

The oxygen requirement of Atlantic salmon (*Salmo salar* L.) in the on-growing phase in sea cages

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SUMMARY

Atlantic salmon aquaculture is a successful and growing industry, with a global production of more than 1.4 million tonnes in 2010. In the on-growing phase, Atlantic salmon are normally kept in sea cages, where both the water temperature and oxygen levels may fluctuate substantially over time and with depth. Oxygen is the main limiting factor of fish metabolism, and sufficient oxygen is therefore essential for all energy-demanding processes, including growth and basic life-supporting functions. Knowledge of the dissolved oxygen (DO) requirement of Atlantic salmon in the on-growing phase, and this species' ability to cope with observed fluctuations in DO (30-120% O₂) is however limited. The overall aim of this thesis was therefore to study the physiology and production performance of Atlantic salmon in response to changes in the oxygen availability, in order to establish knowledge that can be used to assess whether growth and welfare is compromised as a result of insufficient oxygen in the on-growing phase.

Insufficient oxygen supply (environmental hypoxia) is primarily considered to be a problem in summer and autumn, and to occur in short/ frequent rather than prolonged periods. The studies of responses to oxygen fluctuations were therefore performed at 16 °C (a typical autumn temperature along the Western coast of Norway) and with hypoxia occurring in cycles corresponding to the turn of the tidal current, resembling previous observations in sea cages (2 h of hypoxia every 6 h, termed "cyclic hypoxia"). However, the oxygen requirement of fish is known to increase with temperature and with the level of activity. For the determination of the minimum DO requirement of Atlantic salmon, temperatures were therefore set to range between 6 and 18 °C, and the experimental conditions were set to induce activity levels as similar to fish in sea cages as possible.

For the typical autumn temperature of 16 °C, the threshold for optimal feeding of Atlantic salmon post-smolts was ~70% O₂. Feed intake, and presumably also the digestion rate, decreased with increasing hypoxia severity, reaching low levels as DO approached the hypoxia tolerance threshold of 47% O₂. Below this DO threshold, fish were no longer able to maintain their oxygen uptake rate, and resorted to anaerobic ATP production to cover their energy demand. Further, a general stress response was induced at DO equivalent to 51-54% O₂, and a few fish died during the first day of hypoxic periods (40 and 50% O₂). The limited ability to maintain basic functions and the stress and compromised survival at DO below the hypoxia tolerance threshold, demonstrate that this is a limit for acceptable drops in oxygen in Atlantic salmon sea cages, both with regard to production performance and welfare. For post-

smolts with low swimming speed and relatively low growth rates, this threshold increased exponentially with temperature, from 29 to 55% O₂ at temperatures ranging from 6-18 °C. It was estimated that this threshold should be increased by approximately 40% (*i.e.* 41-77% O₂) in order to serve as practical guideline for salmon farming, due to the higher level of activity (feed intake, swimming speed etc.) that can be expected for fish in sea cages.

The reduced feed intake of post-smolts in hypoxic periods was to a large extent compensated for by increased feeding in normoxic periods. This compensation developed after the initial stress response was down-regulated (< 1 week). However, feeding in normoxia did not fully alleviate the negative effects when hypoxia occurred for 2 h every 6 h. As growth reflected feed intake, it was concluded that such frequency and duration of hypoxia reduce the growth and welfare of Atlantic salmon, if DO is reduced below the general threshold of 70% O₂ in hypoxic periods, and that the degree of negative effect depends on the hypoxia severity. The relatively large capacity for normoxic compensation does however suggest that negative effects of moderate hypoxia (DO>hypoxia tolerance threshold) may be fully alleviated if the hypoxic periods are of shorter duration and/or frequency. The hypoxia sensitivity of Atlantic salmon was not considerably reduced as a result of acclimation to cyclic hypoxia, as indicated by the persisting anaerobiosis and inhibited feed intake during the hypoxic periods.

The main conclusions from this thesis is that reductions in DO below the hypoxia tolerance threshold is unacceptable both with regard to production performance and welfare, while negative effects of DO ranging between this threshold and the threshold for maintained feeding depend on hypoxia severity, frequency and duration. Acclimation to hypoxia did not reduce hypoxia sensitivity, while habituation to hypoxic stress was linked to an increased compensatory feeding in normoxic periods. Hypoxia occurring for ~2 h in tidal cycles (~every 6 h) was not fully compensated for, in spite of stress habituation and normoxic feeding.

LIST OF PAPERS

Paper I

Mette Remen, Frode Oppedal, Thomas Torgersen, Albert K. Imsland, Rolf Erik Olsen

Effects of cyclic environmental hypoxia on physiology and feed intake of post-smolt Atlantic salmon: Initial responses and acclimation. *Aquaculture* 326–329, 148–155.

Paper II

Mette Remen, Turid Synnøve Aas, Tone Vågseth, Thomas Torgersen, Rolf Erik Olsen, Albert K. Imsland, Frode Oppedal

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Paper III

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Hypoxia tolerance thresholds for post-smolt Atlantic salmon: Dependency of temperature and hypoxia acclimation. Manuscript ready for submission to *Aquaculture*.

1. INTRODUCTION

In Norway, the number of Atlantic salmon (*Salmo salar* L.) kept in aquaculture sea cages exceeded 330 million by the end of 2010, and the total sales value of farmed salmon exceeded 28 billion NOK the same year (Statistics Norway, 2011). Despite the size and the economic importance of this industry, the knowledge about the environmental requirements of this species is surprisingly scarce. While many years of research have yielded detailed information of growth, sexual maturation, nutritional demands, behaviour, immune system, diseases, vaccine development etc., little is known about the capacity of Atlantic salmon to function properly and to grow in the varying environmental conditions experienced in sea cages during the on-growing phase.

Recent publications have revealed that the oxygen content of the water within Atlantic salmon sea cages fluctuates substantially (30-120% O₂), both temporally (tidal, diurnal and seasonal variation) and spatially (Crampton et al., 2003; Johansson et al., 2006, 2007; Vigen, 2008; Oppedal et al., 2011; Burt et al., 2012). According to previously suggested oxygen minimums for growth of salmonids (68-120% O₂) (Davis, 1975; Barton and Taylor, 1996; Wedemeyer, 1996; EFSA, 2008; Thorarensen and Farrell, 2011), these observations imply that oxygen levels in periods are too low to maintain optimal growth, and that the most severe reductions in oxygen may compromise basic function and survival. However, as little is known about the oxygen requirement of Atlantic salmon in response to factors such as temperature, size, activity level, feeding status and low-oxygen acclimation, it is difficult to assess whether, and to what extent the observed oxygen fluctuations compromise growth and welfare of Atlantic salmon. This hampers decision-making in the salmon farming industry and slows the development of preventive or alleviating measures that may be necessary to avoid negative effects.

2. BACKGROUND

2.1. The oxygen demand of Atlantic salmon

Oxygen (O₂) is essential for the function, growth and welfare of animals, because of the energy which is released when O₂ is reduced to H₂O in the final stage of cellular respiration (Berg et al., 2001). When one glucose molecule is broken down in the presence of oxygen, this results in the formation of ~30 adenosine triphosphates (ATP, the primary energy donor in cells) as opposed to only 6 when oxygen is absent (Berg et al., 2001). Due to this link between oxygen consumption and ATP production, the overall oxygen consumption rate (MO₂) of an animal is often used as a measure of its total energy expenditure, or metabolic rate (Schmidt-Nielsen, 1997).

