18-day respirometry testing period.

3.2.2 Animals rearing conditions

Upon arrival in the laboratory (Ifremer, Brest, France) fish populations were treated identically. They were placed in a 2000-L indoor tank and acclimated for at least 2 months to local temperature (10–20 °C), photoperiod and salinity (30–32 PSU). They were fed via an

automatic feeder (2.5% body weigh daily) with a commercial diet (Neo Start Coul 2, Le Gouessant, France). Two weeks before an experiment started, fish were anaesthetized (MS-222; 100 mg L^{-1}) and implanted subcutaneously with an identification tag (RFID; Biolog-id, France). Fish were unfed for 48 h before experiments. All procedures followed current animal care rules and regulations in France.

3.2.3 Changes in plasma metabolites during hypoxia

Sea bass (n = 50; 33.9 ± 3.1cm, 284.6 ± 12.2 g) were subjected to a HCT on Dec 12, 2013 to determine individual's ILOS (water temperature = 11 °C). The RFID tag allowed individual fish to be characterized as hypoxia sensitive (HS; ILOS > 5.6 % sat.; n=17; Figure 3.2) or hypoxia tolerant (HT; ILOS < 5.6 % sat.; n=18; Figure 3.2). During the second HCT, on Dec 21, 2013, 35 fish were terminally and haphazardly sampled for blood at a 2-h time intervals. Blood samples (1 ml) were withdrawn from the caudal vein using a heparinized syringe and centrifuged (8 min, 8000 rpm, 4°C) to collect the plasma. Samples were frozen in liquid nitrogen before being stored at -20 °C until assayed for lactate and glucose using commercial kits (Biomérieux; reference: 61.192 and 61.269, respectively) and a spectrophotometer (Uvikon 923; λ = 505 nm).

3.2.5 Crude oil exposure effects on cardiorespiratory performances

3.2.5.1 Exposure protocol and PAH bioaccumulation assessment

On April 1st, 2014, fish (n = 161; 11.3 ± 1.1 cm, 17.7 ± 5.0 g) were transported to the CEDRE (distance from Ifremer laboratory = 12 km) and habituated in 2 m^3 tanks supplied with open flow sea water in seasonal water temperature, salinity and photoperiod profiles. Fish were off-feed. The exposure arena was a set of 12 polyethylene tanks (300 L) equipped with home-made oil/water mixing devices comprising a funnel and a submersible bilge pump (Milinkovitch et al., 2011). Two experimental treatments, control and chemically dispersed oil (250 g of oil mixed with 10 g of dispersant), were tested in triplicate. The amount of dispersant (Finasol OSR 52, Total Fluides) applied was in accordance with manufacturer's recommendation.

The oil exposure protocol started with bubbling the oil with air overnight to mimic a 12-h aging of an oil slick at sea (Nordvik, 1995; Mauduit et al., 2016). Fish were then allocated to the various exposure tanks in a way that avoided statistical difference in ILOS among the tanks (27 fish per tank). Crude oil and dispersant was mixed in a bottle before being poured into the tanks. During the 48-h exposure period, DO in the tanks was maintained above 85 % air saturation (% air sat). Following the exposure period (48 h), fish were recovered and briefly bathed in clean seawater, to avoid cross-contamination, and transferred to their original rearing tanks, where they recovered for one week before being transported back to Ifremer. Control fish went through the identical processes without being exposed to oil. No fish mortality occurred during either oil exposure or recovery.

To assess the bioaccumulation of oil and document the detoxification process, 3 fish per tank (n = 9 per treatment) were euthanized to measure the presence of polycyclic aromatic

hydrocarbons (PAH) in the bile and liver 24-h and liver 1-month after exposure. Liver concentrations of 20 PAH (including the components listed by United States Environmental Protection Agency) were assessed by a procedure fully described in Lacroix et al. (2014). Briefly, PAH were extracted from livers samples and concentrations determined using an alkaline digestion combined with stir bar sorptive extraction–thermal desorption–gas chromatography–mass spectrometry (SBSE–GC–MS). Bile polycyclic aromatic hydrocarbons metabolites concentration was determined semi-quantitatively using fluorescence response (Aas et al., 2000). It consisted in measuring the fluorescence with a spectrophotometer with a 5 nm slit width on emission and excitation channels (Jasco FP-6200, Tokyo, Japan). Analyses were performed using three excitation-emission wavelengths i.e., 295-335 (naphthalene-type metabolites); 343–383 nm (four-ringed compounds) and 380–430 nm (benzo[a]pyrene-type metabolites) (Krahn et al., 1987; Lin et al., 1996; Aas et al., 2000).

3.2.5.2 Hypoxia challenge test

Hypoxia challenge tests (HCT) were conducted on February 27th, 2014 (33 days pre-oil exposure; 10°C) and May 20th, 2014 (49 days post-oil exposure; 15°C). During the period fish were exposed to the seasonal variation in photoperiod and water temperature. The HCT (see details in Claireaux et al., 2013) consisted in a rapid decrease of water oxygenation (from nearly 100% to 20% sat. in 40 min) followed by of a slower decrease (0.2% sat. per 10-min) until the end of the experiment. Hypoxia was induced by bubbling nitrogen in the admission of a submersible pump placed in the experimental arena. The bubbling was regulated by a controller and solenoid valve (Oxy-REG; Loligo Systems, Tjele, Denmark) which ensured the repeatability

of the experimental protocol. Water oxygenation was monitored using an oxygen meter (Ponsel Mesure, Caudan, France; accuracy: ± 0.2 % sat.). When a fish lost its ability to maintain balance, it was quickly removed from the experimental arena, identified (RFID tag reading) and placed in a fully aerated tank. The corresponding time and oxygenation level were also recorded. Experiments ended when the last fish was recovered (typically > 2 % sat.). Control and oil-exposed yearling fish were categorized as either hypoxia tolerant (HT; ILOS < 8.25 % sat.; n= 40) or hypoxia sensitive (HS; ILOS ≥ 8.25 % sat.; n = 56) phenotypes according to the bimodal ILOS distribution in the post-oil exposure HCT (Figure 3.2).

3.2.5.3 Respirometry trials

The breadth of ILOS in yearling population (Figure 3.2) was represented in the 18-day respirometry trials. This phase of the experiment was performed starting 167 days post-oil exposure and at 16.5 ± 0.5°C. This comprehensive testing lasted 2-3 days and tested 8 fish simultaneously (Zhang et al., 2016). All respirometry trials were completed within 18 days, so that on average SMR, $\dot{M}O_{2max}$ and O_{2crit} were measured 176 days post-oil exposure. A set of eight automatic intermittent-flow respirometers (volume = 2.1 L each) were submerged in a water reservoir (2 m x 0.6 m x and 0.4 m deep), where aerated water was re-circulated through a temperature regulator (Teco, Seachill TR20) and each of the respirometers (Steffensen, 1989; Svendsen et al., 2016). Computer-controlled flush pumps (Compact 600, EHEIM, Germany), relays and software (AquaResp, University of Copenhagen, Helsingør, Denmark) regulated the individual flow to each respirometer. Another pump mixed the water within the respirometry chamber involved a circulation loop, into which an optical oxygen probe (Robust Oxygen Probe

OXROB3, Pyroscience, Germany & Implantable Oxygen Microsensor, PreSens, Germany) was inserted to continuously monitored DO (% sat.). Hypoxic water was obtained by pumping (Compact 600, EHEIM, Germany) water from the water reservoir to the top of a gas equilibration column where it trickled down through nitrogen gas.

To start with, fish were fasted for 72 h to reach a post-absorptive stage prior to respirometry, because specific dynamic action can last 41 h in a 35 g sea bass fed 3% body mass at 21 °C (Dupont-Prinet et al., 2010). Prior to every respirometry trial, each oxygen probe was calibrated to 0 % sat. (sodium sulfite saturated water) and 100 % sat. (fully aerated water). Each respirometry trial began with a chasing protocol, during which each fish was individually hand chased in a 20-L bucket for 10-min and then given a 2-min air exposure so that $\dot{M}O_{2max}$ could be estimated (Clark and Norin, 2016). Exhausted fish were immediately placed in a respirometer during a flushing cycle and continuous DO monitor began immediately to measure $\dot{M}O_2$ during recovery. During the test period, fish were left undisturbed in individual intermittent-flow respirometer. The first $\dot{M}O_2$ value was available 250 - 370 s after the fish were placed in the respirometer. Each measurement cycle followed the decline in DO when the respirometer was sealed and was followed by a flushing cycle to replenish the respirometer with water from the surrounding tank and a short stabilization period after the respirometer was closed before the next measurement cycle. Short measurement cycles (120 s flush, 30 s stabilization and 220 s measure) captured the high $\dot{M}O_2$ values in exhausted fish while DO remained > 85 % sat.. As $\dot{M}O_2$ declined toward SMR, the cycles were elongated after 2 h (60 s flush, 30 s stabilization and 440 s measure). These measurements continued for 2 or 3 days in a shaded, quiet room, allowing the fish to fully adjust to the respirometer and reach SMR (see Chabot et al., 2016; Zhang et al., 2016).

At the end of this period, I progressively decreased DO in the water reservoir where the respirometers were submerged. DO of the water decreased progressively to 10% sat. over an 8-h period, during which $\dot{M}O_2$ continued to be measured, which allowed us to determine O_{2crit} post-experiment (Section 2.2.5). Animal welfare concerns required that I terminated the respirometry experiment right before fish lost their equilibrium, which required ILOS to be determined by extrapolating ILOS values from post-exposure HCT. Fish recovered quickly in well-oxygenated 16.5 °C water after they were removed from the respirometers. Background $\dot{M}O_2$ of each respirometer chamber was measured without fish for 30-min after each trial. To eliminate microbial respiration, the entire system was disinfected with household bleach for 30-min on completion of each respirometry trial.

3.2.6 Data analysis

I assessed individual SMR (minimal maintenance metabolism in post-absorptive and inactive states; Fry and Hart, 1948; Chabot et al., 2016), $\dot{M}O_{2max}$, absolute aerobic scope (AAS = $\dot{M}O_{2max}$ – SMR; Fry, 1971; Claireaux et al., 2005; Pörtner and Farrell, 2008), factorial aerobic capacity (FAS = $\dot{M}O_{2max}$ / SMR, Clark et al., 2005), critical oxygen level (O_{2crit}; Ultsch et al., 1978; Claireaux and Chabot, 2016), scope for oxygen deficit (SOD), factorial scope for oxygen deficit (FSOD) and accumulated oxygen deficit (AOD) in the post-experiment analyses (see section 3.2.5).

For each determination of $\dot{M}O_2$, the slope of declining DO in the respirometer was calculated by linear regression. Only DO recordings with a linear decline in DO and an $R^2 > 0.94$ were accepted for an accurate measurement of $\dot{M}O_2$. Typically, the first $\dot{M}O_2$ was designated

$\dot{M}O_{2max}$ because it was the highest $\dot{M}O_2$ value after the exhausted fish was placed into the respirometer. However, fish were spontaneously active over the next 2-3 days and in some cases (n=16) a higher active metabolic rate (AMR) was recorded and this higher value was assigned to $\dot{M}O_{2max}$. SMR was determined using a quantile method (p = 0.2 using an R script; Chabot et al., 2016) applied to 2-3 days of continuous $\dot{M}O_2$ measurements excluding the first 12 h of recovery in the respirometer and the $\dot{M}O_2$ obtained during the O_{2crit} test (a total of 326 to 489 $\dot{M}O_2$ values). Using an R script (Claireaux and Chabot, 2016), O_{2crit} was derived from the intersection of SMR and a regression line for the final $\dot{M}O_2$ measurements as a function of DO.

The dynamic relationship between O_{2crit}, ILOS and time was assessed with the SOD (% sat., O_{2crit} – ILOS), the FSOD (dimensionless, $O_{2crit}\,ILOS^{-1}$) and AOD (mg $O_2\,kg^{-1}$, defined below). Because the trait of hypoxic survival was repeatable (Joyce et al., 2016), this enabled us to use the relationship between $\dot{M}O_2$ and DO obtained from the O_{2crit} analysis (Figure 3.3) to calculate the $\dot{M}O_2$ value that corresponded with the DO values in the HCT for each fish. Since HCT provided a relationship between time and DO (Figure 3.3), estimated $\dot{M}O_2$ was plotted against time between the DO values corresponding to O_{2crit} and ILOS (Figure 3.3). Finally, AOD was an integration of $\dot{M}O_2$ as a function of time for the area bounded by SMR and the calculated $\dot{M}O_2$ from O_{2crit} to ILOS (Figure 3.3).

The ILOS between HT and HS phenotypes of plasma sampled fish was analyzed by Student's t-test. Plasma lactate and glucose were analyzed by a two-way (time x phenotype) analysis of covariance (ANCOVA) with body mass as covariate, followed by Tukey-Kramer *post-hoc* tests. Bile fluorescence measurements were compared between control and oil groups by Student's t-test in 290-355 nm, 343-383 nm and 380-430 nm wavelenghes. The sum of 20 PAH components in the livers were compared between control and oil groups at day 2 and day

31 by pairwise t-tests with *Holm-Šídák* correction. Repeatability of hypoxic survival traits throughout the study was assessed with Spearman rank-order correlation coefficient on ILOS in pre- and post- exposure HCT, as well as extrapolated ILOS and measured O_{2crit} in respirometry in control and oil-treated fish, respectively.

In order to correct heterogeneity of variance in O_{2crit}, $\log_{10}$ transformations were applied. To test the dispersed oil as a main effect, Welch one-way ANOVA compared the SMR, $\dot{M}O_{2max}$, AAS, FAS, $LogO_{2crit,}$ ILOS, SOD, FSOD and AOD between control and oil groups. To test the phenotypically specific responses to dispersed oil, Welch one-way ANOVA with Tukey's Kramer *post-hoc* was applied to compare the same suite of indices between control HT, control HS, oil HT and oil HS groups. Statistical analyses were conducted in R (ver.3.2.2; R Development Core Team, 2015) and SPSS v. 23 (SPSS Inc., Chicago, IL, USA). Statistical significance was assigned to $\alpha \leq 0.05$. Values were presented as mean ± s.e.m..

3.3 Results

3.3.1 Plasma glucose and lactate in HT and HS phenotypes

The ILOS of two-year-old fish (n=35) ranged 4.5~7.0 % sat. (Figure 3.2). The HT phenotype was assigned to the ILOS less than 5.6 % sat. and the HS phenotype was assigned to the ILOS larger than 5.6 % sat.. When water oxygen level reached O_{2crit} (approx. 15% sat.), plasma lactate and glucose levels were similar for HT and HS phenotypes. However, below O_{2crit}, plasma lactate and glucose increased significantly faster in the HS phenotype compared with HT phenotype (Figure 3.4). Thus, HS had a significantly higher plasma lactate than HT phenotype (16.8 ± 2.4 vs. 5.8 ± 0.8 mM) when DO had reached 5.7 % sat. $p < 0.001$) at ~7 h into

HCT. As a result, HT phenotype lasted 20% longer than HS phenotype in the HCT. At ILOS, however, both phenotypes displayed similar peak values for plasma lactate and glucose (lactate: HS = 14.7 ± 1.1 mM; HT = 16.8 ± 2.4 mM; Glucose: HS = 1.88 ± 0.10 g L^{-1}; HT = 1.95 ± 0.16 g L^{-1}; Figure 3.4).

3.3.2 Dispersed oil exposure

3.3.2.1 Bio-accumulation of PAH

As expected, 2 days post-exposure, oil-treated fish displayed significantly higher PAH levels in bile than control fish across the three wavelengths tested (Figure 3.5). Specifically, oil-treated fish had a 21-fold higher reading at 343-383 nm (1444 ± 50.9 vs. 69.6 ± 3.3 arbitrary units, $p < 0.001$), 9-fold higher reading at 380-430 nm (223.1 ± 9.9 vs. 24.0 ± 1.3 arbitrary units, $p < 0.001$) and 2-fold higher reading at 290-335 nm (771.2 ± 82.8 vs. 383.2 ± 12.7 arbitrary units, $p < 0.001$). Similarly, 2 days after exposure, liver PAH was 26-fold higher for oil-treated fish than control fish (1508.9 ± 158.6 vs. 58.9 ± 6.3 ng g^{-1} dw, $p < 0.001$). However, PAH detoxification and excretion over a 31-day recovery period reduced liver PAH concentration in oil-treated fish by 47-fold, a value only 8-fold higher than the control fish on day 31 (31.9 ± 11.7 vs. 4.0 ± 0.7 ng g^{-1} dw, $p = 0.02$), but was same as the control fish at day 2 (Figure 3.6).

3.3.2.2 Phenotypes of hypoxia resistance and their repeatability in control fish

The wide breadth of ILOS displayed both during pre-screening (2.6~11.0 % sat.; 33 days before dispersed oil exposure) and 49 days post-exposure (5.1~10.1 % sat.) (Figure 3.2). This

was properly represented in the HS and HT phenotypes used for respirometry trials. As a result, at the second screening, the control HT fish had a 31% lower ILOS than the control HS fish (6.2 ± 0.1 vs. 9.0 ± 0.2 % sat., $p < 0.001$). Subsequently, the respirometry experiments confirmed this the segregation because the control HT had a 24% lower O_{2crit} and 31% lower ILOS than the control HS phenotype (O_{2crit}: 14.0 ± 0.3 vs. 18.4 ± 0.6 % sat., $p = 0.007$; ILOS: 6.2 ± 0.1 vs. 9.0 ± 0.2 % sat., $p < 0.001$). Nevertheless, SOD, FSOD and AOD were the same for the 2 phenotypes ($p \geq 0.25$; Figure 3.8). Moreover, the temporal repeatability of fish's ability to survive hypoxia was examined by assessing the individual rank-order of the three ILOS values and O_{2crit} over 7 months. Specifically, control group had significant correlations between the ranks of O_{2crit} and the three ILOS values ($r \geq 0.718$, $p \leq 0.013$; Table 1). Only one control individual shifted from HS to HT phenotype so that trait of hypoxic survival in control group was stable over the 7-month period (Figure 3.7; $r \geq 0.673$, $p \leq 0.023$; Table 1).

3.3.2.3 Chronic residual effects of chemically dispersed oil

Dispersed oil had no main effect on any of the respirometry metabolic indices ($p > 0.3$; Table 2). When sea bass were segregated into HT and HS phenotypes, dispersed oil also had no residual effect on aerobic metabolic indices (SMR, $\dot{M}O_{2max}$, AAS, FAS) in either phenotype. Nevertheless, chemically dispersed oil diminished the repeatability of hypoxia resistance in pre- and post- exposure HCTs ($r = 0.355$, $p = 0.162$; Table 1). Although ILOS of post-exposure HCT and extrapolated ILOS of respirometry in oil-treated fish were repeatable after the oil exposure ($r = 0.988$, $p < 0.001$; Table 1), the extrapolated ILOS and measured O_{2crit} in respirometry was not correlated ($r = 0.142$, $p = 0.586$). A subtle residual effect of the dispersed oil was evidence of a

reduced hypoxic survival in HT phenotype. Specifically, oil-treated HT fish had a 24% higher O_{2crit} than control HT fish (17.4 ± 0.7 vs. 14.0 ± 0.3 % sat., $p = 0.034$), even though ILOS did not differ between these two groups (6.5 ± 0.2 vs. 6.2 ± 0.1 % sat., $p = 0.8$). Meanwhile, oil-treated HT and HS phenotypes had the same O_{2crit} ($p = 0.673$), while only ILOS of oil-treated HT was 25% lower than the HS counterparts (6.5 ± 0.2 vs. 8.7 ± 0.1 % sat., $p < 0.001$). Therefore, chronic residual effects on the HT phenotype, such as significant elevations of SOD by 40% (10.9 ± 0.5 vs. 7.8 ± 0.3 % sat., $p = 0.035$), FSOD by 17% (2.7 ± 0.04 vs. 2.3 ± 0.06, $p = 0.003$) and AOD by 57% (291.1 ± 28.9 vs. 185.8 ± 13.6 mg O_2 kg^{-1}, $p = 0.008$), were driven by O_{2crit} rather than ILOS. In contrast, acute exposure of HS fish to dispersed oil had no chronic residual effect on O_{2crit}, SOD, FSOD and AOD ($p > 0.226$). Control HT and HS phenotypes, however, had the same SOD, FSOD and AOD ($p \geq 0.25$; Figure 3.8).

3.4 Discussion

The general objective of this work was to examine how an acute chemically dispersed oil exposure chronically affects the repeatability of hypoxic survival traits in European sea bass, hence I can have appreciable understanding of their recovery potential after an oil spill. This goal was pursued using a comprehensive suite of respiratory metabolic indices to assess aerobic capacity and the ability of sea bass to survive hypoxia after oil exposure. To ensure a good representation of hypoxia resistance, I prescreened yearling and two-year-old populations. A wide breadth of ILOS distribution and assigned the two extremes of the distribution as hypoxia tolerant (HT) and hypoxia sensitive (HS) phenotypes. I provided support for the hypothesis that HS phenotype switched on glycolytic metabolism at a higher DO than HT phenotypes, because

HT phenotype accumulated plasma glucose and lactate faster during progressive hypoxia. Hence, I focused on the phenotypically specific responses to the oil exposure. Because the study of the chronic residual effect ran for 7 months, I examined the repeatability of hypoxic survival traits and revealed that chronic residual effects of oil diminished such repeatability and the correlation between O_{2crit} and ILOS. Thus, I distinguished between hypoxia tolerance (O_{2crit}) and hypoxia resistance (ILOS), and evaluated an integration of glycolytic capability and compensatory mechanisms using new indices, such as the scope for oxygen deficit (SOD), factorial scope for oxygen deficit (FSOD) and the accumulated oxygen deficit (AOD). Even though polycyclic aromatic hydrocarbons (PAH) had initially bioaccumulated during the acute oil exposure, and were subsequently either metabolized or excreted during recovery (Tuvikene 1995; Blahova et al. 2013, Dévier et al. 2013), the residual effects were, however, evident in the elevations of $O_{2crit,}$ SOD, FSOD and AOD in the HT phenotype, but not the HS phenotype. The elevation in O_{2crit} is particularly important as it suggests that the oil-exposed HT phenotype now switches to glycolytic metabolism at the same DO as the HS phenotype. The fact that the HT phenotype was still challenged to regain its ability to survive severe and prolonged hypoxia 7-month post-exposure is also of importance as it suggests a differential loss in Darwinian fitness under suboptimal oxygen conditions (Mauduit et al., 2016).