The level of dissolved oxygen in water (DO) which is required to meet the metabolic demands of Atlantic salmon can be expected to depend on a range of factors that affect the total energy expenditure. For example, the metabolic rate (MR) is known to increase with temperature, feeding level, swimming speed and stress (Forsberg, 1997; Deitch et al., 2006; Wilson et al., 2007; Barnes et al., 2011; Folkedal et al., 2012), as well as to decrease with increasing size (Forsberg, 1994). According to the widely accepted, theoretical framework presented by Fry (1947, 1971), the environmental temperature is the main controlling factor of metabolism, and is therefore a key factor to consider with regard to the oxygen demand of a fish. The standard metabolic rate (SMR; the MR of fasted fish at rest) increase exponentially with temperature in salmonids (Brett, 1971; Brett and Groves, 1979), while the maximal metabolic rate (MMR, the MR of fish swimming at maximal speed) increases up to a certain thermal optimum, beyond which MMR falls off and starts to decrease with further temperature increase (Brett, 1971; Fry, 1971; Farrell, 2009). The difference between the SMR and the maximum metabolic rate (MMR, the MR of fish swimming at maximal speed) at a given temperature has been termed the metabolic scope by Fry (1947, 1971), and is a widely used measure of the aerobic performance capacity of fish (Claireaux and Legardère, 1999; Claireaux et al., 2000; Wang et al., 2009; Pörtner, 2010). The metabolic scope, and thus the growth performance of a fish, is at its highest at the thermal optimum, and gradually decrease to zero levels towards both ends of the temperature tolerance range (Farrell, 2009; Pörtner, 2010). For Atlantic salmon, the lower and upper limits of the temperature tolerance range has been found to be 0-7 and 22-28 °C, respectively, and the thermal optimum is thought to lie between 16 and 20 °C (reviewed by Elliott and Elliott, 2010). The MMR may be 6 times

higher than the routine metabolic rate (RMR, the metabolic rate of fasted fish swimming at low speed) in Atlantic salmon (Deitch et al., 2006; Wilson et al., 2007), emphasizing that both the temperature and the level of activity (e.g. feeding and swimming) need to be considered when the DO requirement of Atlantic salmon is determined.

2.2. The oxygen availability in Atlantic salmon sea cages

The level of dissolved oxygen (DO) in sea water depends on 1) the oxygen input, which origin from algal photosynthesis or from mixing/diffusion of oxygen into water from air, 2) the biological oxygen consumption (e.g. bacteria, algae and fish), 3) the vertical and horizontal mixing of water and 4) the solubility of oxygen, which increases with barometric pressure, and decreases with temperature and salinity (Davis, 1975; Bauerfeind, 1985; Skjelvan et al., 2001; Crampton et al., 2003; Johansson et al., 2007; O'Boyle and Nolan, 2010). Thus, the DO of water that enters sea cages, can be expected to vary. Focusing on the surface layers (0-50 m depth) along the Norwegian coast, where 65% of the global Atlantic salmon production took place in 2010 (LaksefaktaTM, 2011), the DO has been found to range between 70 and 140% O₂ (Skjelvan et al., 2001; Johansson et al., 2006, 2007; Vigen, 2008; Aure et al., 2009; Hjelle et al., 2010). Generally, oxygen levels are highest in surface water layers in the summer season, when algal oxygen production is high (Skjelvan et al., 2001; Vigen, 2008; Hjelle et al., 2010; Oppedal et al., 2011). In this season, oxygen levels generally decrease with depth, and reaches more stable levels at 50 m depth (~80% O₂) (Hjelle et al., 2010). Diurnal fluctuations in oxygen occur when algal oxygen production is high, as algae consume oxygen at night (the dark reaction) (Purves et al., 2001). Substantial diurnal oxygen fluctuations have been observed during algal blooms (e.g. 65-90% O₂) (Crampton et al., 2003), but whether, and to what extent diurnal fluctuations are common during the summer season is not known. Recently shown by Stien and Kristiansen (2012), algal blooms may also result in continuous (several days), severe reductions in oxygen (down to 40% O₂) due to an accumulation of dying/dead algae (Tore Kristiansen, *pers. comm.*). When the water column is stratified, oxygen levels are generally lower below the thermocline, due to the restricted transport of oxygen to the lower water layers (Johansson et al., 2007; Vigen, 2008; O'Boyle and Nolan, 2010).

Within Atlantic salmon sea cages, more profound fluctuations in oxygen may arise, as a result of the oxygen consumption of fish (Aure et al., 2009). Any factor that hinder the water flow through rate (e.g. reduced water current speed, increased biofouling and fish

stocking density) and/or increase the oxygen consumption rate of fish (e.g. increased biomass, temperature, stress, swimming speed) (see 2.1), may potentially reduce oxygen levels within sea cages (Levings et al., 1995; Johansson et al., 2006, 2007; Vigen, 2008; Aure et al., 2009; Gansel et al., 2012). Two studies from sea cages on the Western coast of Norway have shown that oxygen levels may fluctuate substantially in autumn, from 80-100% O₂ down to 30% O₂ in extreme cases, and frequent reductions below 60% O₂ (Johansson et al., 2006; Vigen, 2008). The cyclic nature of oxygen reductions (approximately every 6 h) suggest that this is linked to the turn of tidal currents, as water exchange rates, and thus the oxygen supply, may be low during these cyclic turns (Johansson et al., 2007). This “tidal” hypoxia frequency is termed “cyclic hypoxia” in the following. It can be assumed that this is predominantly a problem during autumn when stocking density, growth rates and water temperatures are all high (Oppedal et al., 2011), leading to low oxygen solubility and high oxygen consumptions rates (Brett, 1971; Davis, 1975; Brett and Groves, 1979).

2.3. Responses to reduced oxygen availability

In the sea-water phase, Atlantic salmon are fast-swimming, pelagic fish with high growth rates and oxygen demand. They are opportunistic feeders, and are normally found close to the surface (Hansen and Quinn, 1998). Similar to other salmonids, Atlantic salmon is considered hypoxia-sensitive. They lack specialized mechanisms to deal with severe and long-term hypoxia, such as the metabolic depression, large glycogen stores, and conversion of lactate to ethanol, which is found in the hypoxia tolerant *Carassius* species (e.g. carp and goldfish) (reviewed by Bickler and Buck, 2007; Nilsson and Nilsson, 2008). The life-style and hypoxia sensitivity of salmonids in the sea-water phase is likely to reflect a habitat where oxygen levels normally support high levels of activity and that areas with low oxygen availability can be avoided. Some variation in oxygen levels is however expected (see 2.2), and may be successfully compensated for.

The minimum DO required to support the maximal metabolic rate of a fish can be considered the limit between environmental normoxia and hypoxia (Farrell and Richards, 2009). Above this limit, metabolism is independent of the water oxygen level, while the metabolic scope decreases progressively as oxygen declines within the zone of hypoxia (Farrell and Richards, 2009). When fish encounter hypoxia they may respond to the reduced oxygen availability by reducing both the oxygen demand and -uptake (referred to as oxyconforming), or they may actively try to maintain the oxygen uptake rate over a large

range of hypoxic levels (referred to as oxyregulation) (reviewed by Pörtner and Grieshaber, 1993; Perry et al., 2009). Atlantic salmon, in accordance with other salmonids, have been found to oxyregulate (Stevens et al., 1998; Barnes et al., 2011). The most important means of maintaining the oxygen uptake rate is to increase the ventilation and perfusion of the gills, increasing the gradient and area for oxygen diffusion. The increased equilibration between water and blood at the gills may also result in a respiratory alkalosis, which increases the hemoglobin oxygen affinity through the Bohr effect (Jenssen et al., 1998; Gilmour, 2001; Perry et al., 2009). Although the oxygen consumption rate may be upheld, the depression of the metabolic scope, as well as the increased respiratory energy demand, will gradually reduce the energy available for “voluntary” activities such as feeding and growth (Neill and Bryan, 1991). Thus, the compensatory mechanisms may serve to maintain the functional integrity of the fish, but the production performance is gradually reduced as oxygen declines. Eventually, the cost of increasing gill ventilation becomes exhaustive and counteractive, and fish resort to oxyconformation (reviewed by Perry et al., 2009). The threshold DO where this shift occurs is termed the limiting oxygen saturation (LOS¹) (Neill and Bryan, 1991; Wang et al., 2009).

The LOS represents a threshold below which a range of physiological and behavioral responses are elicited in salmonids, in order to resist and survive the depression of aerobic metabolism. First of all, the rate of anaerobic ATP production increases sharply, and end-products, such as lactate and protons, accumulate (Van den Thillart and Van Waarde, 1985; Vianen et al., 2001; Omlin and Weber, 2010). Survival becomes dependent on the availability of substrates for anaerobic glycolysis (Nilsson and Nilsson, 2008). Further, such reductions in oxygen may induce a release of stress hormones (catecholamines and corticosteroids) (e.g. Reid and Perry, 1994; Van Raaij et al., 1996; Vianen et al., 2001). This may enhance the ability to resist the hypoxic stress, e.g. by increasing the oxygen transport capacity of blood (Kita and Itazawa, 1989; Randall and Perry, 1992; Perry and Reid, 1994; Perry and Gilmour, 1996) and by mobilizing energy (Schreck 1981; Wendelaar Bonga 1997; Mommsen et al., 1999), but over time this response may also become maladaptive. For example, osmoregulation may be compromised during stress (Schreck, 1981; Wendelaar Bonga, 1997) and the substrates for anaerobiosis are more rapidly depleted (Van Raaij et al., 1996).

¹ LOS is equivalent to the limiting oxygen concentration (LOC) described by Neill and Bryan (1991) and the P_{crit} described by Wang et al. (2009), but defined here in units of oxygen saturation because this is considered the most appropriate (see Paper III), and the most comprehensible unit for practical use by aquaculturists.

The LOS is considered the limit between the hypoxic “zone of tolerance” and the “zone of resistance”, as fish are generally able to maintain their functional integrity at DO above this threshold, while function is threatened, and survival is time-limited at DO below this threshold (Hughes, 1981; Fagerlund et al., 1995; Nilsson and Nilsson, 2008). LOS is therefore also referred to as the “hypoxia tolerance threshold” (Barnes et al., 2011). In the following, this limit will be used to differentiate between “moderate” and “severe” hypoxia (e.g., Svendsen et al., 2012).

2.4. Present oxygen thresholds for Atlantic salmon

For salmonids in general, several authors have suggested oxygen thresholds for optimal growth, for example 82-100% O₂ (reviewed by Davis, 1975), 68-82% O₂ (reviewed by Barton and Taylor, 1996) and 77-85% O₂ (reviewed by Wedemeyer, 1996), at temperatures ranging from 8 to 20 °C. There is a general scarcity of studies investigating the DO requirement of Atlantic salmon in particular, but the existing data suggest that the threshold is higher for this species, at least 85% O₂, and possibly up to 120% O₂ (reviewed by Thorarensen and Farrell, 2011). Combined with results from sea cage environment studies (e.g. Crampton et al., 2003; Johansson et al., 2006; Oppedal et al., 2011), these results indicate that the oxygen levels in sea cages may substantially restrict the growth potential of Atlantic salmon during the summer and autumn period when temperature is high (DO ranging from 30-120% O₂). More detailed knowledge of the DO requirement of this species is required in order to evaluate the effect of oxygen fluctuations on the growth of Atlantic salmon sea cages. It is also necessary to take into consideration that oxygen levels may fluctuate substantially on a short-term scale, and that fish may be able to compensate during periods of high oxygen availability. For example, a study by Forsberg and Bergheim (1996), showed that diurnal fluctuations in oxygen from high to low levels of oxygen (~137-47% O₂) at 8 °C did not induce any reduction of the growth performance of post-smolts. Further, it is possible that acclimation to hypoxia alleviates negative effects and that this should be considered when establishing oxygen thresholds. Several studies have showed that the capacity for oxygen uptake- and transport improves in salmonids during prolonged hypoxia (Wood and Johansen, 1972; Soivio et al., 1980; Tetens and Lykkeboe, 1981; Bushnell et al., 1984; Lai et al., 2006), but it is not clear whether such adjustments improve the growth performance of salmonids subjected to hypoxia.

Limited knowledge is also available for the other relevant oxygen threshold, the LOS. The importance of knowing what DO that represents this threshold was emphasized by data recently published by Stien and Kristiansen (2012). This data showed the variation in oxygen and daily mortalities over a 2 month period in autumn in a commercial Atlantic salmon sea cage at the Western coast of Norway. Generally, mortality was low, and oxygen levels ranged between 80-110% O₂. However, during a few days with high algal densities, oxygen declined to low levels (down to 40% O₂ at worst), and a large number of individuals died (up to ~3000 per day). If the oxygen fluctuations in sea cages are followed more closely and the hypoxia tolerance threshold (LOS) is known, measures can be taken to prevent lethal oxygen reductions in the future. As LOS is considered to be a continuum, which increases with the metabolic rate of fish (Fry, 1971; Wang et al., 2009), the effect of temperature and the level of activity (e.g. feeding and swimming) must be taken into consideration when LOS is determined for practical use in salmon farming. A recent study found that the LOS ranged from 28 to 81% O₂ in individual Atlantic salmon post-smolts subjected to 14-22 °C (Barnes et al., 2011), but as fish were fasted and kept in a respirometer during the measurement, these results alone are of limited use as practical guidelines for aquaculture.

2.5. Fish welfare in hypoxia

In order to assess the welfare of Atlantic salmon subjected to fluctuations in oxygen, it is necessary to define fish welfare and to base the assessment on observable indicators that are relevant for welfare. This opens the door to the on-going debate on fish welfare (see Branson, 2008). For example, different welfare definitions are based on varying concerns for the animal, from the ability to function well, feel well or live a natural life (reviewed by Lawrence, 2008). However, as pointed out by Torgersen et al. (2011), the concept of welfare is redundant, if it only concerns the function of a fish, or the nature of its surroundings. For welfare to be a meaningful concept, it should be used to describe the emotional state, or the ‘quality of life’ that is experienced by the individual (Duncan and Fraser, 1997; Huntingford et al., 2006; Volpato et al., 2007). This leads to another controversy, namely whether fish are capable of having a subjective experience of its own welfare (e.g., Rose, 2002; Chandroo et al., 2004; Huntingford, 2006, 2007; Rose, 2007). How, and to what extent fish experience positive and negative emotions cannot be known for certain, but the advanced brain and sensory apparatus, the hormonal “reward system”, and the cognitive abilities of fish, point towards some level of sentience (reviewed by Spruijt et al., 2001; Chandroo et al., 2004;

Braithwaite and Boulcott, 2007). Similar to the standing-point of the Norwegian authorities, the basis for welfare discussion in this thesis is that fish “have a subjective experience of their own mental and physical state” (Anon., 2005), and that welfare has to do with the “quality of life”, as experienced by the individual (Duncan and Fraser, 1997).