3.4.1 Divergent hypoxia resistance phenotypes and their repeatability

The faster accumulation rate of plasma lactate and glucose in the HS phenotype during progressive hypoxia supported the hypothesis that the fish with an onset of glycolytic metabolism at a higher DO than others would result in a faster accumulation rate of the

metabolites. The lower ILOS in HS as compared to HT phenotypes in both two-year-old and yearling sea bass populations, are similar to the lower ILOS and O_{2crit} of the southern compared with the northern subspecies of killifish (*F. heteroclitus heteroclitus*) (McBryan et al., 2016). A divergent hypoxia resistance was also evident among related sculpins species, with LOE_{50} (time for 50% of group lost their equilibrium) of silverspotted sculpins (*Blepsias cirrhosus*) being 37% shorter than staghorn sculpins (*Leptocottus armatus*) and tidepool sculpins (*Oligocottus maculatus*). The lactate accumulation (in brain, liver and muscle tissues), however, was similar when the three species lost equilibrium (Speers-Roesch et al., 2013), as observed for sea bass in the present study. Hence, I assigned left and right portions of the ILOS distribution as HS and HT phenotypes, respectively, in the exposed yearling sea bass population to study the phenotypically specific response to acute chemically dispersed oil exposure.

Furthermore, the fact that accumulation rates of plasma glucose and lactate were differed in the HS and HT phenotypes indicated the repeatability of hypoxia resistance trait, because these tagged two-year-old fish were haphazardly sampled at the second HCT. Whole-animal hypoxia resistance is indeed a temporally stable trait in control European sea bass, as demonstrated both in present and previous studies (Figure 3.7; Joyce et al., 2016). A significant correlation between the HCTs pre- (10 °C) and post- (15 °C) oil exposure (r = 0.691, p = 0.019; Table 1, Figure 3.7) suggested the repeatability was remained when the control fish were measured under seasonal water temperatures. In fact, the change of temperature shifted the left bout of ILOS distribution in the pre-exposure HCT to a higher DO in the post-exposure HCT (Figure 3.2). The traits of hypoxic survival in control fish were also stable under the same water temperature in post-exposure HCT and respirometry (Figure 3.7).

The ILOS of oil-treated fish measured under seasonal water temperature, however, was not repeatable (r = 0.355, p = 0.162; Table 1, Figure 3.7). The rank-order between extrapolated ILOS and measured O_{2crit} in respirometry was also not stable in oil-treated fish (r = 0.142, p = 0.586; Table 1, see Figure 3.7). Hence, the diminished repeatability of hypoxic survival traits could be the consequences of impaired oxygen extraction and delivery abilities (Richards, 2011; Joyce et al. 2016), decreases in arterial blood stores and tissue oxygen extraction efficiency, as well as shifting of SMR induced by either toxicant metabolizing and metabolic depression (Farrell and Richards, 2009; Tierney et al., 2013). Despite the modulations of compensatory mechanisms and oxygen demands collectively affect trait of hypoxic survival, fish must maintain the basic metabolic needs by glycolytic metabolism if DO is under O_{2crit} (Nilsson and Ostlund-Nilsson, 2008). Therefore, I explained the oxygen extraction at gill and the glycolytic metabolism by using individual respirometry to measure aerobic capacity and the ability to survive hypoxia in oil-treated HS and HT phenotypes

3.4.2 Absence of chronic residual effects on aerobic capacity

Contrary to my hypothesis, an acute exposure to chemically dispersed oil did not chronically affect SMR, $\dot{M}O_{2max}$, AAS and FAS of juvenile European sea bass regardless of their hypoxia resistance phenotypes. I used the best practices to measure these aerobic indices over 2-3 days (see Chabot et al., 2016 for SMR; Norin and Clark, 2015 for $\dot{M}O_{2max}$). Indeed, values for $\dot{M}O_{2max}$ (388.4 ± 18.9 and 392.1 ± 16.8 mg O_2 h^{-1} kg^{-1}, respectively) and AAS (296.6 ± 18.0 and 302.0 ± 15.8 mg O_2 h^{-1} kg^{-1}, respectively) of control and oil-treated fish were similar to $\dot{M}O_{2max}$ (432 ± 17 mg O_2 h^{-1} kg^{-1}) and aerobic metabolic scope (AMS) (357 ± 18 mg O_2 h^{-1} kg^{-1})

measured in fasted sea bass (264 g) swum to U_{crit} (Jourdan-Pineau et al., 2015), as were the values for oil-treated sea bass. Therefore, change in SMR did not cause the chronic residual effect of dispersed oil on the reduction of repeatability for hypoxic survival.

Acute effects of oil exposure are detected immediately post-exposure in other U_{crit} tests. For example, McKenzie et al. (2007) found a mixture of organic pollutants (including PAH) impaired the ability of chub (*Leuciscus cephalus*) to repeat a U_{crit} test without affecting AAS, after a 4-week cage exposures in the contaminated river. Likewise, oil-exposed (up to 48 h) mahi-mahi had a 22~37 % reduction of U_{crit} without affecting AMS (Mager et al., 2014). Zebrafish also had a 18% reduction of U_{crit} that was detected 1 year after a 48-h oil exposure (Hicken et al., 2011). Because U_{crit} performance involves both aerobic and anaerobic metabolism, the impaired performance could be possibly caused by the reduced anaerobic capacity.

Previous experiments measure $\dot{M}O_2$ immediately after petroleum hydrocarbons exposure have similarly reported no changes in SMR, AMR and AMS. This is, for example, the case in the golden grey mullet (*Liza aurata*) (Milinkovitch et al., 2012) and in the embryonic/larval mahi-mahi (*Coryphaena hippurus*) (Mager et al., 2014). Chronic (2-6 months) sub-lethal ingestion of PAH in juvenile (2-month-old) and adult (6-month-old) also did not affect SMR, $\dot{M}O_{2max}$ and AAS of zebrafish (*Danio rerio*) (Lucas et al., 2016). Hence, these results suggested that impaired aerobic metabolism is not the cause of the impaired U_{crit}. I proposed that subtle residual effects affected the oxygen extraction (measured by O_{2crit}) and the extent of glycolytic metabolism (estimated by SOD, FSOD and AOD), as discussed below.

3.4.3 Underpinning mechanism of the oil-induced O_{2crit} elevation

The residual effect of chemically dispersed oil was evident as a 24% elevation of O_{2crit} in the HT but not HS phenotype on average of 176 days after oil exposure. Likewise, oil exposure, O_{2crit} of common sole (*Solea solea*) increased by 52% (oil vs. control: 3.5 vs. 2.3 mg O_2 L^{-1}) and by 64% 5 days after oil exposure (oil vs. control: 4.1 vs. 2.5 mg O_2 L^{-1}), respectively (Claireaux et al., 2004; Davoodi and Claireaux, 2007). Richards (2011) summarized that O_{2crit} is influenced by the variation in gill surface area, Hb-O_2 binding affinity and SMR. In present study, Hb-O_2 binding affinity was less likely affected by residual effects, because oil-treated fish had the same $\dot{M}O_{2max}$ as control fish and transfer of oxygen across gill epithelia is perfusion-limited under normoxic conditions (Malte and Weber, 1985; Schmidt et al., 1988). Unless, oil-treated fish had a higher Hb-O_2 binding affinity that was more efficient at transporting O_2 in hypoxia, but I did not have any evidences that suggested this. The residual effect was also absent on SMR, thereby I eliminated that the higher basic metabolic needs drove O_{2crit} higher.

I reasoned that the elevated O_{2crit} corresponded with both oil-induced thickening of gill lamellae and reduction of functional gill surface area (Claireaux et al., 2004; Kochhann et al., 2015). For instance, rainbow trout (*Oncorhynchus mykiss*) displayed the fusion of lamellae tips after 7-day oil exposure (Engelhardt et al., 1981). Likewise, oil-sand-refining wastewater exposed (< 96 h) fathead minnows (*Pimephales promelas*) presented severe edema in the secondary lamellae (Farrell, et al., 2004). Oil-exposed (up to 30 day) tambaqui (*Colossoma macropomum*) had also shown lifting of epithelium, hypertrophy of epithelium and fusion of lamellae (Kochhann et al., 2015). In demersal fish, yellow sole (*Pleuronectes asper*) and rock sole (*Pleuronectes bilineatus*) had gill hyperplasia after a 90-day oil exposure (Moles and

Norcross, 1998). Likewise, 50% of lamellae had shown fusion of lamellae tips in oil-exposed (3-week) juvenile turbot (*Scophthalmus maximus*) (Stephens et al., 2000). Meanwhile, 83% of oil contaminated (5-day) common sole displayed thinning of primary epithelium, as well as aneurysm and complete disappearance of the epithelium on the secondary lamellae (Claireaux et al., 2004).

Gill is extremely plastic to external stimuli and its morphological modifications are expected, but the modifications sometimes come with trade-offs on oxygen uptake. Thickening of gill epithelia in rainbow trout, caused by chloride cell proliferation, could lead to an impairment of oxygen uptake under moderate to severe hypoxia (Thomas et al 1988; Bindon et al., 1994; Greco et al., 1995). Cold acclimation also induces the basal epithelial thickening, decreasing diffusional losses of ions and water across gill lamellae when the oxygen uptake was reduced (Leino and McCormick, 1993). Therefore, I concluded that oil-induced thickening of gill epithelium (Daxboeck et al., 1982) and reduced functional gill surface area predominantly (Julio et al., 2000) compromise the oxygen extraction in hypoxia (Randall, 1982; Farrell and Richards, 2009).

Control HS phenotype had a 31% higher O_{2crit} compared to control HT phenotype. This could be associated with the thicker epithelia of HS phenotype in comparison to HT phenotype, which limited the oxygen diffusion at gill lamellae. I also reasoned that the absence of residual effect on the HS phenotype is also due to their thicker gill epithelia. The thicker barrier, as a protective mechanism, prevents toxicants from diffusing across epithelia (Mitchell and Cech 1983; Post 1983). For instance, fathead minnows had an increased interlamellar cell mass immediately after exposing to oil-sand-refining wastewater (Farrell et al., 2004). Therefore, a new hypothesis is that the thicker gill epithelia protect the HS phenotype from the residual effect

of acute oil exposure, but the thicker water-blood barrier induce the diffusion limitation in hypoxia and make the HS phenotype less tolerant to hypoxia.

3.4.4 Residual chronic effects revealed by new indices of scope for survival

As the consequence of diminished repeatability of hypoxic survival traits in oil-treated fish and the susceptibility of gill to oil exposure in HT phenotype, I discovered that O_{2crit}, other than ILOS, drove estimated magnitude of glycolytic metabolism in hypoxia, resulting in the 40% increase in SOD, 17% increase in FSOD and 57% increase in AOD in oil-treated HT phenotype. Thus, the dispersed oil chronically impaired the ability to extract oxygen from water at low DO, as previously documented in bald notothenid (*Pagothenia borchgrevinki*) immediately after a 7-day oil exposure (Davison et al., 1993). I present theoretical limiting oxygen level curves (Figure 3.9) to illustrate how O_{2crit} can be right-shifted without any effects on SMR and ILOS in the oil-treated HT phenotype, while substantially increases AOD. Specifically, oil-treated HT group switched to glycolytic metabolism at a higher DO than the control counterparts. Meanwhile, the oil-treated HT fish was resistant to hypoxia below O_{2crit} for the longest duration. These collectively led to the highest SOD, FSOD and AOD in oil-treated HT phenotype.

In fact, these results showed that O_{2crit} and ILOS characterize different physiological mechanisms. O_{2crit} measures the ability to extract DO from water into arterial blood (Chapman et al., 2002; Richards, 2011). ILOS is an integrated measure, including loss of brain ATP homeostasis (Speers-Roesch et al., 2013), as well as increased lactate accumulation and depleted glycogen stores in different tissues (Vornanen et al., 2009; Figure 3.4). A great example was that critical oxygen tension of sculpin species had a relatively narrow range of 26 to 54 torr, whereas

their LOE_{50} had a large variation, ranging from 25 to 538 min (Mandic et al., 2013). Further reductions of DO require anaerobic glycolysis to compensate for the energy supply (MacCormack et al., 2006; MacCormack and Driedzic, 2007), which leads to an accumulation of plasma lactate (Pörtner and Grieshaber, 1993), and eventually ILOS is reached (Richards et al., 2007; Nilsson and Östlund-Nilsson, 2008). The loss of equilibrium was possibly caused by the peak threshold levels of plasma lactate and glucose, as observed in present study and related sculpin species (Speers-Roesch et al., 2013). Nevertheless, none of the current indices for hypoxic survival (neither O_{2crit} nor ILOS) describes this physiological process. Thus, I coined SOD, FSOD, AOD to quantify this scope for survival that describes how far fish can be resistant to low DO in a time-dependent fashion.

AOD is a more comprehensive bioenergetic indice, allowing comparisons across individuals, populations and species by taking the time into account for oxygen deficit. Although, no direct comparison study is available for AOD, cumulative oxygen deficit (severity of hypoxic exposure x time) applied the same ideology and quantified that striped bass (*Morone saxatilis*) swam at 50% U_{crit} were over 5-fold less resistant to hypoxia than resting fish (Nelson and Lipkey, 2015). Based on the current study, I propose that a more accurate quantification of glycolytic metabolism should account for both glycolysis activity and its effective time, which is extremely important to understand the full potential of the ability to survive in hypoxia (Fry, 1971, Claireaux and Chabot, 2016).

AOD, however, can only correctly measure the extent of glycolytic metabolism, if rate of glycolytic metabolism is same and metabolic depression is absent. The residual effects of oil were less likely to affect the rate of glycolytic metabolism in HT phenotype, because oil-treated HT phenotype survived in severe hypoxia for the same duration as their control counterparts.

Although I did not quantify metabolic depression, AOD is a window to study it. If metabolic depression occurs, AOD should be larger than excess post-hypoxia oxygen consumption (EPHOC), the area bounded by $\dot{M}O_2$ and SMR when a fish is recovering in normoxia from a hypoxic event. Of course, these validations of AOD are worthy of future studying.

In conclusion, the wide breadth of ILOS distribution discovered here for European seabass represents a divergence of hypoxia tolerance and resistance because the HS phenotype begin to accumulate lactate at a higher DO than the HT phenotype. Residual effect of the chemically dispersed oil did not impair the aerobic capacity of sea bass, regardless of their hypoxia resistance phenotypes, however, it diminished the repeatability of hypoxic survival trait. Oil-treated HT, but not HS fish, had elevations of O_{2crit}, SOD, FSOD and AOD as chronic residual effects, despite an average of 176-day recovery from a 48-h oil exposure. The HT phenotype of sea bass contains an important gene pool for the species to thrive in periodically hypoxic habitats. Even subtle residual effects on this phenotype are certainly of great concern for the population dynamics, recruitment and production, when the HT sea bass may not pay off a larger oxygen deficit aerobically during hypoxic episodes.

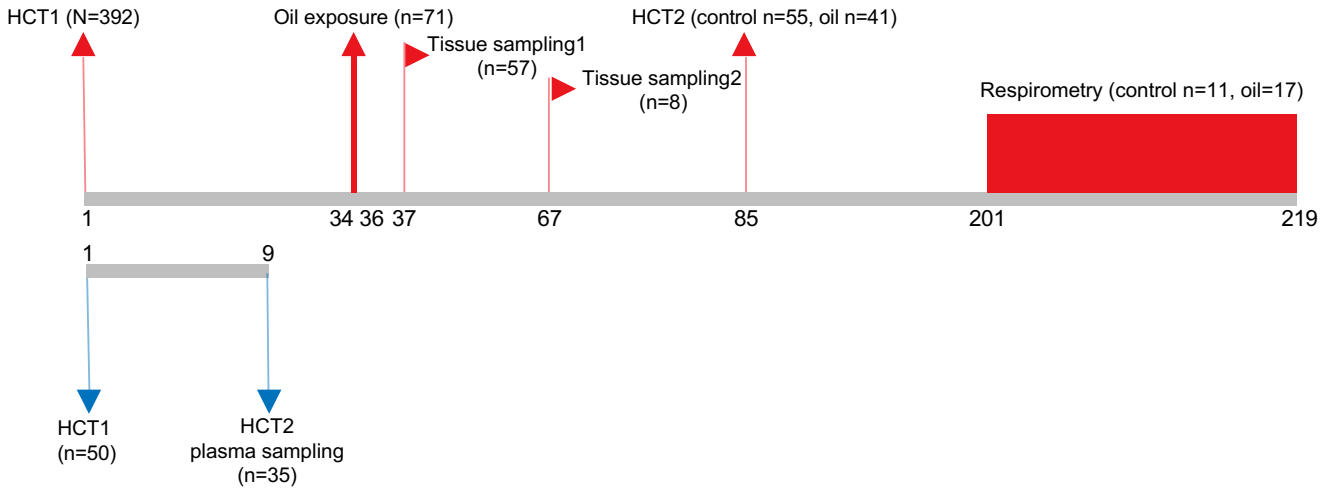


Figure 3.1 Timeline of the experimetns in the 2-year-old (blue) population of European sea bass (*Dicentrarchus labrax*) for plasma glucose and lactate sampling and the yearling population of European sea bass for oil contamintion study (red). HCT refers to hypoxia challenge test.

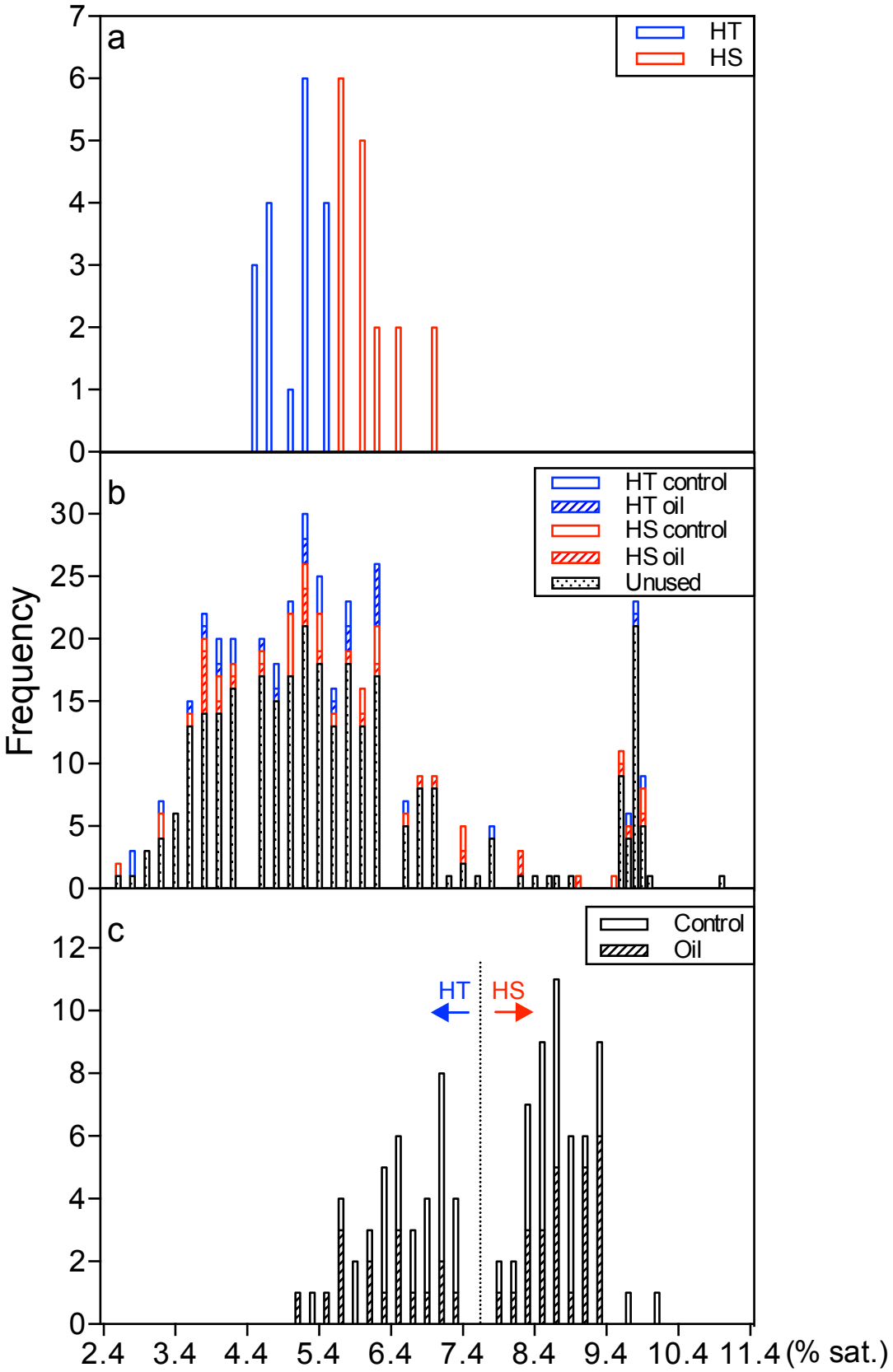

a
HT
HS
b
HT control
HT oil
HS control
HS oil
Unused
c
Control
Oil
HT
HS
Frequency
(% sat.)
2.4
3.4
4.4
5.4
6.4
7.4
8.4
9.4
10.4
11.4

Figure 3.2. Frequency distribution of incipient lethal oxygen saturation (ILOS) of European sea bass (*Dicentrarchus labrax*) measured in plasma metabolites sampling (a, n = 35, December, 2013), pre- (b, N = 392, February 2014) and post- (c, n = 96, May 2014) chemically dispersed oil exposure. In plasma metabolites sampling, I categorized the hypoxia sensitive (HS) phenotype as the first 18 fish that lost their equilibrium (a, ILOS > 5.6 % sat.), and the rest of 17 fish were hypoxia tolerant (HT) phenotype (a, ILOS < 5.6 % sat.). The breadth of ILOS in yearling population were represented in respirometry sampled fish as HT control, HT chemically dispersed oil exposed (oil), HS control and HS oil. The HT and HS phenotypes were categorized by the left bout (c, ILOS < 8.25 % sat.) and right bout (c, ILOS ≥ 8.25 % sat.) in post-exposure, respectively. The control oil groups were also labelled in panel c.