As it is not possible to measure the emotional state of a fish, an assessment of welfare relies on indicators that can be linked to the emotional state (Bracke et al., 1999; Fraser and Duncan, 1998). It is generally accepted that emotions have evolved in order to motivate and direct the physiology and behavior towards a fulfillment of the animals’ needs and avoidance of harm (Duncan and Fraser, 1997; Berridge, 2004). Thus, an inability to fulfill important needs (“welfare needs”), such as respiration, thermoregulation or nutrient uptake, can be expected to induce negative emotions and reduce welfare (Bracke et al., 1999). In conjunction to the effect of environmental variables on fish welfare, an investigation of physiological and behavioral parameters, such as the nutritional status, the level of stress hormones in blood or swimming behavior (e.g. changes in speed/pattern), may therefore be used to assess whether fish are deprived of such needs (Bracke et al., 1999; Stien et al., 2007; Huntingford and Kadri, 2008). For example, a prolonged depression of feed intake caused by hypoxia can be expected to negatively influence the welfare of Atlantic salmon, because feeding can be considered an important welfare need of this fast-growing species in the sea-water phase. Further, if hypoxia is severe, it can be assumed that the inability to maintain the oxygen uptake rate, as well as the struggle to maintain other, fundamental needs (e.g. osmoregulation and cardiac function) is equivalent to poor welfare. By performing a multidisciplinary assessment of physiology and behaviour, the welfare may be estimated on a range from good to poor depending on the degree of need fulfillment (Bracke et al., 1999; Stien et al., 2012).

3. AIMS OF THE THESIS

The overall aim of this thesis was to study the physiology and production performance of Atlantic salmon in response to changes in the oxygen availability. Such knowledge is necessary in order to assess whether, and to what extent, observed oxygen reductions in sea cages compromise the growth performance and/or welfare of Atlantic salmon in the on-growing phase. Five main objectives were defined:

1. To investigate the effect of increasing hypoxia severity (70-40% O₂) on feed intake and to identify the oxygen level where feed intake is initially reduced at a typical autumn temperature of 16 °C (Paper I).
2. To identify the onset of anaerobiosis and stress in post-smolts subjected to hypoxic periods of increasing severity (70-40% O₂) at 16 °C (Paper I).
3. To investigate the effects on physiology (the oxygen consumption rate and parameters of stress), feed intake and feed utilization when oxygen fluctuates between hypoxic and normoxic levels (cyclic hypoxia), and to see whether compensation occurs in normoxic periods (Paper I, II).
4. To determine the relationship between temperature (6, 12 and 18 °C), the oxygen consumption rate (MO₂) and the hypoxia tolerance threshold in post-smolts kept under production-like conditions (Paper III).
5. To investigate whether acclimation to cyclic hypoxia alleviates negative effects on feed intake (Paper I, II) and/or physiological status (Paper I), or lower the hypoxia tolerance threshold (III).

In Paper I and II, a typical summer/autumn temperature for the Western coast of Norway was used (16 °C) (Johansson et al., 2007; Vigen, 2008; Oppedal et al., 2011), and the frequency and duration of hypoxic periods was set to resemble the “tidal” fluctuations in oxygen observed by Johansson et al. (2006) and Vigen (2008). The studies were limited to post-smolts <1 kg in order to reduce the number of factors that affect the DO requirement.

4. ABSTRACT OF PAPERS

Paper I

Effects of cyclic environmental hypoxia on physiology and feed intake of post-smolt Atlantic salmon: Initial responses and acclimation.

Mette Remen, Frode Oppedal, Thomas Torgersen, Albert K. Imsland, Rolf Erik Olsen

In order to investigate the effects of hypoxic periods on the feeding behaviour and physiology of Atlantic salmon (*Salmo salar* L.) post-smolts (237 ± 7 g), fish were kept at constant 90% O₂ (control) or subjected to cyclic reductions in oxygen, from 90% O₂ (termed normoxia) to 40, 50, 60 or 70% O₂ (termed hypoxia) for 2 h every 6 h for 23 days at 16 °C (groups are denoted as 90:40, 90:50, 90:60, 90:70 and 90:90). Fish were fed to satiation three times per day, twice in hypoxia and once in normoxia. Blood samples were drawn during hypoxic periods on day 0, 7, 14 and 21 and analyzed for hematocrit, haemoglobin concentration and plasma cortisol, chloride, lactate and glucose concentrations. During first exposure to hypoxia (day 0), plasma lactate was increased in 90:60, 90:50 and 90:40 groups, plasma cortisol was increased in 90:50 and 90:40 groups and plasma chloride was increased in the 90:40 group, indicating oxygen shortage and stress at oxygen saturations below 60% O₂. On day 7, 14 and 21, there were no signs of stress, but plasma lactate levels remained increased in hypoxic periods in 90:40, 90:50 (all days) and 90:60 groups (day 7 and 14), suggesting that the stress response was down-regulated in spite of a persisting oxygen dept. Feed intake was reduced according to hypoxia severity in groups subjected to 40-60% O₂ during the first meal of the day, and in all experimental groups (40-70% O₂) during the third meal of the day. The effect of hypoxia on feed intake persisted throughout the experiment, but total daily feed intake was substantially increased in 90:40 and 90:50 groups during week 2 and 3 due to compensatory feeding in the normoxic feeding period. Present results suggest that 70% O₂ may represent a threshold for reduced growth and that 60% O₂ represents a minimum O₂ saturation considering welfare of Atlantic salmon post-smolts subjected to cyclic hypoxia at 16 °C.

Paper II

Production performance of Atlantic salmon post-smolts in cyclic hypoxia, and following compensatory growth.

Mette Remen, Turid Synnøve Aas, Tone Vågseth, Thomas Torgersen, Rolf Erik Olsen, Albert Imsland, Frode Oppedal

The present study investigated growth performance of post-smolt Atlantic salmon (*Salmo salar*) subjected to cyclic environmental hypoxia, resembling observed tidal cycles in sea cages during late summer and autumn. Triplicate groups (N=955, initial weight=385±3 g, mean±SE) were kept in tanks at constant 80% O₂, (control, termed 80:80) or subjected to four daily reductions in oxygen saturation (1 h 45 min), to either 70, 60 or 50% O₂ (80:70, 80:60 and 80:50) at 16 °C for 68 days. Feed was provided twice daily in normoxia. After the cyclic hypoxia period, 1/3 of the fish were kept further for 30 days at 17 °C and 90% O₂ in order to study possible compensatory growth (termed the post-hypoxia period). Cyclic hypoxia did not induce any changes in oxygen uptake rates of fish in hypoxic or normoxic periods. Feed intakes were reduced by 13 and 6% in fish in the 80:50 and 80:60 groups during the cyclic hypoxia period, respectively, with corresponding reductions in specific growth rate. Feed conversion and apparent digestibility were not affected by hypoxia, but whole body contents of energy and lipid were reduced in the 80:50 group. This group also displayed compensatory growth during the post-hypoxia period, but the fish did not reach the same weight as fish in the control group. The main conclusions were that feeding in normoxia alleviates some, but not all of the negative effects of cyclic hypoxia on growth performance, and that the minimum oxygen saturation for maintained growth lies between 60 and 70% O₂.

Paper III

Hypoxia tolerance thresholds for post-smolt Atlantic salmon: Dependency of temperature and hypoxia acclimation

Mette Remen, Frode Oppedal, Albert K. Imsland, Rolf Erik Olsen, Thomas Torgersen

In order to establish hypoxia tolerance thresholds for Atlantic salmon (*Salmo salar*) in the on-growing phase, the effect of temperature (6, 12 and 18 °C) and hypoxia acclimation (33 days of hypoxic periods occurring every 6 hours at 16 °C) on the oxygen consumption rate (MO_2) and limiting oxygen concentration (LOC; referred to as the hypoxia tolerance threshold) was investigated in fish kept under production-like conditions (fed, undisturbed and freely swimming fish in tanks). Further, the effects of temperature and oxygen on the relationship between gill ventilation frequency (Vf) and MO_2 were studied in order to evaluate Vf as an indicator of MO_2 . Both MO_2 and LOC were found to increase exponentially with temperature ($Q_{10} = 2.7$ for MO_2 and 1.4 for LOC), while hypoxia acclimation resulted in a tendency for reduced MO_2 , but no lowering of LOC. The mean LOC at 6, 12, 16 and 18 °C were 2.9, 3.4, 3.8 and 4.3 mg L⁻¹, respectively. A strong correlation between MO_2 and LOS (LOC given in units of oxygen saturation) was found ($R^2=0.93$), regardless of temperature, suggesting that measurements of MO_2 can be used to estimate the LOS of post-smolts. Vf was considered a reliable estimator of MO_2 in normoxic conditions, but not during reductions in oxygen, due to the increasing Vf, and relatively stable MO_2 as oxygen declined towards LOC.

5. METHODOLOGICAL CONSIDERATIONS

5.1. Experimental conditions

Aiming to provide salmon farmers with knowledge that can be used to assess the effects of observed oxygen fluctuations on growth and welfare, three laboratory studies were carried out. The experimental conditions were kept as close to sea cage conditions as possible in all three studies, primarily by keeping freely swimming fish in groups and feeding them to satiation. However, the difference between swimming freely and feeding in a sea cage, and being confined to the small volume of a fish tank can be expected to be of relevance for the energy expenditure, and thus for the oxygen requirement. For example, the low water current speed, and the limited area for movement in the small tanks (~460 L) used in Paper I and III, can be expected to result in lower swimming speeds than typical for sea cages, and social interactions may influence the stress level and feed intake (Jobling, 1985; Metcalfe et al., 1995; Cubitt et al., 2008). The effect of experimental conditions on the reliability of the obtained hypoxia tolerance threshold was discussed in Paper III, concluding that the thresholds were lower than what can be expected for similar temperatures in sea cages. By using the relationship between MO_2 and LOS, it was however possible to estimate LOS that may serve as practical guidelines for salmon in sea cages (see Paper III and 6.1.4). As post-smolts were kept in the same tanks in Paper I, it may be suggested that the oxygen threshold for growth at 16 °C found in Paper I may also have been underestimated. Nonetheless, results from this experiment are considered more reliable for use in practical guidelines for commercial production, as fish were generally not disturbed (except for a rapid collection of 5 fish once every week) and had higher growth rates than the post-smolts in Paper III.

In the large tanks used in Paper II (~5600 L), the higher water current speed, the larger area for movement, exploration and avoidance can be considered to be more similar to the sea cage environment, and to yield oxygen thresholds that are more reliable for use as practical guidelines in sea cages. However, the experimental conditions used in Paper II induced sexual maturation, predominantly in males. This unexpected effect was later confirmed to be caused by the combination of high temperature (16 °C) and continuous lighting in a study by Fjelldal et al. (2011). Continuous light was primarily used to avoid the stress response linked to sudden transitions from light to dark (Folkedal et al., 2010), but the occurrence of sexual maturation clearly showed that this was not a useful strategy for a long-term growth study at

high temperature. Light/dark cycles that are more similar to the natural environment of sea cages, with gradual transitions between light and dark, are preferable in future studies.

5.2. Open respirometry

The measurement of oxygen consumption rates (MO_2) in Paper II and III were performed using fish tanks, with (~460 L; Paper III) or without lids (~5600 L; Paper II and III), and was referred to as “open respirometry”. Experimental conditions were set to resemble production conditions as much as possible (*i.e.* undisturbed, fed fish swimming freely; see 5.1.), and MO_2 was calculated based on measurements of water flow-through rate and the oxygen content of water entering and leaving the tanks (see Paper III). This procedure deviate from the generally used procedure for MO_2 measurements, where individual, fasted fish are placed in small, concealed chambers with controlled water current speeds (closed respirometry) (e.g., Deitch et al., 2006; Wilson et al., 2007). The generally used method is indispensable for determination of the metabolic scope (measurement of SMR/RMR and MMR) in various environmental conditions, but does not yield information about the oxygen requirement of fish kept under production-like conditions. The open respirometry was found to be better suited for this purpose, as this approach enables an observation of undisturbed, feeding fish.

Measuring the oxygen consumption rate of a fish group gives no information about the variation in metabolic rates between individuals. As shown by Ott et al. (1980) and Barnes et al. (2011), such variation may be substantial (SD of ± 13 -19% in the study of Barnes et al.), and is important to consider when general oxygen thresholds are established and used. However, as closed respirometry is time-consuming (one fish per respirometer per day in general), the number of replicates in such studies is generally low. Together, the few observations and large individual variation may prohibit a detection of the treatment effect. For example, the relationship between temperature (14-22 °C) and LOS was not clear from a previous study of Atlantic salmon in a closed respirometer (Barnes et al., 2011), while the results from the open respirometry in Paper III yielded a clear, exponential relationship between these two variables. In order to elucidate overall treatment effects, the open respirometry is therefore better suited, while an estimation of the individual variation requires closed respirometry.

During the measurement of LOS, the oxygen content of water progressively declined, as a result of minimal water exchange and oxygen consumption by the fish. This increases the gradient for oxygen diffusion over the water surface, and may thus influence the measurement

of MO_2 . As described in Paper III, the flux of oxygen over the water surface was investigated in empty tanks and controlled for in the calculation of MO_2 (found necessary for Exp II only). Whether the diffusion constant, k , is equal in empty tanks and in tanks with fish, depend on the extent to which the fish increases vertical mixing and surface turbulence. In Exp II in Paper III, the effect of fish on k was considered to be small because fish generally continued to swim against the water current and seldom broke the water surface during the decline in DO. Thus, the oxygen consumption rate measured by using open respirometry was considered reliable.

6. GENERAL DISCUSSION

6.1. Physiological responses to hypoxia of increasing severity

The results from Paper I-III provided an insight into the gradual effect of declining oxygen levels on the physiology and production performance of Atlantic salmon. A distinction can be made for the effects of reducing oxygen to levels within the zone of tolerance and the zone of resistance.

6.1.1. The zone of tolerance

Similar to previous observations in Atlantic salmon and other salmonids (Ott et al., 1980; Ultsch et al., 1980; Stevens et al., 1998; Barnes et al., 2011), post-smolts maintained their MO_2 relatively stable over a large DO range, in agreement with being oxyregulators (Papers II and III). As oxygen declined within this range, the gill ventilation frequency increased (Paper III), suggesting that the hypoxic ventilatory response is important for upholding MO_2 in Atlantic salmon, as it is in other oxyregulators (reviewed by Perry et al., 2009). The increased pumping of water over the gills does however come with a cost, which eventually exceeds the benefit of the O_2 so obtained (Perry et al., 2009). Below a threshold oxygen level, hyperventilation was therefore exhausted, and ventilation frequency started to decrease with further reduction in DO (Paper III). This threshold was termed the limiting oxygen saturation (LOS) (Paper III; Neill and Bryan, 1991). The oxygen level where ventilation peaked corresponded to the LOS, in accordance with previous observations in rainbow trout, as well as other oxyregulators (reviewed by Perry et al., 2009).

For oxygen declining within the zone of environmental hypoxia, the range of DO where MO_2 is kept stable has been termed the “zone of tolerance” (Hughes, 1981). Generally, physiological disturbances were small in post-smolts for DO within this range. At 16 °C, the only measurable change in blood parameters was a gradual increase in plasma lactate levels, which started at 60% O_2 (Paper I). Accumulation of lactate at DO above the LOS has previously been observed in rainbow trout (Vianen et al., 2001), and may be connected to the increased respiratory energy demand, while aerobic ATP production is stable. If other, energy-demanding functions are not sufficiently down-regulated (e.g. feed intake and digestion, see 6.2), an increase in anaerobic ATP production may be necessary.