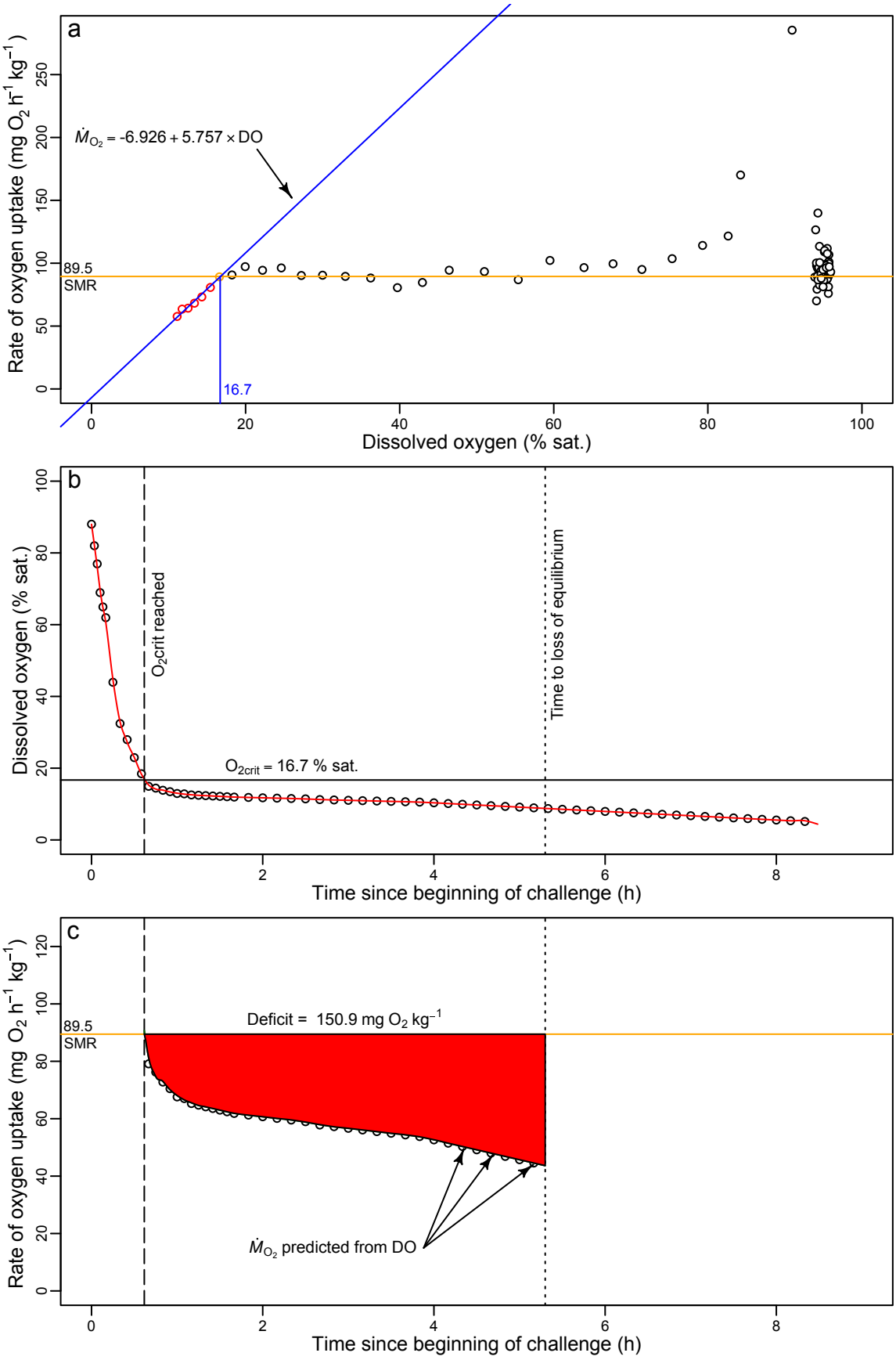

a
$\dot{M}_{O_2}$ = -6.926 + 5.757 × DO
89.5
SMR
16.7
Rate of oxygen uptake (mg O_2 h^{-1} kg^{-1})
Dissolved oxygen (% sat.)
b
O_{2crit} reached
Time to loss of equilibrium
O_{2crit} = 16.7 % sat.
Dissolved oxygen (% sat.)
Time since beginning of challenge (h)
c
Deficit = 150.9 mg O_2 kg^{-1}
89.5
SMR
$\dot{M}_{O_2}$ predicted from DO
Rate of oxygen uptake (mg O_2 h^{-1} kg^{-1})
Time since beginning of challenge (h)

Figure 3.3. Representative calculation of accumulated oxygen deficit (AOD) of a European sea bass (*Dicentrarchus labrax*) in a hypoxia challenge test (HCT). The $\dot{M}O_2 \sim$ DO regression equation obtained from (a) used dissolved oxygen level in (b) to calculate the rate of oxygen uptake ($\dot{M}O_2$) in (c). AOD was then an integration of $\dot{M}O_2$ as a function of time for the area bounded by standard metabolic rate (SMR) and the estimated $\dot{M}O_2$ between critical oxygen level (O_{2crit}) and incipient lethal oxygen saturation (ILOS).

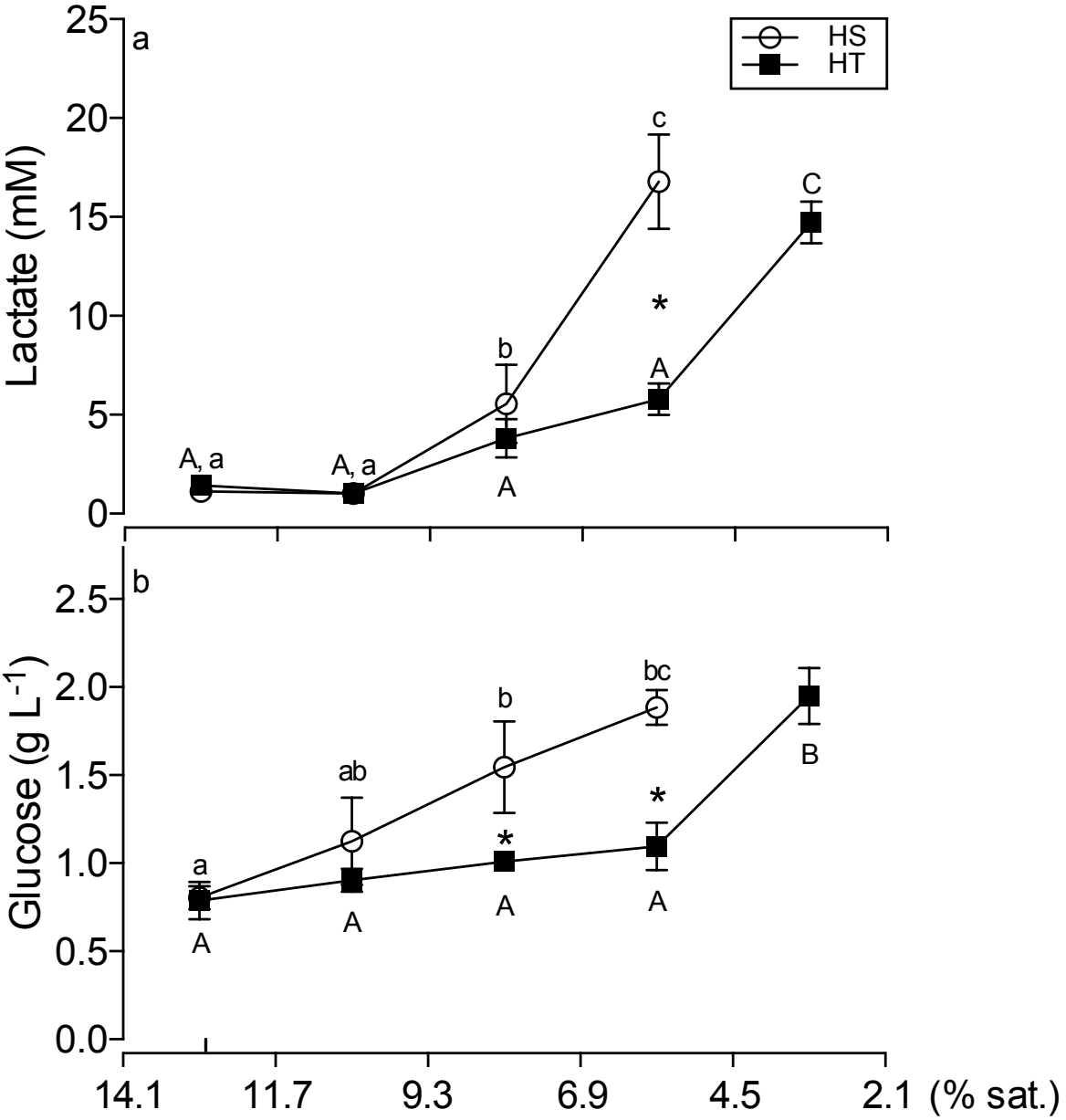

a
HS
HT
Lactate (mM)
25
20
15
10
5
0
A, a
A, a
b
A
c
*
A
C
b
Glucose (g L-1)
2.5
2.0
1.5
1.0
0.5
0.0
a
A
ab
A
b
*
A
bc
*
A
B
14.1
11.7
9.3
6.9
4.5
2.1
(% sat.)

Figure 3.4. a) Plasma lactate and b) plasma glucose concentrations of European sea bass (*Dicentrarchus labrax*) as a function of water air saturation (% sat.) during a hypoxia challenge test (HCT) on December 21, 2013. The fish were randomly sampled every 2 hours. To identify HT (n=17) and HS (n=18) phenotypes. Different upper case letters indicate the statistical significance of sampling time in hypoxia tolerant (HT) phenotype. Different lower case letters indicate the statistical significance of sampling time in the hypoxia sensitive (HS) phenotype. Asterisk indicate the significant differences between HT and HS phenotypes at the same sampling interval. Statistical significance ($P < 0.05$) was detected by two-way ANOVA followed by Tukey's Kramer *post-hoc* comparisons. N-values were 2–7 and error bars represent s.e.m.

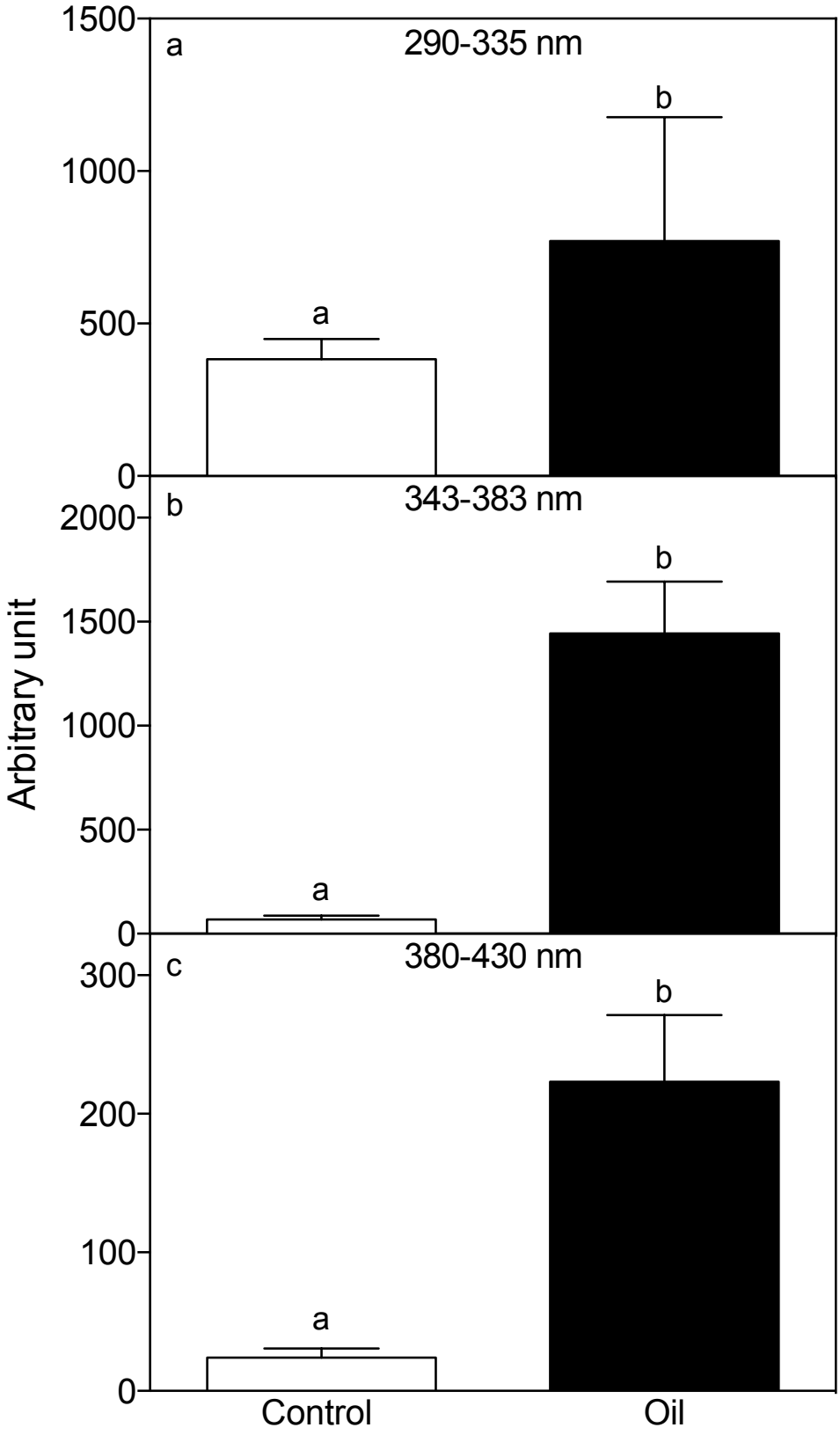

1500
1000
500
0
a
290-335 nm
b
a
b
343-383 nm
2000
1500
1000
500
0
b
b
a
c
380-430 nm
300
200
100
0
b
a
Arbitrary unit
Control
Oil

Figure 3.5. Fluorescence measured (arbitrary unit) polycyclic aromatic hydrocarbons at 48-h post chemically dispersed oil exposure at a) 290-355 nm b) 343-383 nm c) 380-430 nm wavelengths in bile of control (n=27) and chemcially dispersed oil exposed (oil) (n=24) European sea bass (*Dicentrarchus labrax*). Different letters indicated the significant differences ($P < 0.05$) between control and chemically dispersed oil (oil) groups by Student's t-test. Values were mean ± s.e.m..

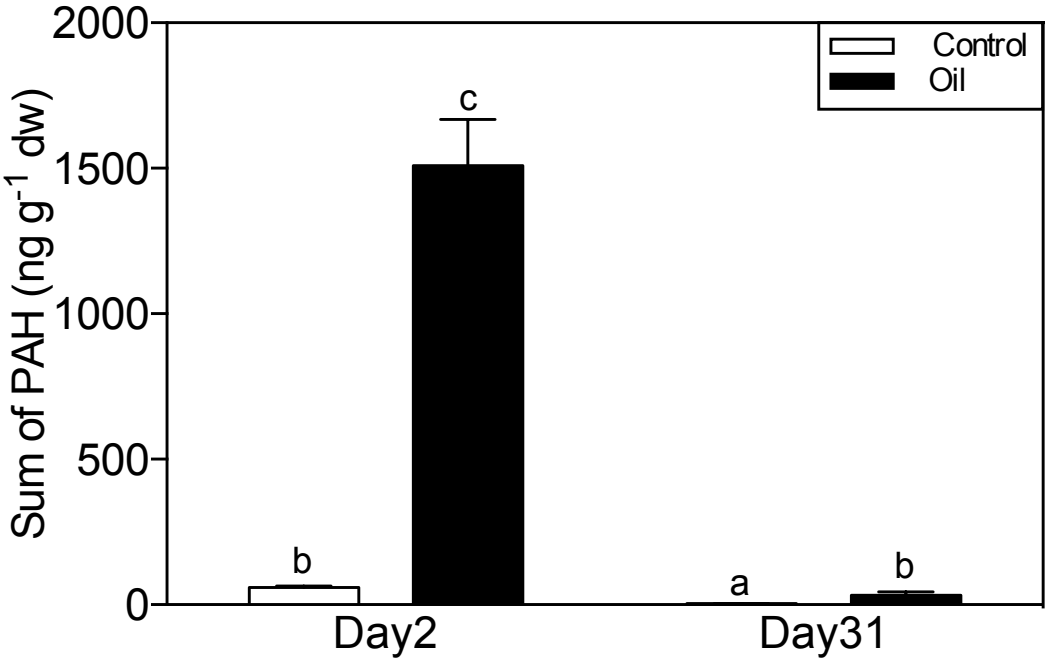


Figure 3.6. Sum of 20 polycyclic aromatic hydrocarbons components including the components listed by United States Environmental Protection Agency, in the livers of control and chemically dispersed oil (oil) exposed European sea bass (*Dicentrarchus labrax*) in day 2 and 31. Different letters indicated the statistical differences ($P < 0.05$) by pairwise t-tests with *Holm-Šídák* correction. N-values were 3–5 and error bars represent s.e.m..

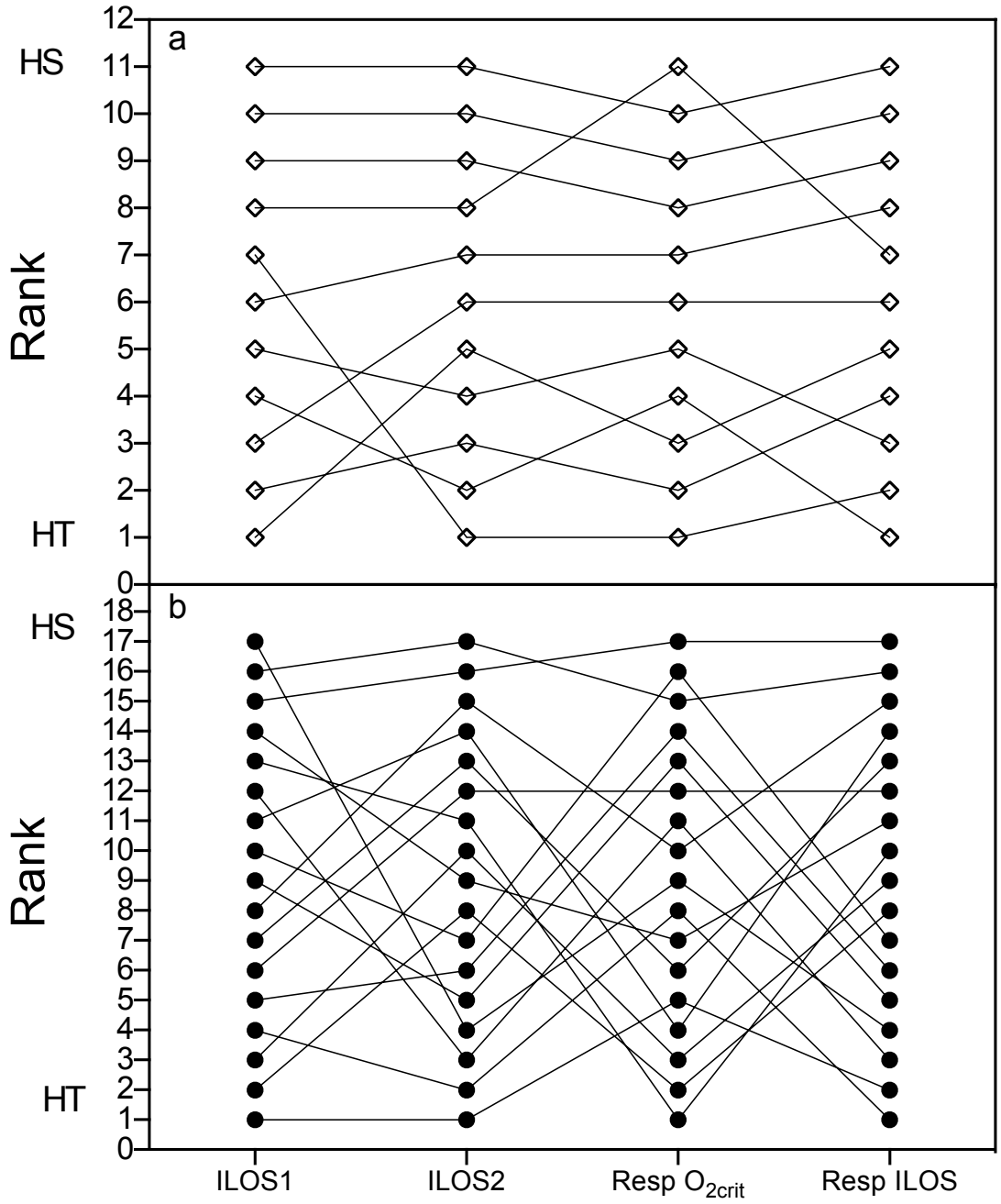

a
b
HS
HT
Rank
ILOS1
ILOS2
Resp O_{2crit}
Resp ILOS

Figure 3.7. Individual repeatability of hypoxic survival traits in control (a) and chemically dispersed crude oil exposed (b) European sea bass (*Dicentrarchus labrax*). The hypoxia sensitive (HS) and hypoxia tolerant (HT) fish were measured for incipient lethal oxygen saturation (ILOS) in pre- and post- exposure hypoxia challenge test (ILOS1 and ILOS2), as well as the extrapolated ILOS and critical oxygen level (O_{2crit}) measured in respirometry (Resp).

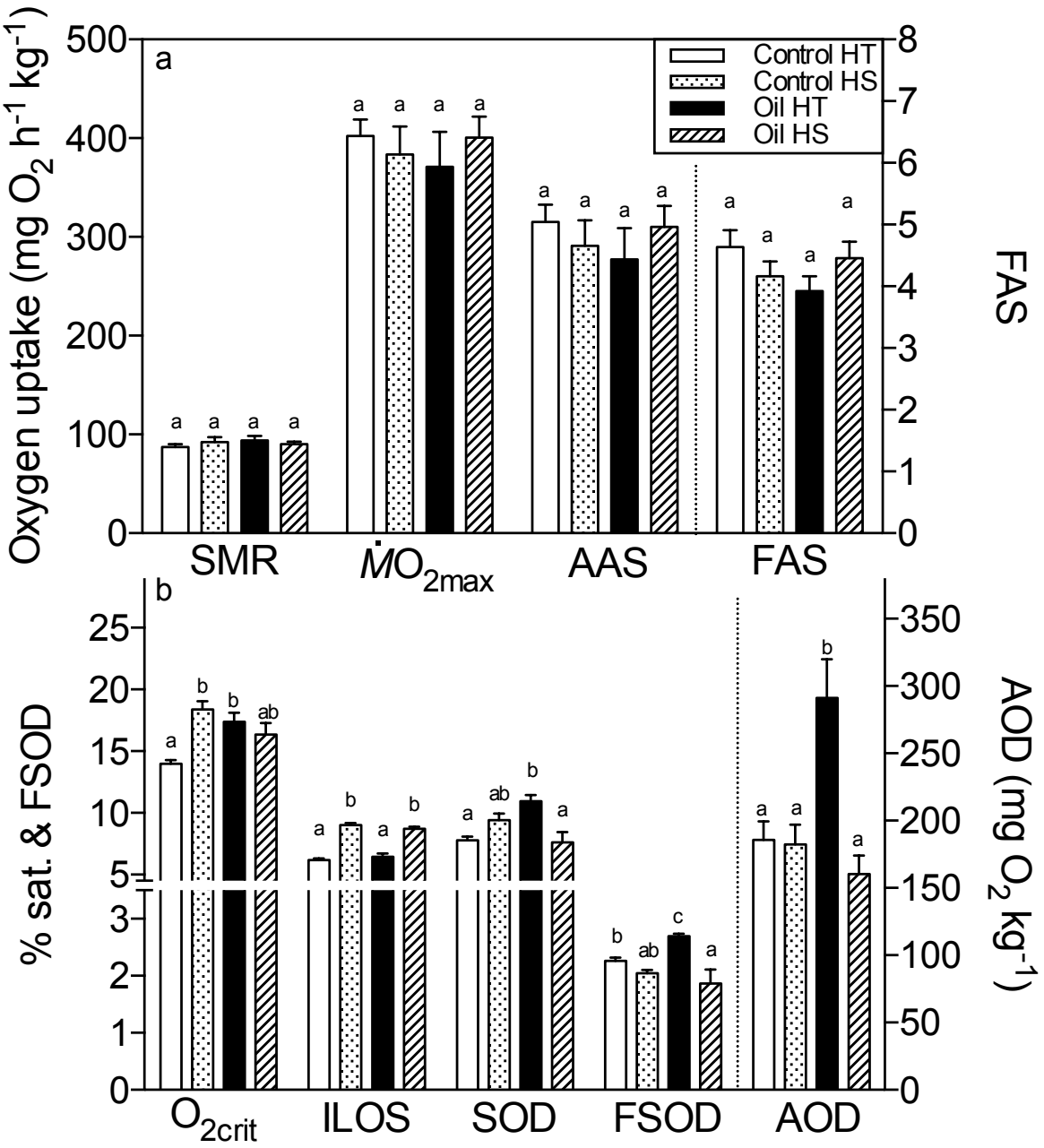

a
Control HT
Control HS
Oil HT
Oil HS
Oxygen uptake (mg O_2 h^{-1} kg^{-1})
FAS
SMR
$\dot{M}O_{2max}$
AAS
FAS
b
% sat. & FSOD
AOD (mg O_2 kg^{-1})
O_{2crit}
ILOS
SOD
FSOD
AOD

Figure 3.8. A) Standard metabolic rate (SMR), maximal rate of oxygen uptake ($\dot{M}O_{2max}$), absolute aerobic scope (AAS), factorial aerobic scope (FAS); B) critical oxygen level (O_{2crit}), incipient lethal oxygen saturation (ILOS), scope for oxygen deficit (SOD), factorial scope for oxygen deficit (FSOD) and accumulated oxygen deficit (AOD) of control hypoxia tolerant (HT) (n=5), control hypoxia sensitive (HS) (n=6), dispersed oil contaminated (oil) HT (n=7) and oil HS (n=10) European sea bass (*Dicentrarchus labrax*). Different letters indicated the significant differences ($P < 0.05$) between groups within a respiratory index by one-way Welch ANOVA Tukey's Kramer *post-hoc*. Values were mean ± s.e.m.

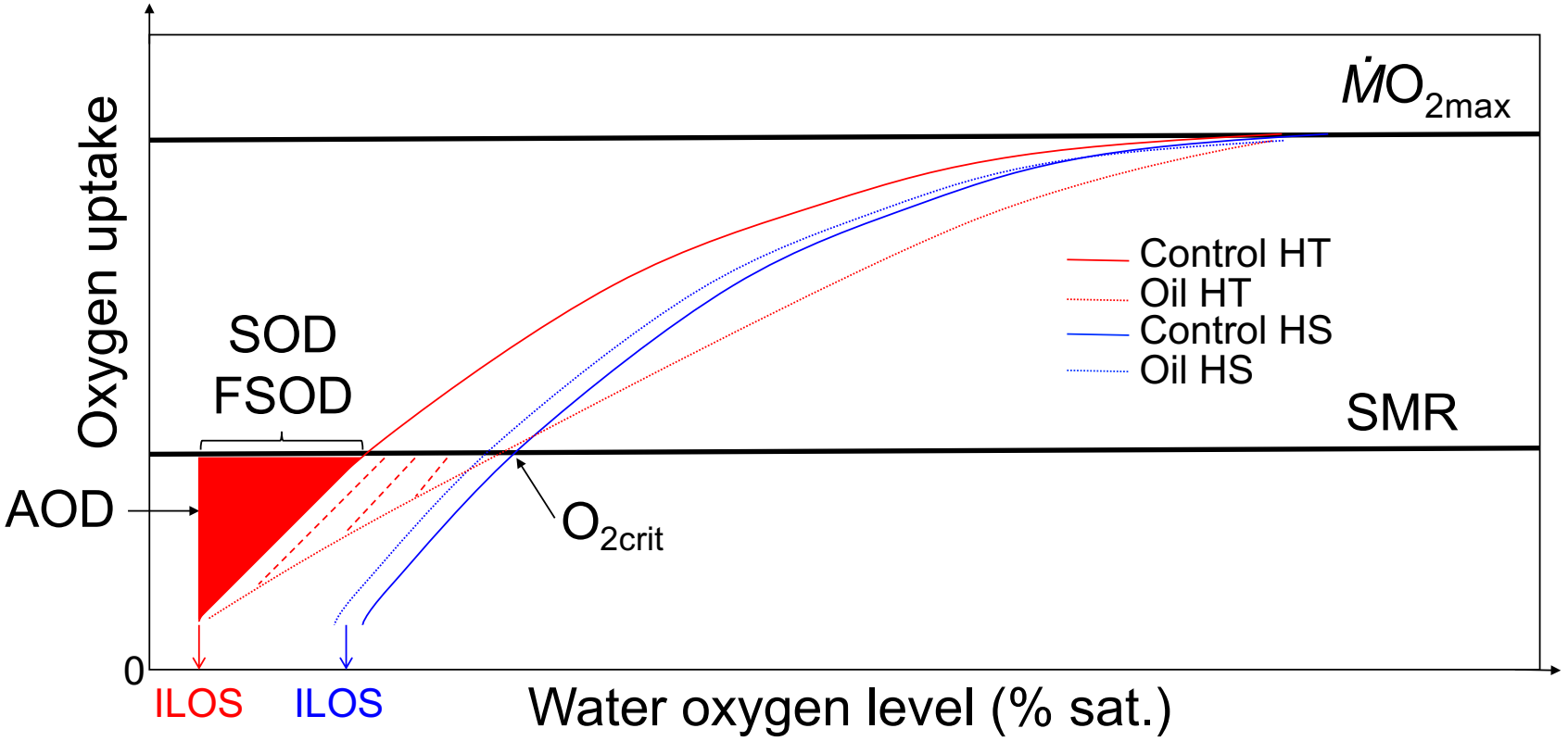


Figure 3.9. Schematic illustration of the theoretical limiting oxygen level (LOL) curves would distinguish the two phenotypes of hypoxia resistance (hypoxia tolerant, HT; hypoxia sensitive, HS) demonstrated here for European sea bass (*Dicentrarchus labrax*). Control (solid lines) and after exposure to chemically dispersed oil (Oil, dash lines) illustrated how I envisage O_{2crit} (the intersection of standard metabolic rate and the curves) without $\dot{M}O_{2max}$, SMR and ILOS changing as a result of the exposure. Scope for oxygen deficit (SOD), factorial scope for oxygen deficit (FSOD) and accumulated oxygen deficit (AOD) were labeled.

Table 3.1 Spearman's correlation for critical oxygen level (O_{2crit}) and incipient lethal oxygen saturation (ILOS) of the yearling population European sea bass (*Dicentrarchus labrax*) over 7-month experiments, including pre- & post- exposure hypoxia challenge test (HCT1 & HCT2) and respirometry (Resp).

		HCT1 ILOS	HCT2 ILOS	Resp ILOS	Resp O_{2crit}
HCT1 ILOS	Control		*0.691 P=0.019	*0.673 P=0.023	*0.718 P=0.013
	Oil-treated		0.355 P=0.162	0.375 P=0.138	0.365 P=0.149
HCT2 ILOS	Control			*0.973 P<0.001	*0.900 P<0.001
	Oil-treated			*0.988 P<0.001	0.120 P=0.646
Resp ILOS	Control				*0.809 P=0.003
	Oil-treated				0.142 P=0.586

For each correlation, Spearman's correlation coefficient is shown in the first row, P value is in the second row.

Table 3.2. The summary of chemically dispersed oil as a main effect on standard metabolic rate (SMR), maximal metabolic rate ($\dot{M}O_{2max}$), absolute aerobic scope (AAS) and factorial aerobic scope (FAS), critical oxygen level (O_{2crit}), incipient lethal oxygen saturation (ILOS), scope for oxygen deficit (SOD), factorial scope for oxygen deficit (FSOD) and accumulated oxygen deficit (AOD) between control and dispersed oil exposed (oil) European sea bass (*Dicentrarchus labrax*). The indices were from pre- and post- hypoxia challenge test (Pre- and Post-HCT), as well as respirometry experiment (Resp). Statistical significance was assigned to $P < 0.05$ in Student's t-test and Welch one-way ANOVA. Values were mean ± s.e.m..

		Dimensions	Control	Oil	F-value	*p*-value
Pre-HCT	ILOS	% sat.	5.8 ± 0.1	-	-	-
10 °C	N=392	hour	6.1 ± 0.1	-	-	-
Post-HCT	ILOS	% sat.	7.7 ± 0.2	7.8 ± 0.2	-	0.7
15 °C	n=96	hour	6.2 ± 0.1	6.1 ± 0.2	-	0.7
	SMR	mg O_2 h^{-1} kg^{-1}	90.1± 3.0	91.8 ± 2.3	0.22	0.65
	$\dot{M}O_{2max}$	mg O_2 h^{-1} kg^{-1}	392.1 ± 16.8	388.4 ± 18.9	0.02	0.89
Resp	AAS	mg O_2 h^{-1} kg^{-1}	302.0 ± 15.8	296.6 ± 18.0	0.05	0.82
16.5 °C	FAS		4.4 ± 0.2	4.2 ± 0.2	0.28	0.60
control n=11	O_{2crit}	% sat.	16.4 ± 0.8	16.8 ± 0.6	0.18	0.68
oil n=17	SOD	% sat.	8.7 ± 0.4	9.0 ± 0.7	0.18	0.67
	FSOD		2.1 ± 0.1	2.2 ± 0.1	0.26	0.62
	AOD	mg O_2 kg^{-1}	183.9 ± 9.6	214.2 ± 21.2	21.7	0.21

Chapter 4: DISCUSSION AND CONCLUSION

The primary objective of my thesis was to build a respirometry assessment paradigm (RAP) that combines the existing suite of respirometry metabolic indices and enhances the estimation of true metabolic scope. Besides measuring aerobic capacity (SMR, $\dot{M}O_{2max}$, AAS, FAS) and recovery (EPOC), RAP explicitly distinguishes the indices of hypoxia tolerance (O_{2crit}) from hypoxia resistance (ILOS) by introducing SOD, FSOD and AOD to quantify how fish survive in the 'scope for survival' during hypoxia (Hochachka, 1990). Using automatic intermittent-flow respirometry systems, RAP simultaneously measured 10 metabolic indices of 8 fish in a test lasting a minimum of 3 days. Theoretically, more fish could be measured simultaneously simply by replicating the systems, but this might beyond the capacity of a single investigator. I believe RAP goes a long way to meet the goal of providing a high-throughput testing system that can address issues relevant to the anthropocene.

To test whether RAP is an effective methodology of evaluating the fish's performance, I validated it by testing a long-standing hypothesis that fish respiratory physiology and bioenergetics significantly affect functional integrity (Fry, 1971) in two scenarios: aquaculture and an oil spill. Therefore, I successfully measured the rate of oxygen uptake ($\dot{M}O_2$) as a proxy for metabolic rate in both normoxic and hypoxic conditions and with applications to resource-rich and resource-threatened environments. I suggest that RAP can reliably assess whole-animal respiratory robustness across a wide spectrum of circumstances.

4.1 Advanced knowledge by RAP and perspectives

4.1.1 Impaired athletic robustness and cardiorespiratory plasticity in domesticated Atlantic salmon

World-leading Norwegian aquaculture industry suffers 15–20% mortality rate of smolts at the first 90-day of grow-out in the sea sites (Kristensen et al., 2012a). Good evidence suggests that growth-oriented selective breeding strategy and domesticated rearing environment might have led to cardiovascular aberrations (Mercier et al., 2000; Poppe et al., 2003; Gjedrem and Baranski, 2009). Dysfunction of the cardiovascular system impairs performance of the cardiorespiratory system as a whole (Claireaux et al., 2004). Therefore, I tested whether RAP could be applied as a whole-animal diagnosis to detect cardiorespiratory impairments in domesticated salmonids.

RAP discovered that approximately ten generations of selective breeding in Norwegian aquaculture compromised athletic robustness of domesticated (*Bolaks*) strain of Atlantic salmon which compared to a wild (*Lærdal*) strain, and attenuated, but did not prevent from gaining potential physiological benefits of exercise training. Specifically, the domesticated strain when compared with the wild strain had a significantly lower $\dot{M}O_{2max}$, AAS and FAS, results that were supported by a lower CS activity in white muscle of the domesticated strain. Hence, the domesticated strain appeared to be athletically less robust than the wild strain. In summary, the domesticated Atlantic salmon strain, as expected, had an impaired aerobic capacity (Gamperl and Farrell, 2004; Claireaux et al., 2005) when compared to the wild strain. This might be due to the previously observed cardiovascular anomalies (Mercier et al., 2001; Poppe and Taksdal, 2000;

Poppe et al., 2002, 2003) and reduced enzymatic capacity in swimming muscle in the domesticated salmonids (Anttila et al., 2008a; Anttila and Mänttäri, 2009).

As a result of exercise training, the wild strain had a significantly higher $\dot{M}O_{2max}$, AAS and EPOC, whereas the domesticated strain only had a higher CS activity in red muscle. Exercise training also increased the CS activity in red muscle of the wild strain to a larger extent compared with the domesticated strain. Hence the novel conclusion here was that domestication reduced cardiorespiratory plasticity, however the underpinning mechanisms are still unclear and a set of hypotheses are needed to distinguish genotypic and phenotypic influences. One hypothesis might be that the lower cardiorespiratory plasticity of the domesticated strain is linked to a more homogenous genetic profile suited to captive environment (Christie et al., 2016). An alternative hypothesis is that captive environment compromises athleticism of the wild strain during developmental stages, and exercise-training protocol helps the wild strain regain athletic robustness. Future research, therefore, should test these two hypotheses.

Notably, enzymatic and metabolic data illustrate that exercise-induced enhancements of enzyme activities in swimming muscle link to the improvements of whole animal aerobic capacity. A higher CS activity in red muscle of trained fish, as discovered in this study, could aid aerobic ATP production during swimming and recovery (Kieffer et al., 1998; Anttila et al., 2006). A higher lactate dehydrogenase (LDH) activity in white muscle, as observed in wild strain after training, could enhance recovery by promoting lactate clearance, resulting in a higher EPOC (Wood, 1991; Milligan et al., 2000; Anttila et al., 2010). Hence, I concluded that the incremental exercise-training regime enhances the capacity of wild strain to recharge oxygen stores on hemoglobin and myoglobin, replenish high-energy phosphates, as well as eliminate

accumulated lactate and resynthesize glycogen (Gaesser and Brooks, 1984; Wood, 1991; Scarabello et al., 1991).

4.1.2 Chronic residual effects of oil on hypoxia resistance in European sea bass

I discovered that even though PAH had initially bioaccumulated during the acute oil exposure and was subsequently either metabolized or excreted (Tuvikene 1995; Blahova et al., 2013; Dévier et al., 2013), chronic residual effects (on average of 176 days later) were absent for indices of aerobic metabolism (SMR, $\dot{M}O_{2max}$, AAS, FAS) after an acute exposure to chemically dispersed oil. Good evidence suggests that oil chronically impaired the U_{crit} without affecting aerobic capacity (Mckenzie et al., 2007b; Mager et al., 2014). In fact, a decline in U_{crit} potentially relates to a limitation of oxygen uptake at the gills in both exercise and hypoxia (Daxboeck et al., 1982), requiring an increased glycolytic support of energy demands (Wang et al., 1994; Farrell and Clutterham 2003). Supported by my result, a residual elevation of O_{2crit}, SOD, FSOD and AOD was present only in the oil-treated HT and not the HS phenotype. The increased O_{2crit} meant an impaired ability to extract oxygen at low ambient oxygen tension, which agrees with the previously documented acute effects of oil exposure on bald notothen (Davison et al., 1993) and common sole (Claireaux et al., 2004; Davoodi and Claireaux, 2007). If HS sea bass had a thicker gill epithelium than HT sea bass, this would protect them from the residual effect of oil (Mitchell and Cech 1983) but made them less tolerant of hypoxia (Bindon et al., 1994; Greco et al., 1995).

A chronic residual effect on hypoxia resistance can be harmful to productivity, growth and stability of aquatic populations (e.g., European sea bass; Heintz et al., 2000; Heintz 2007;

Claireaux et al., 2013), but it is barely studied. This is because traditional respiratory metabolic indices do not have enough resolution to identify the subtle chronic residual effect.
Hence, RAP advances the resolution of existing suite of respirometry metabolic indices by introducing SOD, FSOD and AOD. AOD, particularly, is a more sensitive bioenergetics index, because it takes both the glycolytic activities and effective time to quantify the extent of glycolytic metabolism. Furthermore, a novel discovery is that ILOS of HT sea bass remained unaffected, despite the O_{2crit} of HT phenotype was elevated. This challenges the incorrect notion that O_{2crit} and ILOS are both the indices of hypoxia tolerance; more correctly O_{2crit} is a measure of hypoxia tolerance, while ILOS is a measure of hypoxia resistance.

In summary, a concern of aquaculture is that selective breeding and growth-optimization in a captive environment trades off the athletic robustness and reduces the survival rate of smolts post-seawater transfer. Meanwhile, conservationists are seeking for comprehensive understanding about the chronic residual influences of oil spill on fishes. Because respiratory robustness is great interest for both applications, I validated the RAP framework in aquaculture and oil contamination scenarios.

4.1.3 Perspectives of RAP

The fundamental contribution of RAP is providing easily measured and comprehensive framework to estimate metabolic scope by converging aerobic and anaerobic scopes. The oxygen and capacity-limited thermal tolerance (OCLTT) hypothesis is, by far, the most comprehensive framework to predict how climate change will affect fish population and biogeography (Pörtner and Farrell, 2008), but OCLTT solely focuses on the aerobic component of metabolic scope and

overlooks its anaerobic component. Although scientists have started to acknowledge the importance of anaerobic metabolism in predicting the influence of climate change (Claireaux and Chabot, 2016; Ejbye-Ernst et al., 2016), measuring excess post-hypoxia (or post-anoxia) oxygen consumption, the current indices of using $\dot{M}O_2$ to quantify the glycolytic metabolism, is time consuming and low-throughput (Cox et al., 2011; Svendsen et al., 2011). RAP solves this issue by incorporating the measures of SOD, FSOD and AOD with the established intermittent-flow respirometry that yields accurate aerobic metabolism indices. Thus, RAP enhances the estimation of metabolic scope by providing quicker measures of glycolytic metabolism, which then increases the power of respirometry indices in predicting the influence of climate change in fishes. Therefore, RAP can potentially be a standard respirometry experimental protocol for scientists to address the environmental stressors on fishes and further explore how respiratory robustness governs ecological performances and biogeography.