The low level of physiological disturbance does however not mean that such levels of hypoxia are tolerated without any negative effects for the performance of Atlantic salmon. As

oxygen declines within the zone of tolerance, the metabolic scope is gradually reduced, reducing the energy available for “voluntary activities” such as feeding, digestion or swimming. At LOS, excess activity is reduced to zero levels (Fry, 1971; Brett and Groves 1979; Neill and Bryan, 1991). The gradual depression of post-smolt feed intake when oxygen declined from 70% O₂ to the LOS of 47% O₂ at 16 °C agree with such a gradual reduction of the metabolic scope (Paper I, III). Thus, the zone of tolerance represents a range of oxygen levels that can be tolerated on a short-term basis, but can be expected to impair the nutritional status and production performance of Atlantic salmon if hypoxia is frequent or prolonged. This is further discussed in 6.2.1.

6.1.2. The zone of resistance

When oxygen is reduced below the LOS, fish enter the “zone of resistance” (Hughes, 1981). Generally, reductions in oxygen to levels around or below the LOS found in Paper III (~47% O₂ at 16 °C), challenged the internal steady-state, or homeostasis, of post-smolts (Paper I). The increasing plasma concentration of lactate at oxygen levels ranging from 60 to 40% O₂ indicated that post-smolts were increasingly dependent on anaerobic ATP production to cover basic energy demands (Paper I), in agreement with the reduced oxygen available for aerobic metabolism (Paper III). This is a well-known response in rainbow trout subjected to DO below its LOS (Burton and Heath, 1980; Van Raaij et al., 1996; Vianen et al., 2001; Omlin and Weber, 2010), and may enable a relatively high rate of ATP-production in spite of the depressed aerobic metabolism. However, as the ATP-yield from anaerobiosis is low, carbon fuels are rapidly consumed, and end-products, such as lactate and protons, accumulate (Bickler and Buck, 2007). Survival-time in the “zone of resistance” can therefore be expected to be limited by the amount of fuels available, together with the severity and duration of hypoxia (Nilsson and Nilsson, 2008). The mortalities observed during the first days of cyclic hypoxia to levels around or below LOS (40 and 50% O₂) emphasize that mortalities may occur, also as a result of relatively short periods (2 hours) of such reductions (Paper I). Further, an unintended reduction in oxygen down to levels of approximately 30% O₂ showed that oxygen approached acute lethal levels, as ~30% of the fish died within 1-2 h (Paper I).

The initial reductions in oxygen to levels close to the LOS (51-54% O₂) also resulted in a sharp increase in the concentration of cortisol in plasma of post-smolts (Paper I), suggesting that a general stress response was induced (Barton, 2002; Wendelaar Bonga, 1997). The release of stress hormones into circulation at DO around or below LOS agrees with previous observations in rainbow trout (Perry and Reid, 1994; Perry and Gilmour, 1996;

Van Raaij et al., 1996), and is known to initiate a range of physiological and behavioral responses that may increase the ability to cope with a stressor (Barton, 2002; Wendelaar Bonga, 1997). However, the observed increase in plasma cortisol in post-smolts at DO close to the LOS was connected to osmoregulatory problems (elevated chloride levels), and a few mortalities during the first days of cyclic hypoxia (Paper I). This supports Van Raaij et al. (1996), who found that the stress response, and a concurrent increase in activity levels, reduces the coping ability when severe hypoxia cannot be escaped. Overall, the effects of reducing DO to levels below LOS indicated that such depression of aerobic metabolism is challenging for Atlantic salmon post-smolts, and that the coping ability and survival time is reduced if hypoxia induces a general stress response.

The distinction between the general responses to reductions in DO above and below LOS is important in order to understand the rapid development of severe physiological disturbance as DO falls below the LOS, but it should be emphasized that the LOS for a given temperature (e.g. 47% at 16 °C; Paper III) does not represent the LOS for all individuals. The LOS is a continuum which increases with the activity level (e.g. feeding, stress and swimming) and may thus vary largely between individuals (Ott et al., 1980; Barnes et al., 2011) (see 6.1.4 for further discussion). The large variation in plasma cortisol levels between post-smolts during the first reduction in oxygen down to levels of 51-54% O₂ may at least in part be related to such variation (Paper I).

6.1.3. Acclimation and habituation in cyclic hypoxia

Based on the positive effect of hypoxia acclimation on the oxygen uptake- and transport capacity of other salmonids (Soivio et al., 1980; Tetens and Lykkeboe, 1981; Lai et al., 2006), it was hypothesized that post-smolts increase their hypoxia tolerance over time, and that possible negative effects of cyclic hypoxia to some extent are alleviated. However, results from Paper I show that the accumulation of lactate and depression of feed intake was relatively stable during 21 days of cyclic hypoxia, and results from Paper III show that the DO representing the LOS of post-smolts did not change as a result of 33 days of acclimation to cyclic hypoxia. Thus, possible physiological adjustments did not seem to alleviate the limiting effect of oxygen on metabolism. As acclimatory processes were not studied in detail, it is not clear whether the continued hypoxia sensitivity was due to a lack of physiological adjustments, or if physiological adjustments occurred without considerable alleviating effects. According to recent studies of Atlantic cod (*Gadus morhua*), the latter may be the case. Peterson and Gamperl (2010, 2011) found that the metabolic capacity and -scope was not

improved as a result of hypoxia acclimation, in spite of several physiological adjustments that may improve the capacity for oxygen uptake and -utilization (e.g. increased blood hemoglobin concentration, increased ventilatory effort and enhanced tissue oxygen extraction). The simultaneous finding of reduced cardiac performance in hypoxia-acclimated fish lead the authors to conclude that improved oxygen uptake and -utilization only served to reduce the negative effects of impaired cardiac function on the cardio-respiratory function.

In spite of the continued limiting effect of oxygen on metabolism of post-smolts, the stress response observed during the initial hypoxic periods (DO=40-54% O₂) was rapidly down-regulated. Within 4-6 days, the release of cortisol to water was reduced to levels of the control (Fig. 6.1; Paper I). Down-regulation of stress has been observed previously in rainbow trout subjected to severe hypoxia (Bernier and Craig, 2005), and can be explained by a gradual habituation to the frequent hypoxic stress (Liebermann, 2000). Habituation to frequent stress increase the overall fitness as the stress response re-allocates energy away from growth, immune function and reproduction (Wendelaar Bonga, 1997). When the stressor is severe hypoxia, this is particularly beneficial, as stress the mobilization of energy and increased oxygen consumption (Folkedal et al., 2012; Mommsen et al., 1999) are counteractive when oxygen availability is restricted (Van Raaij et al., 1996). The down-regulation of stress may explain the low mortality of post-smolts observed after the first days of cyclic hypoxia (Paper I).

To sum up, Atlantic salmon post-smolts continued to be sensitive to the limiting effect of hypoxia on metabolism, but the stress induced at DO around or below their LOS disappeared within a few days. Thus, if Atlantic salmon post-smolts are subjected to an acute reduction in oxygen below the LOS, more severe effects can be expected than if fish are customized to fluctuations in oxygen.

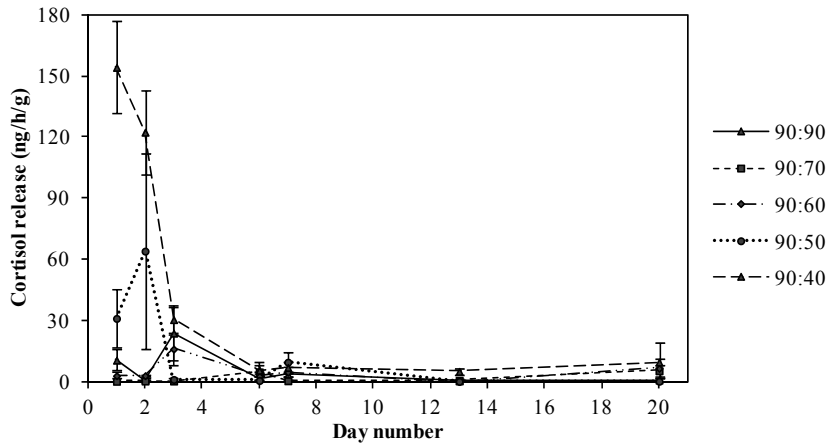


Fig. 6.1. Cortisol release rates ($\text{ng g}^{-1} \text{h}^{-1}$) of Atlantic salmon post-smolts, measured in water from the tank outlets 1 h after reducing oxygen levels in the afternoon hypoxic periods, during 20 days of cyclic hypoxia (2 h every 6 h; group names in legend represent oxygen saturation in normoxia: hypoxia) at 16 °C. All samples were taken at the same time (18:00). Data points are means \pm S.E.M (n=3). Unpublished data from the experiment presented in Paper I.