4.2 **Justifications of RAP**

I argue that RAP is a coherent framework that provides the most reliable measures of aerobic capacity, capability to recover from exhaustion and measures of the capacity to survive hypoxia in a short timeframe. Although RAP still needs a minimum of 3 days to measure up to 10 indices, it can easily measure 12 fish (a statistically useful treatment group) with the appropriate sets of the automatic intermittent-flow respirometry. The experimental duration is mostly decided by the fact that fishes take a minimum of 2 diurnal cycles to habituate to the respirometer and reach SMR (Chabot et al., 2016). An excellent measure of SMR is paramount

to deriving reliable indices of aerobic and recovery capabilities (AAS, FAS and EPOC), as well as to calculate the indices of hypoxic survival (O_{2crit}, SOD, FSOD and AOD) (Figure 4.1.)

Some researchers hold the notion that $\dot{M}O_{2max}$ derived by chasing fish to exhaustion might be different from the one obtained from swimming fish to fatigue (Reidy et al., 1995). The two methods yield same evaluation of $\dot{M}O_{2max,}$ if the experiments were done properly (Claireaux and Chabot, 2016). $\dot{M}O_{2max}$ has been widely adapted for intermittent-flow respirometry to explicitly measure the highest $\dot{M}O_2$ (Clark et al., 2013). The handling of fish prior to the hand-chase procedure might raise a concern that fish cannot reach the $\dot{M}O_{2max}$ post-stress. Salmonids, however, can reach $\dot{M}O_{2max}$ and maximum cardiac output in the second U_{crit} test without full recoveries of $\dot{M}O_{2,}$ cardiac output and plasma lactate, presumably because the arterial and venous blood content can be replenished rapidly (Jain et al., 2003; Eliason et al., 2013). Hence, $\dot{M}O_{2max}$ derived by chasing fish to exhaustion should be valid.

The ideology underpinning the established hypoxia challenge experiment is to make fishes recruit compensatory mechanisms during the moderate hypoxia in the first 40-min before reaching O_{2crit}. The slow decrease of DO lets fish establish compensatory mechanisms for severe hypoxia and the fish are hypoxemic. Below O_{2crit}, the duration of survival is predominately decided by a balance of energetic substrate supply and basic metabolic needs, as well as handling of metabolic wastes and buffering blood acidosis until fish reach ILOS (Richards et al., 2009). Thus, the HCT protocol allowed me to use SOD, FSOD and AOD to estimate the energetic supply by glycolytic metabolism.

During my applications of RAP, I realize that whole-animal respiratory robustness is a dynamic concept that involves an integration of multiple physiological systems. Hence, this proposes a challenge of justifying what a superior respiratory robustness is. I propose that the

improvement at any component of oxygen cascade would contribute to a superior respiratory robustness, and such evaluation has to be constrained within one species. This is because animals adapt to environment and achieve Darwinian fitness with a variety of strategies. Thus, within one species, the slight advantages at either aerobic capacity, hypoxic survival or recovery from oxygen deficits can translate into a higher Darwinian fitness (Mauduit et al., 2016). Meanwhile, an animal is a highly coordinated physiological system, change of one index can enhance other multiple indices. A great example is that a lower SMR can increase AAS/FAS but decrease O_{2crit} or even ILOS. This means the fish is more efficiency at the energy utilization so that both aerobic capacity and hypoxic survival are improved. Of course, the RAP is still at its infancy and the understandings of respiratory robustness will improve with more applications of RAP.

Despite all the justifications and the scientific advancements as summarized above, this thesis still revealed the limitations of RAP and pitfalls of adapting well-established physiological principles into the aquaculture production, as stated below.

4.3 Limitations and future improvements of RAP

As illustrated in chapter 3, the sensitivity of RAP could decrease when there is a large individual variation. Given an eco-physiology study phenotypically specific responses to the environment, I recommend conducting preliminary screening experiments to understand the level of variation in the population and segregate the potential phenotypes. For instance, Chapter 3 screened hundreds of sea bass with a HCT both pre- and post-oil exposure, discovering HT and HS phenotypes and by acknowledging these two phenotypes, RAP revealed a subtle residual effect of oil exposure in HT phenotype. Therefore, depending on the research questions, I

recommend conducting other screening tests prior to RAP: for example, a swimming test of a school of fish to identify the divergence of swimming performance or a maximum critical temperature experiment to identify the segregation of the thermal tolerance.

Meanwhile, RAP only measures the overarching performance of all the underpinning mechanisms. For example, the residual effects of oil exposure, elevated O_{2crit}, SOD, FSOD and AOD, only provide preliminary understanding of the dynamic relationship between hypoxia tolerance and hypoxia resistance under oil contamination. In general, RAP is a diagnostic method prior to diving into the search of a causative relationship between stressors and ecological performance. Therefore, RAP could be incorporated with manipulative experiments for the more comprehensive understandings about the physiological mechanisms.

In theory, a calorimetry experiment is still necessary to validate whether AOD truly measures oxygen deficit by recording metabolic heat and AOD simultaneously, then measures EPHOC in normoxia. EPHOC the integrated area bounded by $\dot{M}O_2$ and SMR when fish are recovering aerobically after a HCT (Svendsen et al., 2011). If there is a metabolic depression, measured by a reduction in metabolic heat, AOD should be larger than EPHOC. Otherwise, AOD and EPHOC should be the same. This idea still needs to be tested in future research.

4.4 Dysfunctions of screening protocol

Although superior swimmers of Atlantic salmon smolts that had minimally 55% higher U_{max} than inferior swimmers, as quantified by an incremental acceleration protocol, the two groups had the same routine metabolic rate, $\dot{M}O_{2max}$ and active aerobic scope (Anttila et al., 2014a). Using RAP, I examined whether a constant acceleration screening protocol can segregate

the anaerobic metabolism between inferior and superior athletic robustness in wild and domesticated Norwegian Atlantic salmon strains. Contrary to expectations, the screening protocol did not distinguish inferior and superior athletic robustness in both strains according to any of these metabolic and enzymatic indices. To preclude the artifact of bias sampling (top vs. bottom 20%), an exploratory three-way MANOVA model that restricted the fastest 10% swimmers ($140 \leq U_{max} \leq 145$ cm s^{-1}) and slowest 10% swimmers ($60 \leq U_{max} \leq 67.5$ cm s^{-1}) revealed that numerical difference in EPOC between top and bottom 10% swimmers of both strains approached statistical significance (898.5 ± 63.8 vs. 729.8 ± 57.4 mg O_2 kg^{-1}, three-way ANOVA, $F = 3.9$, $p = 0.06$). Thus, the constant acceleration screening protocol, which was up to 57% higher than the U_{crit} (Farrell, 2008), used here appeared to be better at distinguishing anaerobic rather than aerobic capacities. Furthermore, given that there was no screening effect on LDH activities in white muscle of either strain, it would seem that the fast component of EPOC, resynthesizing ATP and replenishing blood and myoglobin oxygen content (Wood, 1991; Farrell and Clutterham, 2003), was favoured by the screening protocol. In fact, the aerobic swimming screening protocol, that measures U_{crit}, effectively segregated the superior swimmers from inferior swimmers in hatchery-reared rainbow trout, showing that superior swimmers had a 19% higher $\dot{M}O_{2max}$ and a 30% higher maximal cardiac output than inferior swimmers (Claireaux et al., 2005). Future studies should, therefore, focus on developing screening protocols and apparatus that assesses aerobic capacity on a commercial scale to accommodate large numbers of fish.

4.5 The refinement and adaptation of exercise-training in aquaculture production

Although previous exercise training regimes enhanced cardiorespiratory robustness in salmonids (Castro et al., 2013; Anttila et al., 2014a), I still have to question whether the training regime used here has sufficient duration to reveal the full plasticity of the cardiorespiratory system, particularly for the domesticated strain (Davison, 1997; Gamperl and Farrell, 2004). I proposed that a refined training-regime, perhaps with a higher exercising-load, might significantly improve the aerobic metabolism in the domesticated stain, because the domesticated strain showed a promising enhancement of CS activity in red muscle after a short 18-day exercise regime.

The industry can achieve a higher exercising-load by either extending the exercise-training period or increasing the exercise intensity. As told, reducing the angle of the water inlet spray bars against the wall of the circulatory tanks can increase the current velocity without additional electricity cost, thereby the industry theoretically can exercise Atlantic salmon during all the pre-smolt rearing stages in hatchery. If the extra pumping capacity is necessary to generate a higher current velocity to achieve sufficient exercise intensity, the industry needs to further investigate whether the potential economic gains of reduced smolts mortality and a higher growth will exceed the cost of pumping. Hence, I exploited a short training period and discovered that the 18-day training period did not enhance respiratory robustness. This ultimately will be a foundation to determine the lowest cost of obtaining the training benefits.

A recovery period between exercise training and seawater transfer practically reduces the stress of exercise and conditioning the fish for transport. Meanwhile, an ideal exercise-training regime should produce training effects that maintain not only throughout the recovery period, but

also the grow-out stages in the ocean. As suggested in this study, a 14-day period prior to smolts transportation is sufficient to recover from an 18-day incremental exercise training. Eventually, grow-out trials and tagging projects are inevitable to prove that the enhanced athletic robustness directly improves the smolts survival in ocean, which is of great interest for both aquaculture and conservation sectors (Jensen et al., 2016).

4.6 Conclusion

My thesis explored whether RAP, measuring $\dot{M}O_2$ as a proxy of metabolic rate in normoxic and hypoxic conditions, can evaluate the respiratory robustness and metabolic scope in a scale and accuracy that scientists will have proper methodology to address challenges involved with the rapid rate of extinction in the anthropocene. The benefits of RAP include high throughput of respiratory metabolic indices, comprehensive examination of aerobic and anaerobic capacities, as well as rapid screening test for industrial and conservation applications. The limitations, however, involve indirect measure of metabolic rate, lack of mechanistic explorations and reduction of detective power when data has a large variation. Of course, only time will tell whether or not physiologists will broadly adapt and apply RAP to address their research questions.

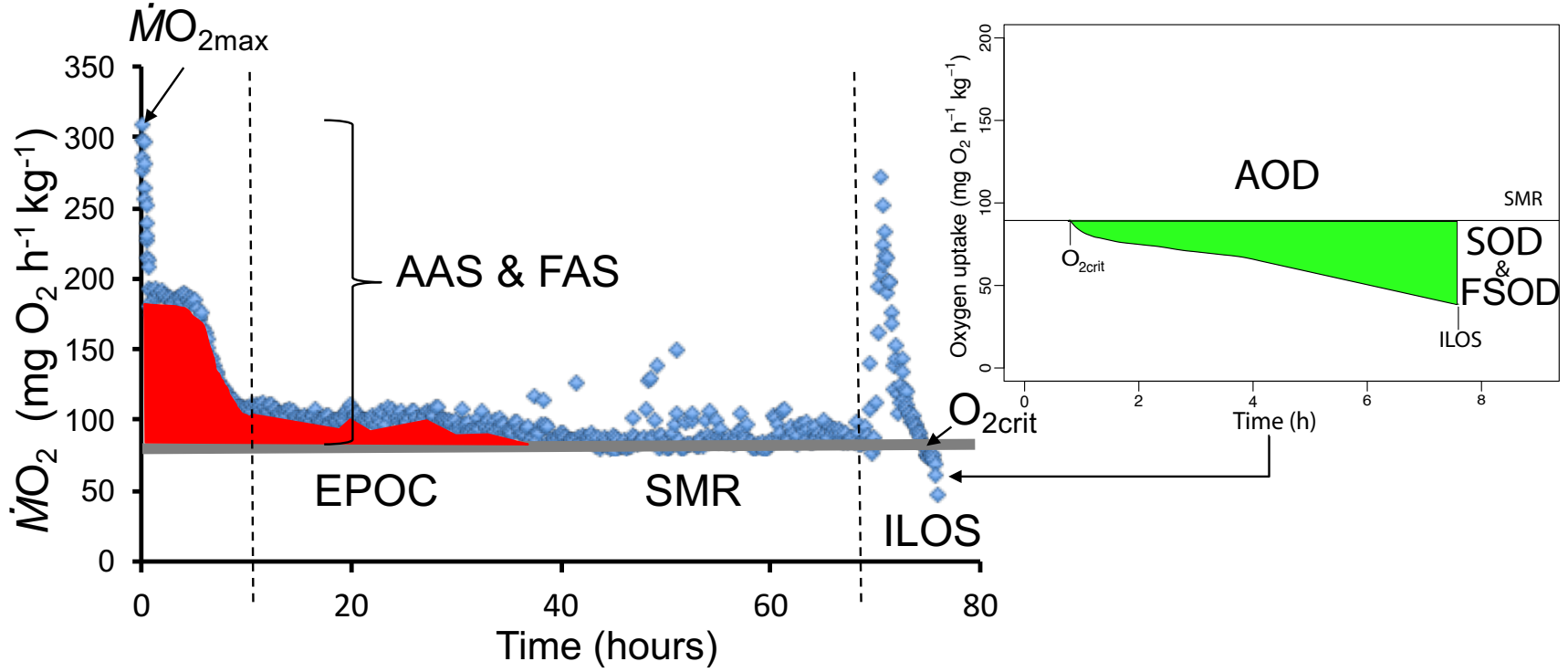

$\dot{M}O_{2max}$
$\dot{M}O_2$ (mg O_2 h^{-1} kg^{-1})
350
300
250
200
150
100
50
0
0
20
40
60
80
Time (hours)
AAS & FAS
EPOC
SMR
ILOS
O_{2crit}
Oxygen uptake (mg O_2 h^{-1} kg^{-1})
200
150
100
50
0
AOD
SMR
SOD
&
FSOD
O_{2crit}
ILOS
0
2
4
6
8
Time (h)

Figure 4.1. Representative trace of a 3-day RAP experimental protocol. Hand-chase stimulated maximum rate of oxygen uptake ($\dot{M}O_{2max}$). The fish then was recovered in the intermittent-flow respirometer and reached quiescent state after first 10 h (the first dash vertical line). The rate of oxygen uptake ($\dot{M}O_2$) still continuously decreased until 40 h. The data between two dash vertical lines was used to calculated standard metabolic rate (SMR). Then absolute aerobic scope (AAS), factorial aerobic scope (FAS) and excess post-exercise oxygen consumption (EPOC; red shade) are computed. A hypoxia challenge test starts after the second dash vertical line. A spike of $\dot{M}O_2$ showed hyperventilation and stress responses prior reached critical oxygen level (O_{2crit}) and incipient lethal oxygen saturation (ILOS). The insert demonstrates the derivations of SOD (scope for oxygen deficit), FSOD (factorial scope for oxygen deficit) and AOD (accumulated oxygen deficit; green shade) from O_{2crit}, and ILOS (time).

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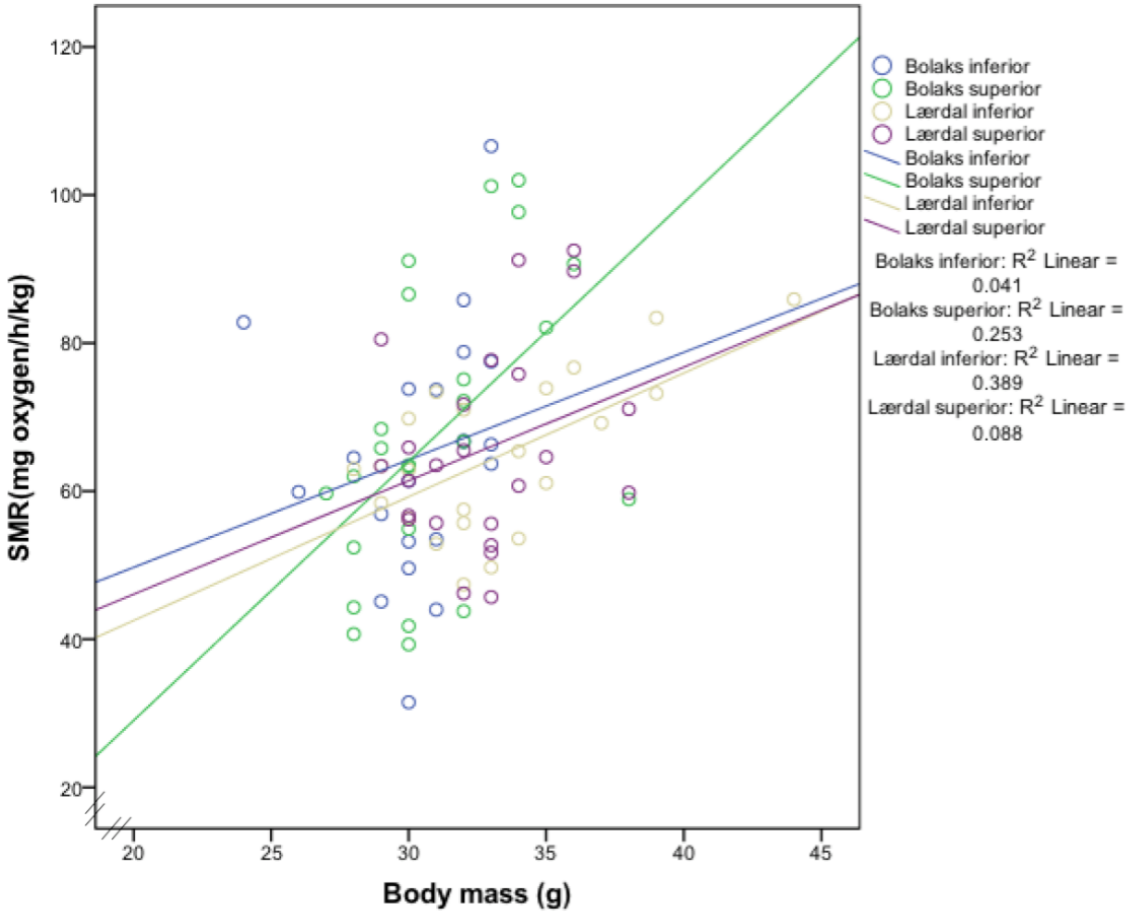
Appendices

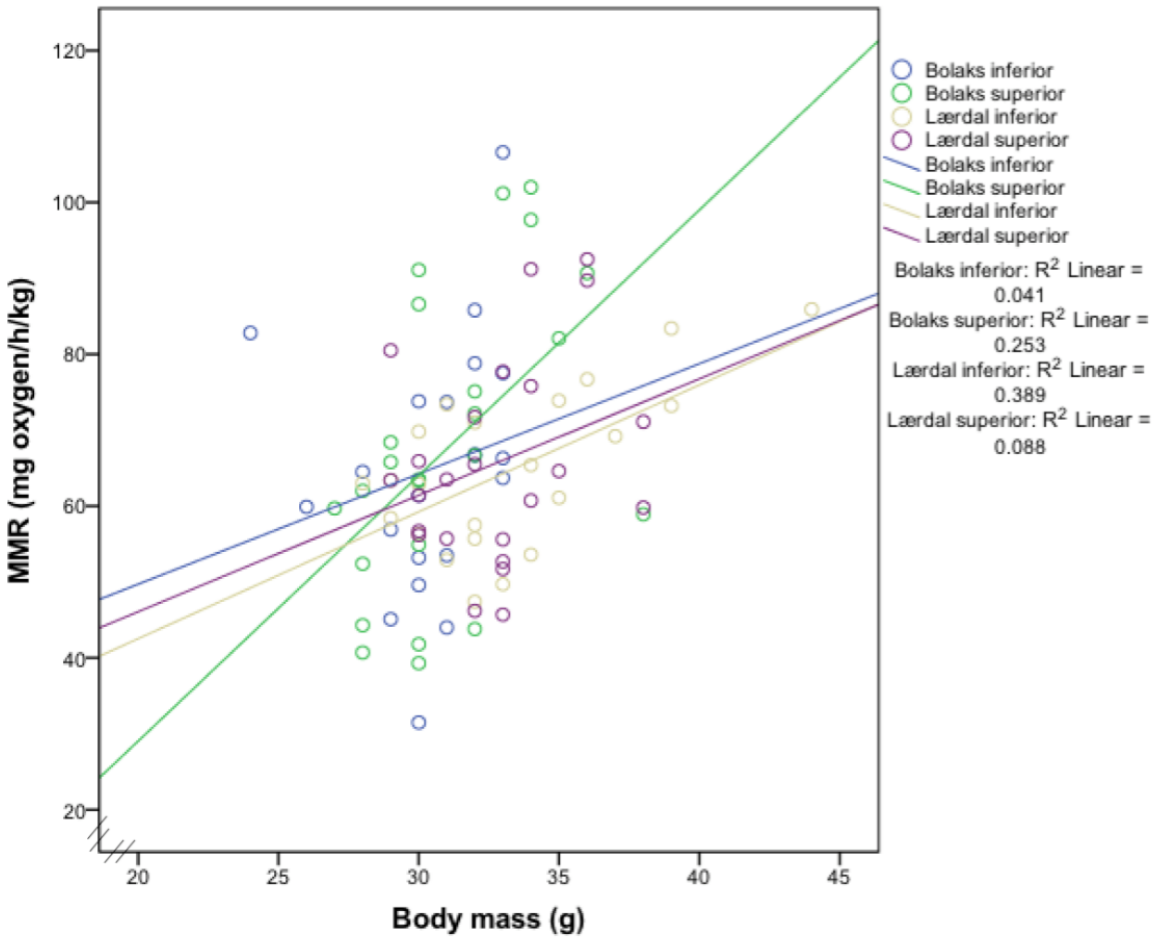
Appendix A Supplementary materials of Chapter 2

A.1 Scatter plots of the A) standard metabolic rate (SMR); B) maximum rate of oxygen uptake ($\dot{M}O_{2max}$); C) absolute aerobic scope (AAS); D) factorial aerobic scope (FAS); E) excess post-exercise oxygen consumption (EPOC); F) critical oxygen level (O_{2crit}); G) incipient lethal oxygen saturation (ILOS); and H) maximum swimming capcacity (U_{max}) with body mass of *Bolaks* inferior, *Bolaks* superior, *Lærdal* inferior and *Lærdal* superior swimmers in Atlantic salmon (*Salmo salar*). R^2 values were obtained by linear regression analysis.

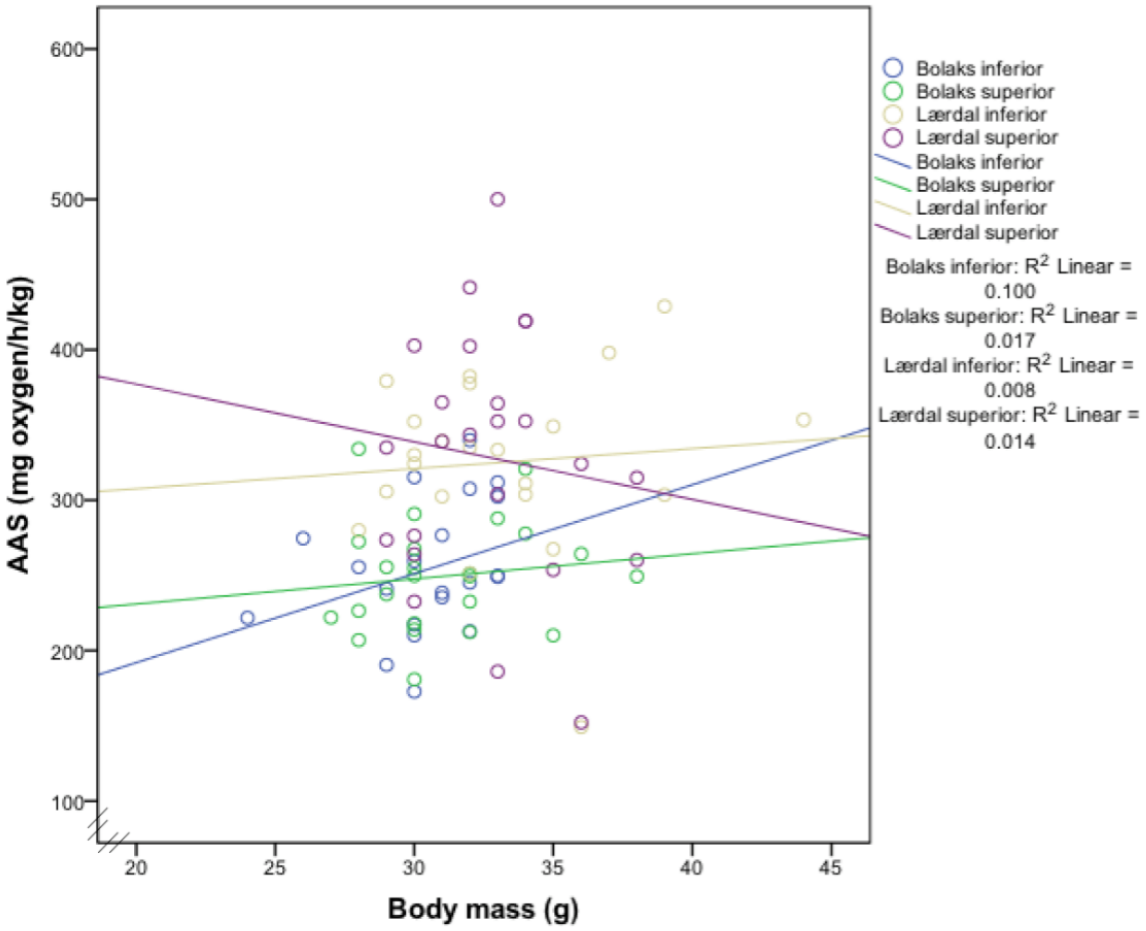
A


SMR(mg oxygen/h/kg)
120
100
80
60
40
20
20
25
30
35
40
45
Body mass (g)
Bolaks inferior
Bolaks superior
Lærdal inferior
Lærdal superior
Bolaks inferior
Bolaks superior
Lærdal inferior
Lærdal superior
Bolaks inferior: R2 Linear = 0.041
Bolaks superior: R2 Linear = 0.253
Lærdal inferior: R2 Linear = 0.389
Lærdal superior: R2 Linear = 0.088

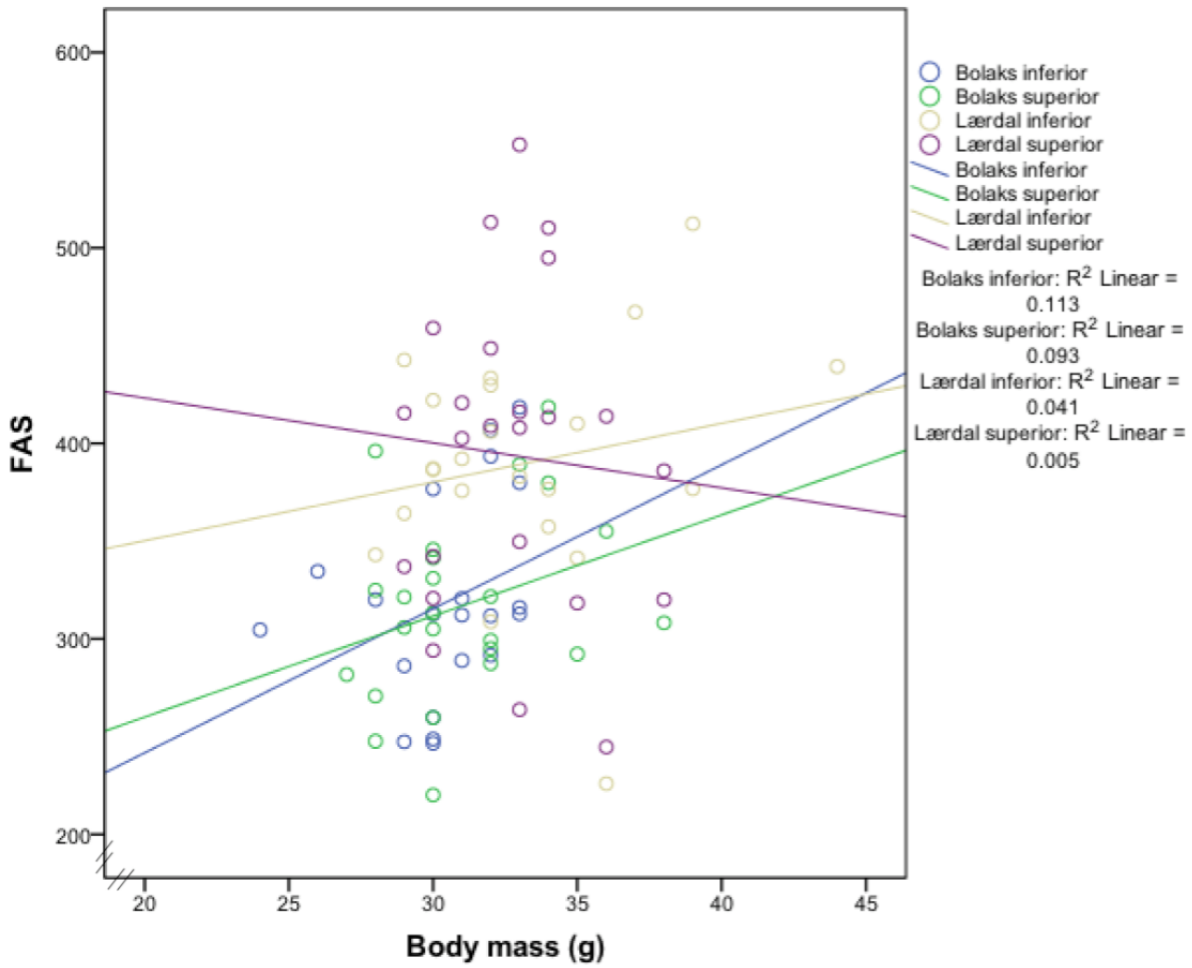
B


MMR (mg oxygen/h/kg)
120
100
80
60
40
20
20
25
30
35
40
45
Body mass (g)
Bolaks inferior
Bolaks superior
Lærdal inferior
Lærdal superior
Bolaks inferior
Bolaks superior
Lærdal inferior
Lærdal superior
Bolaks inferior: R2 Linear = 0.041
Bolaks superior: R2 Linear = 0.253
Lærdal inferior: R2 Linear = 0.389
Lærdal superior: R2 Linear = 0.088

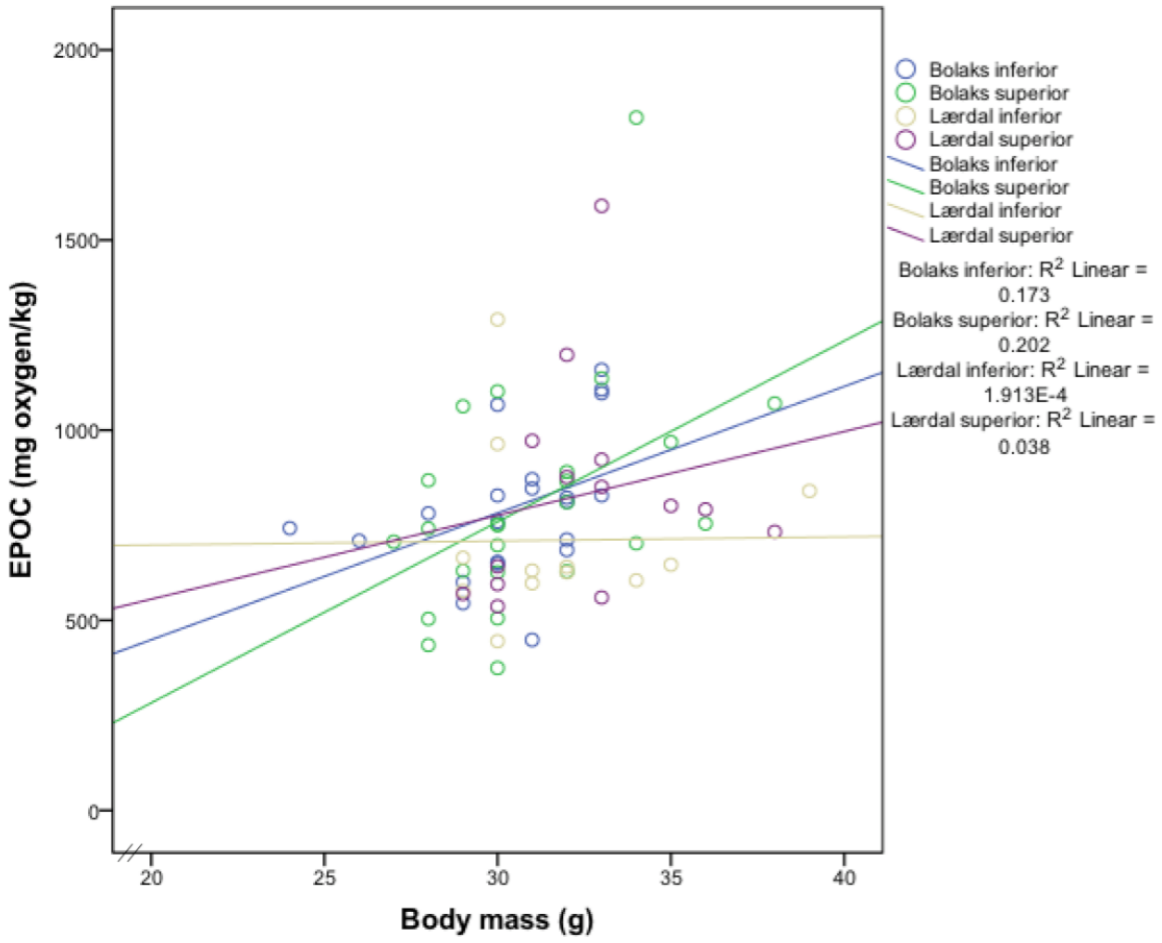
C


600
500
400
300
200
100
AAS (mg oxygen/h/kg)
20
25
30
35
40
45
Body mass (g)
Bolaks inferior
Bolaks superior
Lærdal inferior
Lærdal superior
Bolaks inferior
Bolaks superior
Lærdal inferior
Lærdal superior
Bolaks inferior: R2 Linear = 0.100
Bolaks superior: R2 Linear = 0.017
Lærdal inferior: R2 Linear = 0.008
Lærdal superior: R2 Linear = 0.014

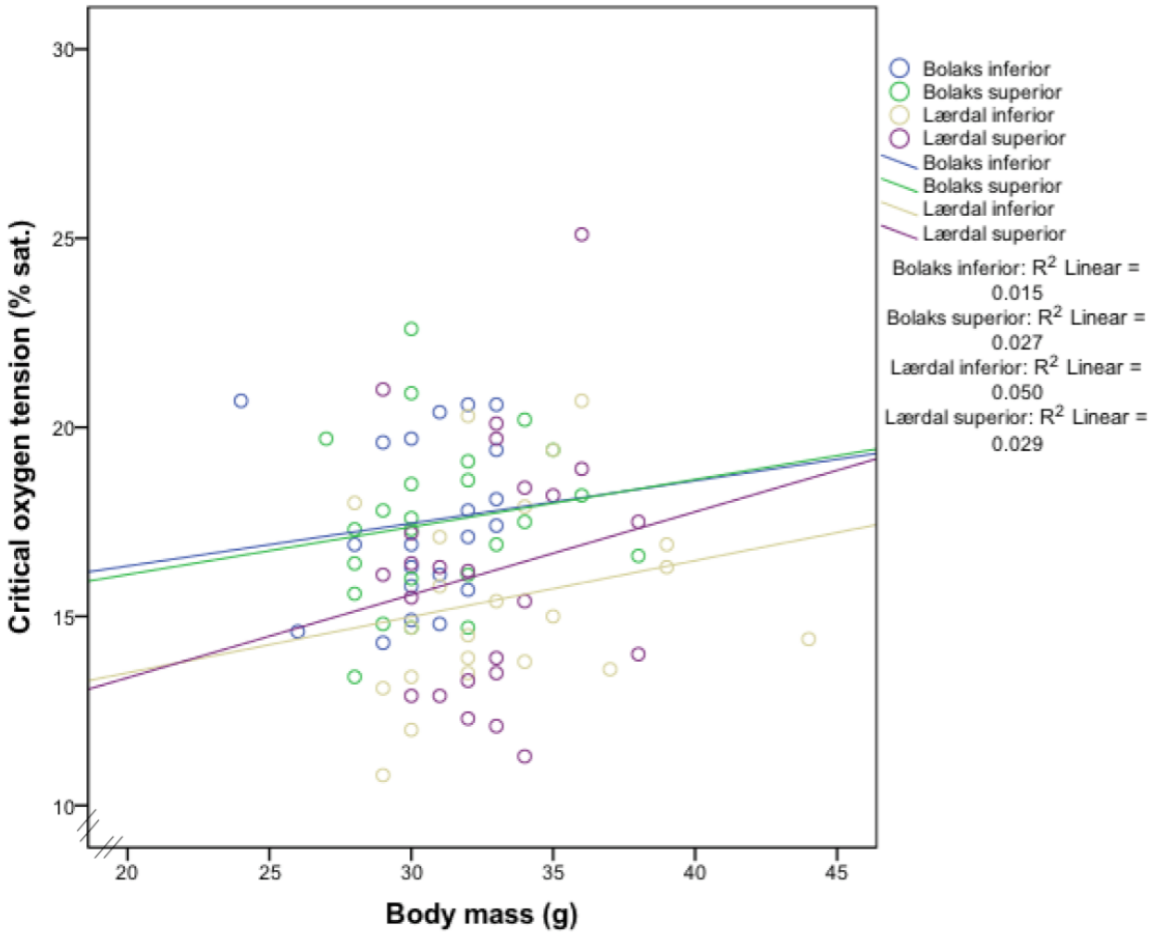
D


600
500
400
300
200
FAS
20
25
30
35
40
45
Body mass (g)
Bolaks inferior
Bolaks superior
Lærdal inferior
Lærdal superior
Bolaks inferior
Bolaks superior
Lærdal inferior
Lærdal superior
Bolaks inferior: R2 Linear = 0.113
Bolaks superior: R2 Linear = 0.093
Lærdal inferior: R2 Linear = 0.041
Lærdal superior: R2 Linear = 0.005

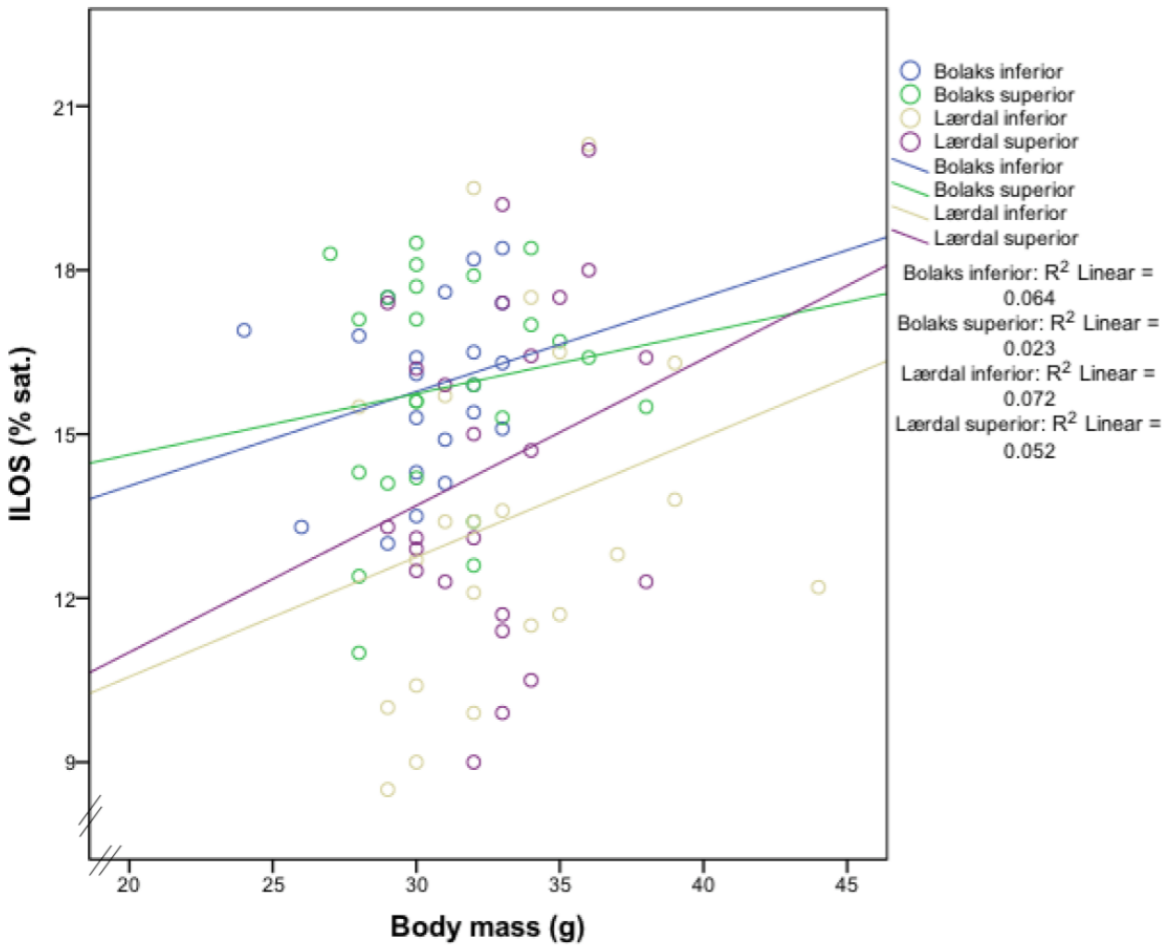
E


EPOC (mg oxygen/kg)
2000
1500
1000
500
0
20
25
30
35
40
Body mass (g)
Bolaks inferior
Bolaks superior
Lærdal inferior
Lærdal superior
Bolaks inferior
Bolaks superior
Lærdal inferior
Lærdal superior
Bolaks inferior: R2 Linear = 0.173
Bolaks superior: R2 Linear = 0.202
Lærdal inferior: R2 Linear = 1.913E-4
Lærdal superior: R2 Linear = 0.038