6.1.4. Hypoxia tolerance thresholds

As LOS represent a threshold below which feeding ceases, and where oxygen shortage challenge the basic function and survival of post-smolts (Paper I, II, III), it is reasonable to advise that such levels should be avoided in the on-growing phase in Atlantic salmon sea cages. LOS is termed the hypoxia tolerance threshold, as it represents the lower limit of the zone of tolerance. Generally, any factor that increases the metabolic rate can be expected to increase the LOS (Neill and Bryan, 1991; Wang et al., 2009), implying that the water temperature, swimming activity, fish size, feeding status and stress level are important determinants of the LOS (Brett and Groves, 1979; Forsberg, 1997; Wilson et al., 2007; Folkedal et al., 2012). Results from Paper III confirmed that the LOS of post-smolts increases with temperature, at an exponential rate. LOS ranged from 29 to 55% O_2 for temperatures ranging from 6-18 °C and increased by a factor of 1.65 for every 10 °C increase in temperature (Paper III). This is the first study to show such a clear relationship between temperature, oxygen consumption rates and the DO representing the hypoxia tolerance threshold for Atlantic salmon.

At any given temperature, the LOS may however vary substantially in response to the activity level (Neill and Bryan, 1991; Wang et al., 2009) In order to provide

aquaculturists with hypoxia tolerance thresholds that are suitable for salmon in sea cages, it is therefore important to find LOS for fish under “normal” production conditions (excess feeding and swimming speed ranging from low to moderate levels). The metabolic rate of post-smolts in Paper III was considered low compared to what can be expected in a sea cage, as the oxygen consumption rates were lower than previous observations in post-smolts kept under production-like conditions (Bergheim et al., 1991; Fivelstad and Smith, 1991; Forsberg, 1994; Atkins and Benfey, 2008; Castro et al., 2011). Thus, the LOS suggested for temperatures between 6 to 18 °C is likely to be underestimated. As a strong linear relationship between the oxygen consumption rate and the LOS was found in both Paper III and a recent study by Barnes et al. (2011), and because these relationships were remarkably similar between the two studies, it was suggested that LOS can be estimated from MO_2 , regardless of temperature and experimental conditions (Paper III). Thus, the LOS for post-smolts in commercial production conditions was estimated based on the above-mentioned measurements of post-smolt MO_2 . The results suggested that LOS should be increased by at least 40%, resulting in hypoxia tolerance thresholds spanning from 41 to 77% O_2 at temperatures ranging from 6 to 18 °C (Fig. 6.2; Paper III). This is the first attempt to suggest hypoxia tolerance thresholds for post-smolts in the on-growing phase in sea cages. The individual variation in LOS observed in rainbow trout (Ott et al., 1980) and Atlantic salmon (Barnes et al., 2011), emphasize that the hypoxia tolerance thresholds suggested here must be viewed as a mean threshold for a group of fish. Higher levels of O_2 may be required to avoid anaerobiosis and hypoxic stress in all individuals.

A hypoxia tolerance threshold of 77% O_2 at 18 °C may seem high, as it has been suggested that Atlantic salmon are able to maintain optimal feed intake at DO above 70% O_2 (EFSA, 2008). However, as Paper III is the first to show the exponential relationship between temperature and LOS for Atlantic salmon kept under production-like conditions, the sharp increase in oxygen demand at high temperatures have not been implemented in earlier reviews of the oxygen requirement of Atlantic salmon (EFSA, 2008; Thorarensen and Farrell, 2011). This exponential increase in LOS with temperature, combined with thermal limitations of the cardio-respiratory system at high temperatures (Farrell, 2009; Farrell et al., 2009; Pörtner, 2010), emphasize that high levels of water oxygen saturation may be required to avoid hypoxic stress at temperatures above the thermal optimum of 16-20 °C (Elliott and Elliott, 2010). Results of Hevrøy et al. (2012) and unpublished results presented by Tom Hansen (*pers. comm.*) showed that a temperature of 19 °C and an oxygen saturation of 90% O_2 resulted in low feeding capacity and growth as well as heart damages (necrosis in the compact

myocardium), indicating that 90% O₂ represented hypoxic levels and/or that 19 °C is at the upper end of the thermal tolerance range for Atlantic salmon adults. According to the relationship between temperature and LOS estimations (Paper III), it is conceivable that environmental hypoxia contributed to the negative effects, as LOS can be estimated to be 83% O₂ for post-smolts at 19 °C. This would suggest that 90% O₂ represent a DO at the lower end of the zone of tolerance, which results in a considerable reduction of the metabolic scope, and a severe restriction of feeding and growth.

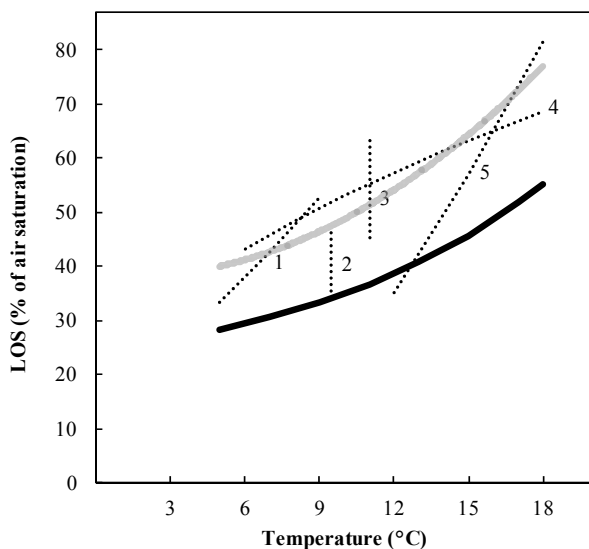


Fig. 6.2. The limiting oxygen saturation (LOS, % of air saturation) of fed Atlantic salmon post-smolts at temperatures ranging from 6-18 °C obtained in the present study (black line), compared to LOS estimated from MO₂ measured in other studies of Atlantic salmon smolts/post-smolts fed to satiation and swimming at various speed (~0.4-1.7 body lengths, BL, s⁻¹) (broken lines). LOS was estimated using $LOC=9.785*MO_2+17.873$. Based on this comparison, it is suggested that LOS should be increased by at least 40% (grey line) in order to serve as a practical guideline for the salmon farming industry. The number next to broken lines indicate the literature reference; 1) Fivelstad and Smith, 1991 (200-800 g post-smolts, swimming speed ~0.4-0.7 body lengths, BL, s⁻¹), 2) Bergheim et al., 1991 (520-800 g post-smolts, ~1.2-1.7 BL s⁻¹), 3) Castro et al., 2011 (100-200 g post-smolts, 0.7 BL s⁻¹), 4) Forsberg, 1994 (500 g post-smolts, 1 BL s⁻¹) and 5) Atkins and Benfey, 2008 (~150 g parr/smolt, swimming speed unknown). Adapted from Paper III.