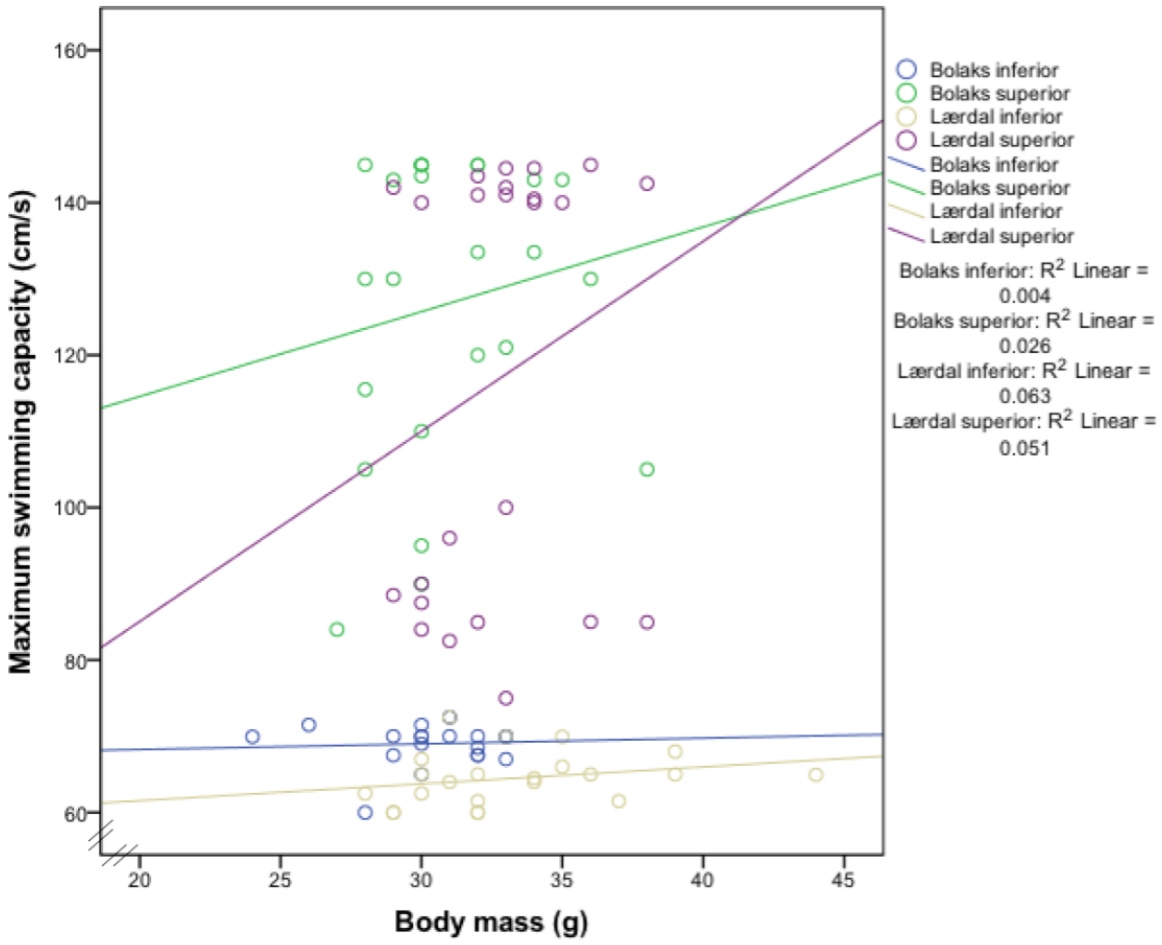
F


Critical oxygen tension (% sat.)
30
25
20
15
10
20
25
30
35
40
45
Body mass (g)
Bolaks inferior
Bolaks superior
Lærdal inferior
Lærdal superior
Bolaks inferior
Bolaks superior
Lærdal inferior
Lærdal superior
Bolaks inferior: R2 Linear = 0.015
Bolaks superior: R2 Linear = 0.027
Lærdal inferior: R2 Linear = 0.050
Lærdal superior: R2 Linear = 0.029

G

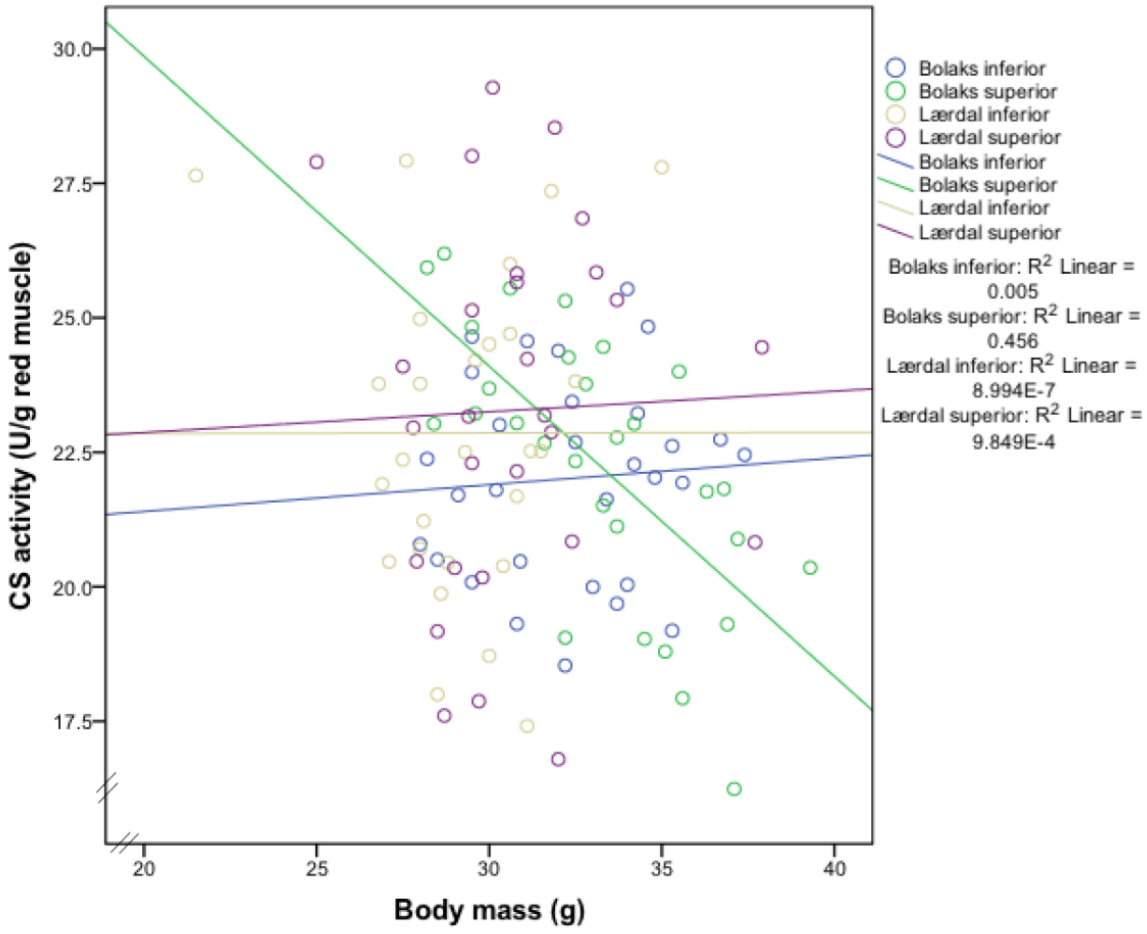

ILOS (% sat.)
21
18
15
12
9
20
25
30
35
40
45
Body mass (g)
Bolaks inferior
Bolaks superior
Lærdal inferior
Lærdal superior
Bolaks inferior
Bolaks superior
Lærdal inferior
Lærdal superior
Bolaks inferior: R2 Linear = 0.064
Bolaks superior: R2 Linear = 0.023
Lærdal inferior: R2 Linear = 0.072
Lærdal superior: R2 Linear = 0.052

H

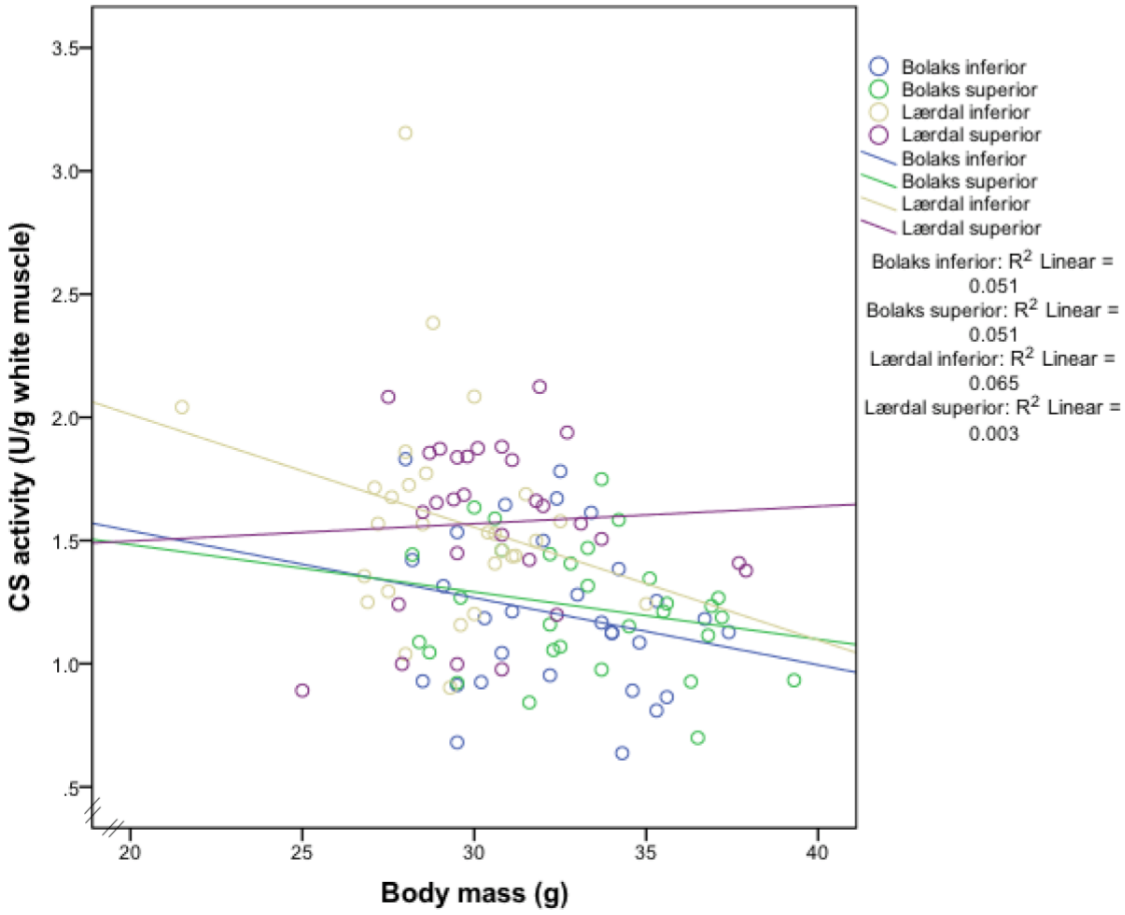

Maximum swimming capacity (cm/s)
160
140
120
100
80
60
20
25
30
35
40
45
Body mass (g)
Bolaks inferior
Bolaks superior
Lærdal inferior
Lærdal superior
Bolaks inferior
Bolaks superior
Lærdal inferior
Lærdal superior
Bolaks inferior: R2 Linear = 0.004
Bolaks superior: R2 Linear = 0.026
Lærdal inferior: R2 Linear = 0.063
Lærdal superior: R2 Linear = 0.051

A.2 Scatter plots of the activity of A) citrate synthase (CS) in red muscle; B) CS in white muscle; C) lactate dehydrogenase (LDH) in red muscle and; D) LDH in white muscle with body mass of *Bolaks* inferior, *Bolaks* superior, *Lærdal* inferior and *Lærdal* superior swimmers in Atlantic salmon (*Salmo salar*). R^2 values were obtained by linear regression analysis.

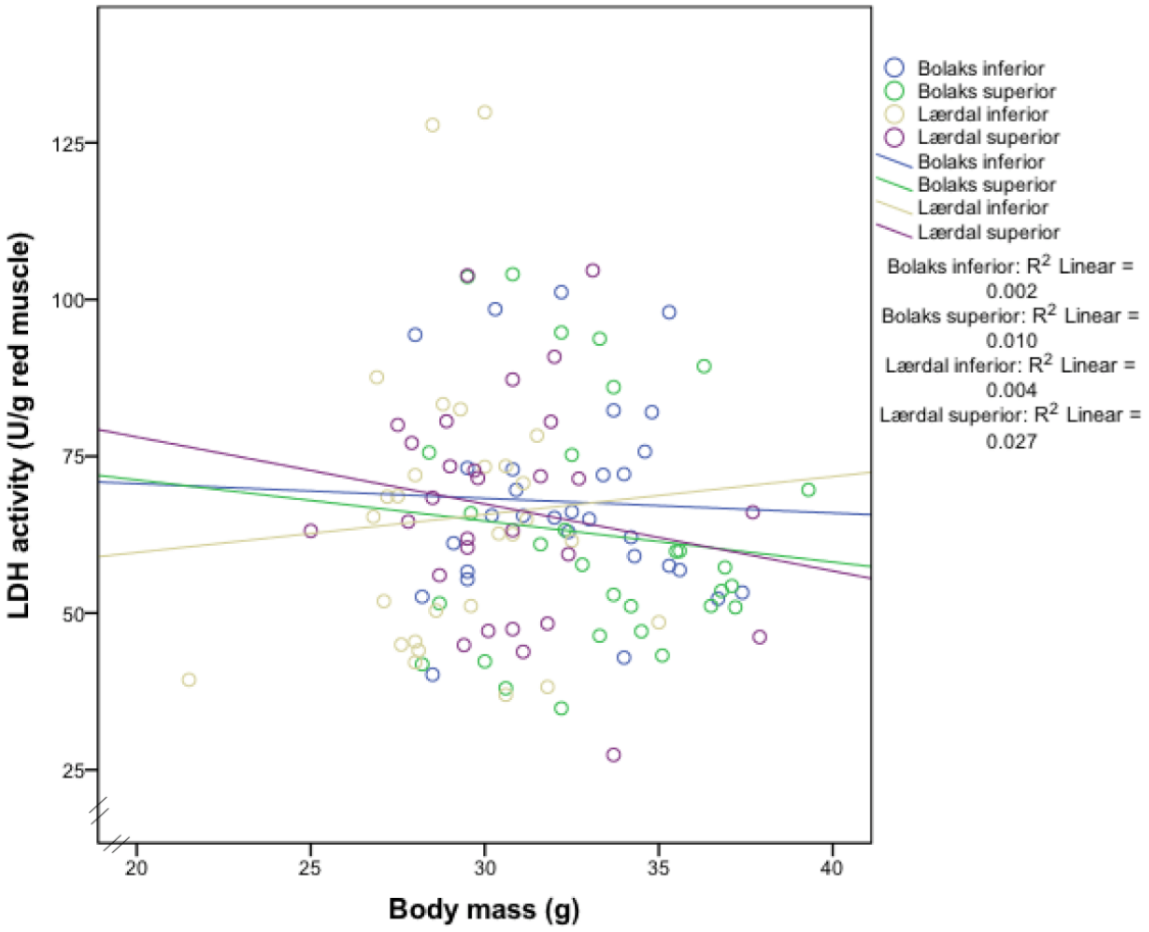
A


30.0
27.5
25.0
22.5
20.0
17.5
CS activity (U/g red muscle)
20
25
30
35
40
Body mass (g)
Bolaks inferior
Bolaks superior
Lærdal inferior
Lærdal superior
Bolaks inferior
Bolaks superior
Lærdal inferior
Lærdal superior
Bolaks inferior: R2 Linear = 0.005
Bolaks superior: R2 Linear = 0.456
Lærdal inferior: R2 Linear = 8.994E-7
Lærdal superior: R2 Linear = 9.849E-4

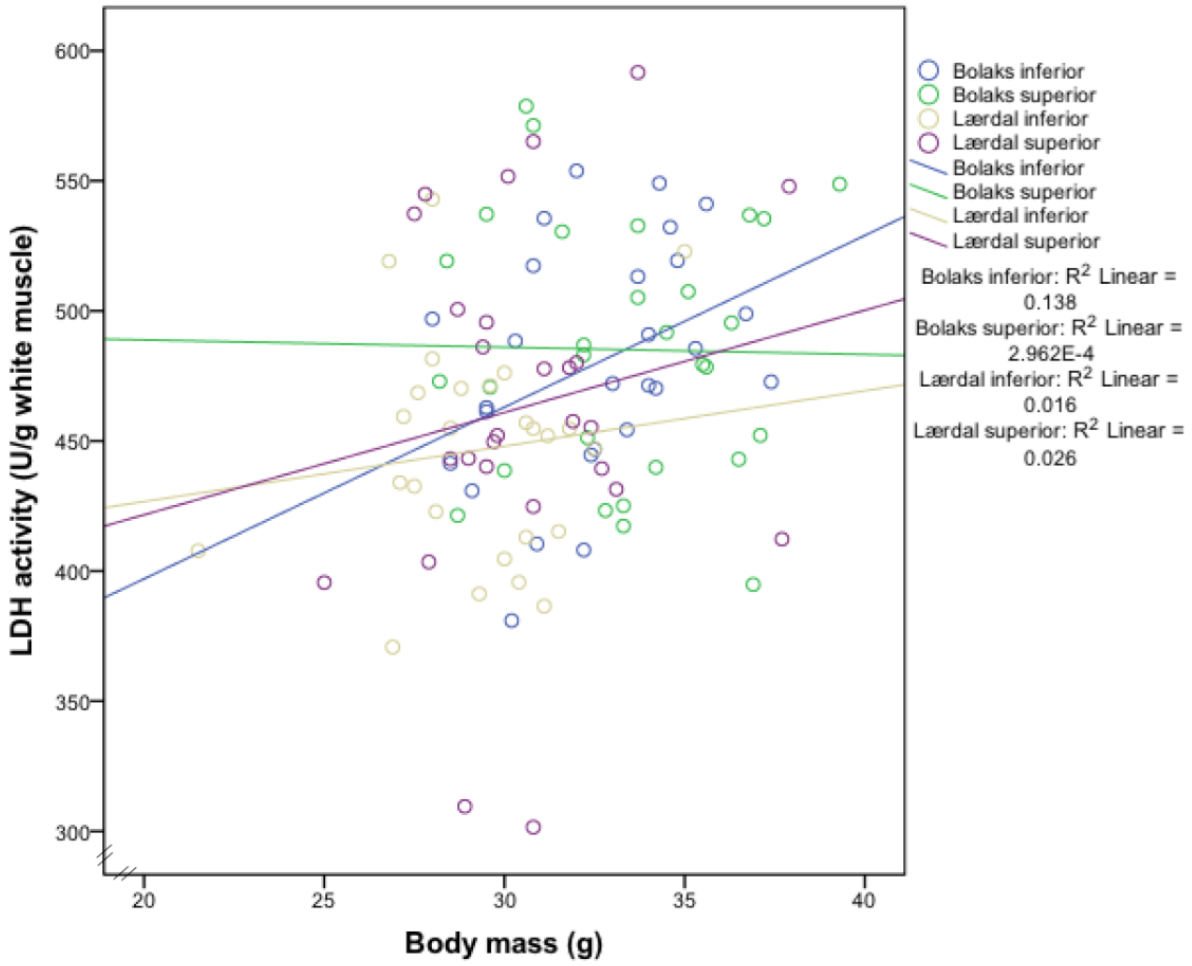
B


CS activity (U/g white muscle)
3.5
3.0
2.5
2.0
1.5
1.0
.5
20
25
30
35
40
Body mass (g)
Bolaks inferior
Bolaks superior
Lærdal inferior
Lærdal superior
Bolaks inferior
Bolaks superior
Lærdal inferior
Lærdal superior
Bolaks inferior: R2 Linear = 0.051
Bolaks superior: R2 Linear = 0.051
Lærdal inferior: R2 Linear = 0.065
Lærdal superior: R2 Linear = 0.003

C

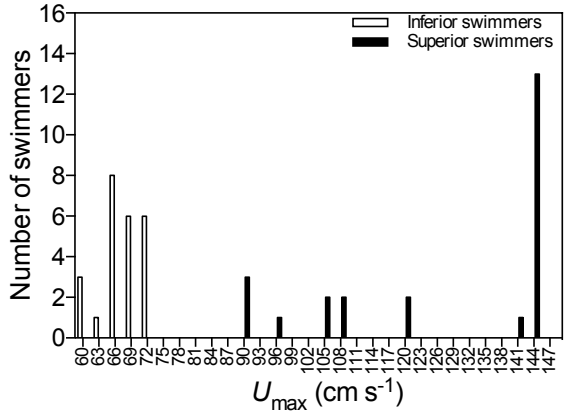

LDH activity (U/g red muscle)
125
100
75
50
25
20
25
30
35
40
Body mass (g)
Bolaks inferior
Bolaks superior
Lærdal inferior
Lærdal superior
Bolaks inferior
Bolaks superior
Lærdal inferior
Lærdal superior
Bolaks inferior: R2 Linear = 0.002
Bolaks superior: R2 Linear = 0.010
Lærdal inferior: R2 Linear = 0.004
Lærdal superior: R2 Linear = 0.027

D

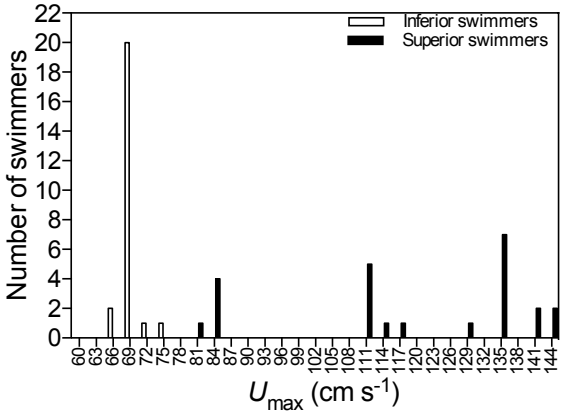

LDH activity (U/g white muscle)
600
550
500
450
400
350
300
20
25
30
35
40
Body mass (g)
Bolaks inferior
Bolaks superior
Lærdal inferior
Lærdal superior
Bolaks inferior
Bolaks superior
Lærdal inferior
Lærdal superior
Bolaks inferior: R2 Linear = 0.138
Bolaks superior: R2 Linear = 2.962E-4
Lærdal inferior: R2 Linear = 0.016
Lærdal superior: R2 Linear = 0.026

A.3 Histogram of absolute U_{max} distribution of screening trials and a sub-group for respirometry in *Bolaks* strain

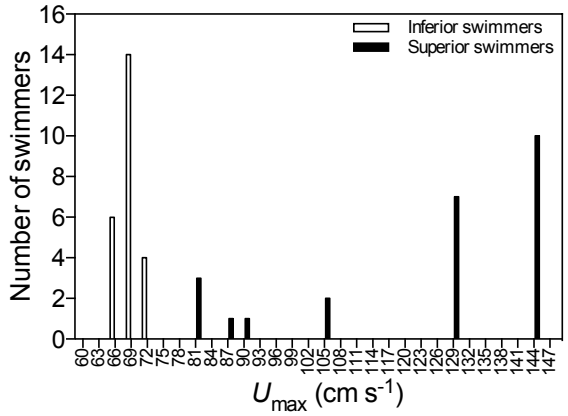
Screening trial 1



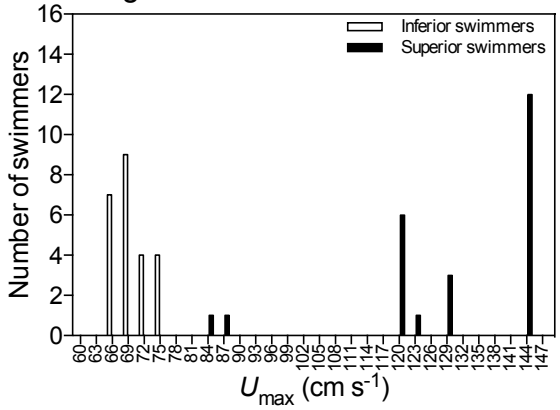
Screening trial 2



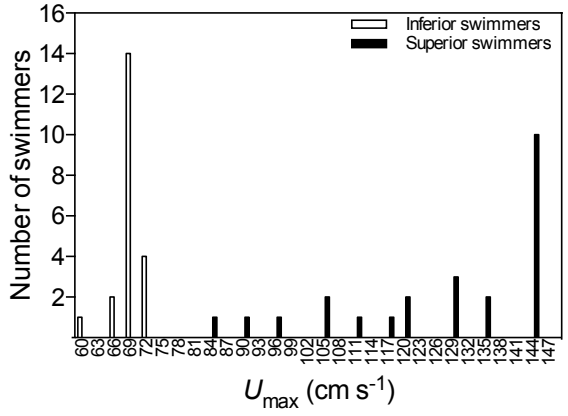
Screening trial 3



Screening trial 4

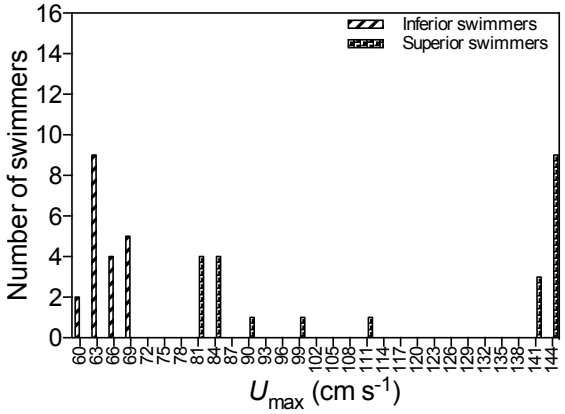


Randomly sampled fish for respirometry

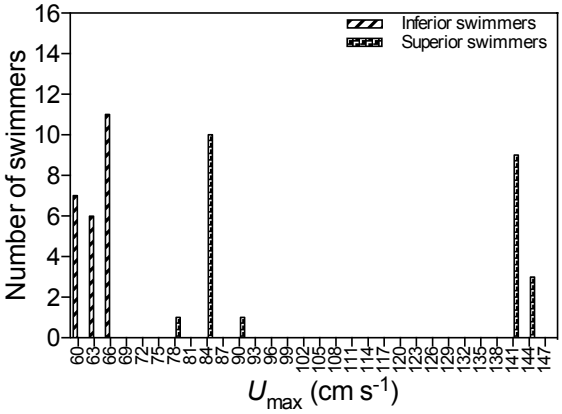


A.4 Histogram of absolute U_{max} distribution of screening trials and a sub-group for respirometry in *Lærdal* strain

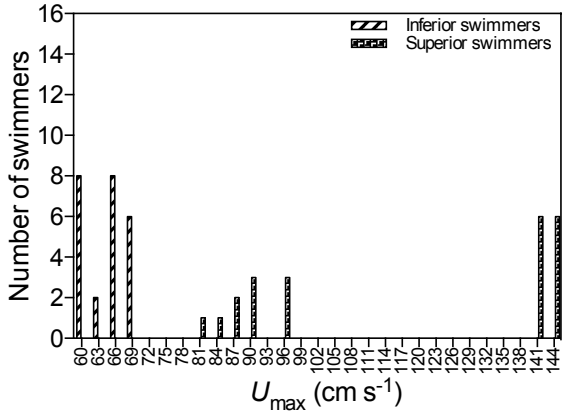
Screening trial 1



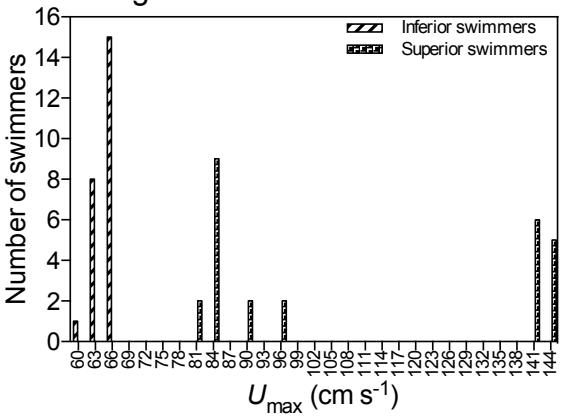
Screening trial 2



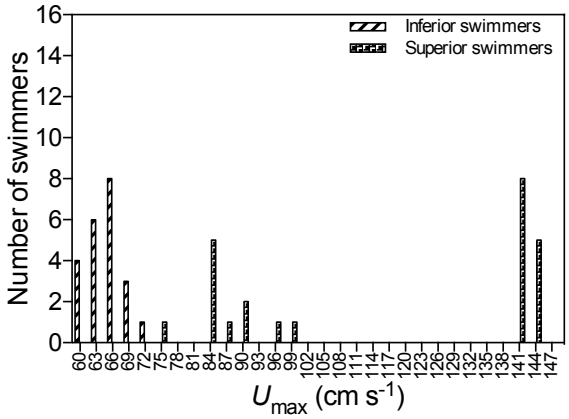
Screening trial 3



Screening trial 4

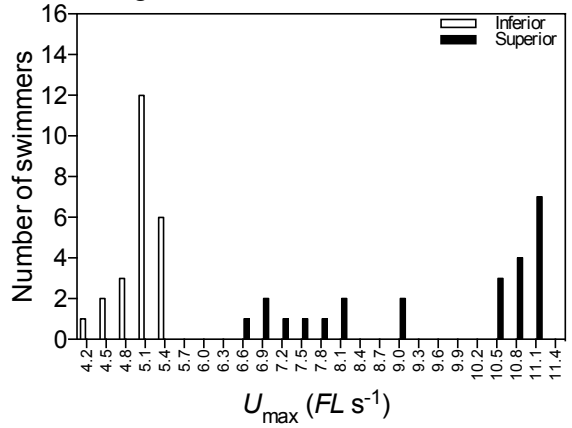


Randomly sampled fish for respirometry

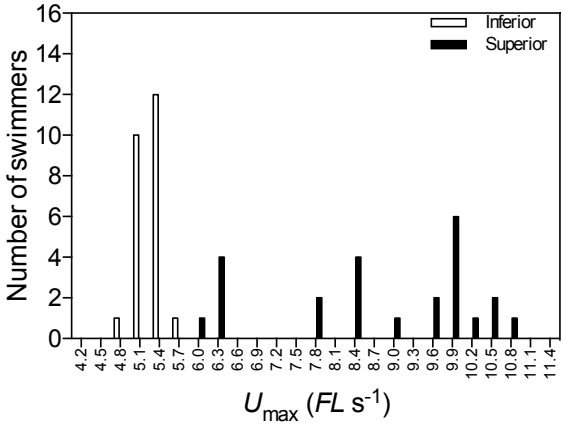


A.5 Histogram of relative U_{max} distributions of screening trials and a sub-group for respirometry in *Bolaks* strain

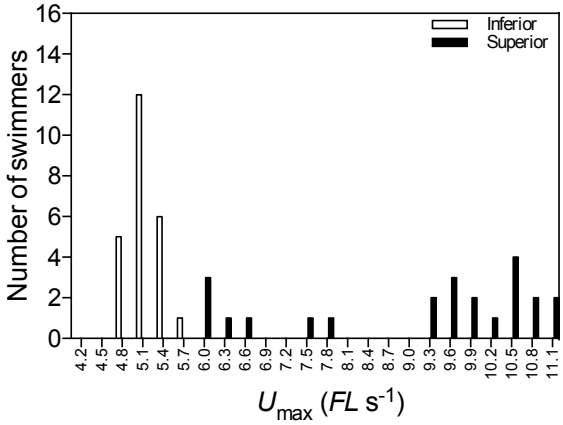
Screening trial 1



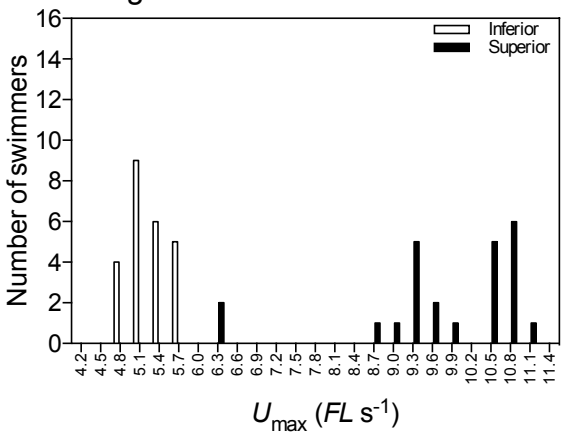
Screening trial 2



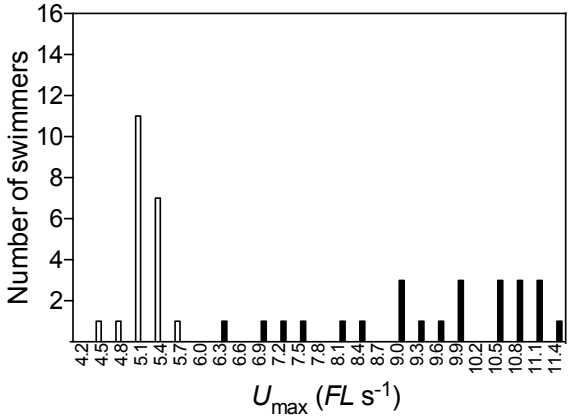
Screening trial 3



Screening trial 4

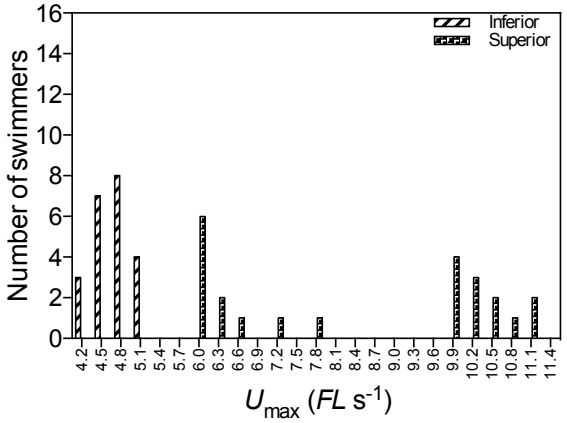


Randomly sampled fish for respirometry

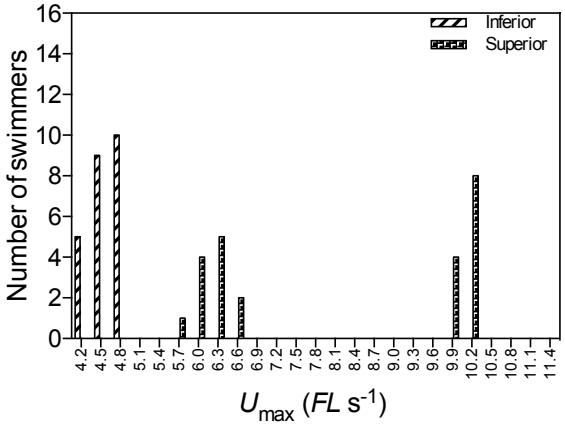


A.6 Histogram of relative U_{max} distributions of screening trials and a sub-group for respirometry in *Lærdal* strain

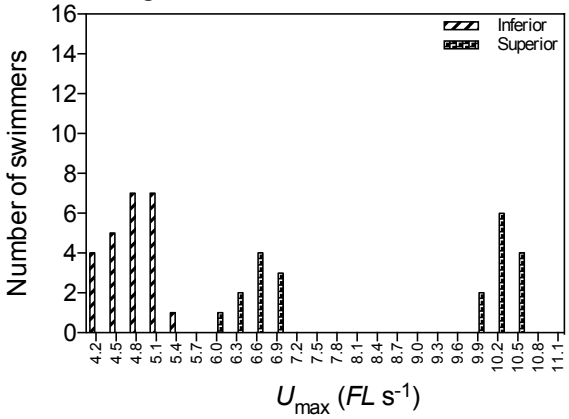
Screening trial 1



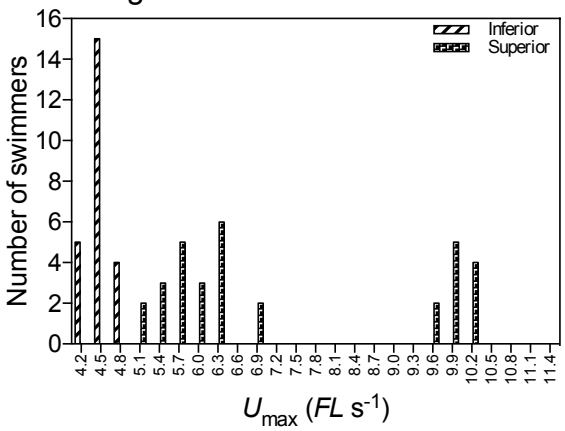
Screening trial 2



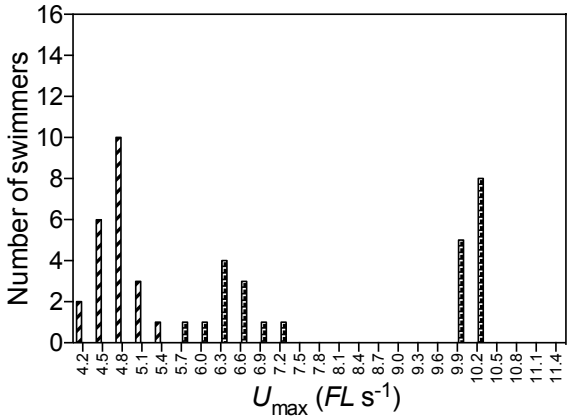
Screening trial 3



Screening trial 4



Randomly sampled fish for respirometry



A.7 Body weight and fork length in eight groups of Atlantic salmon (*Salmo salar*) used for the respirometry experiment. Different superscript letters in a column indicate significant differences ($P<0.05$) in body mass and fork length, detected by three-way ANOVA Tukey HSD *post-hoc* test. Different superscript letters in a column indicate significant differences ($P<0.05$) in body mass and fork length, regarding strain, training, and initial swimming capacity, were made using a three-way ANOVA with a Tukey HSD *post-hoc* test.

Strain	Initial swimming capacity	Training	Body mass (g)	Fork length (cm)
Bolaks	Inferior	$Control_{(n=12)}$	38.6 (1.9)[a]	14.4 (0.1)[a]
Bolaks	Superior	$Control_{(n=12)}$	38.3 (0.9)[a]	14.7 (0.1)[a]
Bolaks	Inferior	$Trained_{(n=9)}$	31.6 (1.3)[a]	14.2 (0.2)[a]
Bolaks	Superior	$Trained_{(n=12)}$	32.8 (1.3)[a]	14.3 (0.2)[a]
Lærdal	Inferior	$Control_{(n=11)}$	33.4 (0.8)[a]	13.8 (0.1)[a]
Lærdal	Superior	$Control_{(n=12)}$	34.0 (0.9)[a]	13.9 (0.1)[a]
Lærdal	Inferior	$Trained_{(n=11)}$	30.9 (1.1)[a]	14.2 (0.2)[a]
Lærdal	Superior	$Trained_{(n=11)}$	31.9 (0.5)[a]	14.2 (0.2)[a]

Values are mean (s.e.m.)

A.8 Standard metabolic rate (SMR, mg O_2 h^{-1} kg^{-1}), maximum rate of oxygen uptake ($\dot{M}O_{2max}$, mg O_2 h^{-1} kg^{-1}), absolute aerobic scope (AAS, mg O_2 h^{-1} kg^{-1}), factorial aerobic scope (FAS), excess post-exercise oxygen consumption (EPOC, mg O_2 kg^{-1}) and critical oxygen level (O_{2crit}, % sat.) and incipient lethal oxygen saturation (ILOS, % $O_{2sat.}$) in inferior and superior swimmers of domesticated (*Bolaks*) and wild (*Lærdal*) strains of Atlantic salmon (*Salmo salar*) parr (R^2>0.8). The values were calculated using the Low10 approach. Values in a row with different superscript letter were significantly different (*P*<0.05), detected by three-way MANOVA followed by multiple ANOVAs with Tukey HSD *post-hoc*.

Variables	*Bolaks*				*Lærdal*			
	Inferior swimmer		Superior swimmer		Inferior swimmer		Superior swimmer	
	Control (N=12)	Trained (N=9)	Control (N=12)	Trained (N=12)	Control (N=11)	Trained (N=11)	Control (N=12)	Trained (N=11)
SMR	64.5 (4.5)^{a}	65.3 (5.2)^{a}	70.2 (4.5)^{a}	65.2 (4.5)^{a}	60.9 (4.7)^{a}	68.5 (4.7)^{a}	63.6 (4.5)^{a}	67.7 (4.5)^{a}
$\dot{M}O_{2max}$	312.1 (16.4)^{c}	327.1 (18.9)^{bc}	317.7 (16.4)^{c}	316.5 (16.4)^{c}	378.9 (17.1)^{abc}	401.2 (17.1)^{ab}	347.5 (16.4)^{bc}	440.4 (16.4)^{a}
AAS	247.6 (15.7)^{c}	261.8 (18.1)^{bc}	247.5 (15.7)^{c}	251.4 (15.7)^{c}	318.0 (16.4)^{ab}	332.7 (16.4)^{ab}	283.8 (15.7)^{bc}	372.7 (15.7)^{a}
FAS	5.1 (0.4)^{b}	5.2 (0.5)^{ab}	4.8 (0.4)^{b}	5.1 (0.4)^{b}	6.4 (0.4)^{ab}	5.9 (0.4)^{ab}	5.6 (0.4)^{ab}	6.9 (0.4)^{a}
EPOC	739.0 (66.8)^{ab}	872.6 (77.1)^{ab}	716.4 (66.8)^{b}	900.5 (66.8)^{ab}	629.3 (87.4)^{b}	824.4 (103.4)^{ab}	700.1 (73.1)^{ab}	1159.3 (115.7)^{a}
O_{2crit}	17.5 (0.8)^{ab}	17.5 (0.9)^{ab}	18.3 (0.8)^{b}	16.7 (0.8)^{ab}	16.4 (0.8)^{ab}	14.6 (0.8)^{a}	16.1 (0.8)^{ab}	16.2 (0.8)^{ab}
ILOS	15.7 (0.4)^{ab}	16.1 (0.6)^{ab}	16.5 (0.6)^{b}	15.2 (0.6)^{ab}	14.2 (0.8)^{ab}	12.7 (1.1)^{a}	14.2 (0.8)^{ab}	14.2 (0.9)^{ab}

Three-way (strain*swimming capacity*training) MANOVA: strain F=12.1, p<0.001; swimming capacity F=0.67, p=0.7; training F=4.3, p<0.001

Values are means (s.e.m.)

Sample sizes of EPOC in wild trained inferior and superior swimmers were 5 & 4, respectively.

A.9 Body weight, fork length and muscle tissue sample mass in eight sampling groups of Atlantic salmon (*Salmo salar*) for citrate synthase (CS) and lactate dehydrogenase (LDH) enzyme activities measurements. n=15 per group. Different superscript letters in a column indicate significant differences ($P<0.05$) in body mass and fork length, regarding strain, training, and initial swimming capacity, were made using a three-way ANOVA with a Tukey HSD *post-hoc* test.

Strain	Initial swimming capacity	Training	Body mass (g)	Fork length (cm)	Red muscle sample mass (mg)	White muscle sample mass (mg)
Bolaks	Inferior	Control	34.3 (0.7)[b]	14.3 (0.1)[a]	16.8 (0.6)	29.2 (2.2)
Bolaks	Superior	Control	36.3 (0.8)[b]	14.8 (0.1)[a]	18.4 (0.7)	38.3 (2.3)
Bolaks	Inferior	Trained	30.3 (0.8)[a]	14.0 (0.1)[a]	19.8 (0.8)	37.2 (2.4)
Bolaks	Superior	Trained	31.6 (0.6)[a]	14.1 (0.1)[a]	17.0 (0.8)	34.9 (2.1)
Lærdal	Inferior	Control	28.7 (0.5)[a]	14.1 (0.1)[a]	15.0 (1.0)	29.3 (2.7)
Lærdal	Superior	Control	30.1 (0.6)[a]	14.3 (0.1)[a]	14.9 (0.6)	30.6 (2.2)
Lærdal	Inferior	Trained	30.1 (0.9)[a]	14.0 (0.1)[a]	13.6 (0.5)	29.6 (2.1)
Lærdal	Superior	Trained	31.1 (0.9)[a]	14.4 (0.2)[a]	15.1 (0.7)	29.4 (1.9)

Values are mean (s.e.m.)

The End