6.2. Production performance in cyclic hypoxia

The general advice of avoiding reductions in oxygen below LOS in sea cages is sensible, due to the physiological challenges, and impaired production performance at such

levels of oxygen (Paper I, III). If oxygen declines to less severe levels (within the zone of tolerance), general advice is less straight-forward, as the severity, frequency and duration of hypoxic periods can be expected to determine whether the production performance is compromised. As oxygen tends to fluctuate, rather than to remain at low levels for long periods of time (Johansson et al., 2006; 2007; Vigen, 2008; Oppedal et al., 2011; Burt et al., 2012), an important objective in Paper I and II were to investigate the ability of Atlantic salmon post-smolts to feed and digest the feed during frequent alternations between hypoxia and normoxia.

6.2.1. Effects on feed intake

The feed intake of post-smolts corresponded to the experienced oxygen level (Paper I), in accordance with previous observations of Atlantic salmon and other salmonids subjected to graded hypoxia (Brett, 1979; Brett and Blackburn, 1981; Pedersen, 1987; Crampton et al., 2003; WEALTH, 2008). The depression of feed intake started when oxygen was reduced to levels of about 70% O₂ at 16 °C, and developed gradually with declining DO. This agrees with a gradual reduction of the metabolic scope as oxygen declines within the zone of tolerance (Fry, 1971; Farrell and Richards, 2009). Further, the low levels of feed intake at DO below the hypoxia tolerance threshold of ~47% O₂ (Paper I, III), fits with the expected loss of aerobic capacity below this threshold (Paper I; Neill and Bryan, 1991; Wang et al., 2009). It is plausible that individual differences in basic oxygen requirements is the reason why some feed was still ingested even at DO as low as 40% O₂. The depression of appetite was less pronounced when fish had not been fed for 18 h (*i.e.*, during the first meal of the day), demonstrating that Atlantic salmon sensitively regulate their feed intake during reductions in DO, balancing the demand for nutrients with the need to lower the oxygen demand (Paper I). This agrees with previous findings in Atlantic cod, turbot (*Scophthalmus maximus*) and European sea bass (*Dicentrarchus labrax*) (Claireaux et al., 2000; Pichavant et al., 2000, 2001), and agree with Claireaux et al. (2000), who conclude that the sensitive regulation of feed intake may be a way to maximize the metabolic scope when oxygen limits metabolism.

When oxygen fluctuated in tidal cycles (2 h of hypoxia every 6 h), and fish were fed both in hypoxia (twice per day) and normoxia (once per day), it was evident that reductions in DO below the threshold for maintained feeding (70% O₂) in hypoxic periods resulted in a depression of the total daily feed intake (Paper I). This effect was most pronounced when DO was reduced to levels close to 40 and 50% O₂ (Fig. 6.3; Paper I). However, the depression of the total daily feed intake can be expected to be smaller than if hypoxia was continuous, as

fish increased their ability to feed in normoxic periods over time. In post-smolts subjected to 40 and 50% O₂ in hypoxic periods, the total daily feed increased from 25-48% to 70-78% of control group levels within 20 days (Fig. 6.3; Paper I).

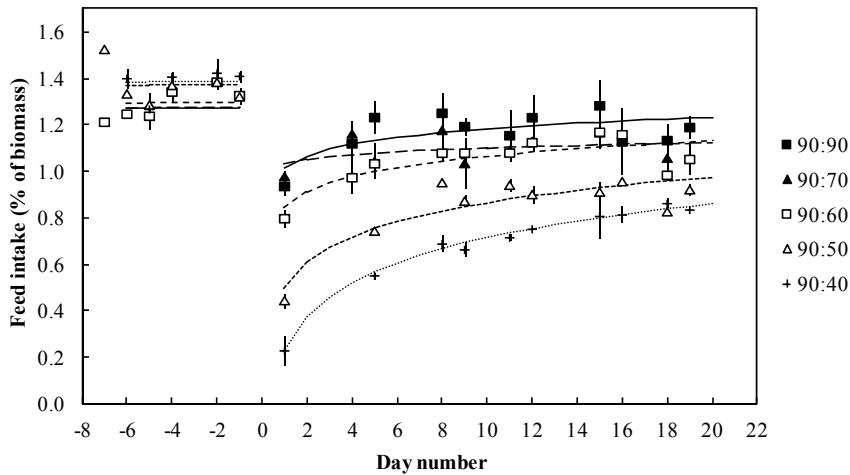


Fig. 6.3. Total daily feed intake (% of biomass) of post-smolts subjected to cyclic hypoxia (2 h every 6 h) of varying severity (group names in legend indicate % oxygen saturation in normoxia: hypoxia). Fish were fed to satiation twice in hypoxia and once in normoxia per day. Data points represent means \pm S.E.M (n=3). Three-parameter asymptotic curves were fitted to the data, and the level of line punctuation reflects hypoxia severity (whole line=normoxia). Data from Paper I.

As the severe depression of appetite in hypoxia periods persisted, this resulted in a remarkable change in the daily feeding pattern, with low levels of feed intake during the two hypoxic feeding periods, and higher levels than the control during the normoxic feeding period (Fig. 6.4; Paper I). The increased normoxic feeding is likely to be connected to the down-regulation of stress, as stress effectively inhibits appetite (Paper I; Bernier and Craig, 2005; Folkedal et al., 2012). Further, the low feed intake during the first part of the experiment may have reduced the levels of stored lipids, which again may have stimulated the appetite of fish during the later part of the experiment (Paper III; Ali et al., 2003).

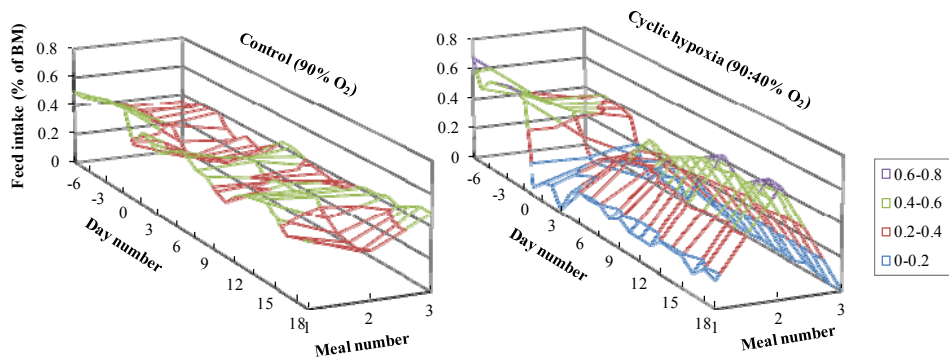


Fig. 6.4. Average feed intake (% of biomass) per meal per day, in triplicate tank groups of Atlantic salmon post-smolts kept at constant 90% O₂, or kept at 90% O₂ and subjected to cyclic hypoxic periods (2 h of 40% O₂ every 6 h) at 16 °C. Meal 1 and 3 were given in hypoxic periods and meal 2 was given in normoxia. Fish were fed to satiation. The data presented in the figure is background material for the figures of feed intake presented in Paper I.

The remarkable ability of post-smolts to feed in normoxic periods, in spite of the frequent and severe hypoxia, indicated that feeding in normoxia (only) might enable Atlantic salmon to maintain optimal feed intake, providing that they are given sufficient time to habituate (Paper I). This hypothesis was investigated in Paper II, where post-smolts were subjected to similar cyclic hypoxia, but fed in normoxia only. In accordance with the findings in Paper I, the total daily feed intake of post-smolts started to decrease if DO was reduced below 70% O₂ in hypoxic periods. However, the magnitude of negative effect was reduced: 50% O₂ in hypoxic periods yielded a 13% reduction of the daily feed intake compared to fish kept at constant 80% O₂ (Paper II). This is considerably lower than the 22-52% reduction of feed intake observed when fish were subjected to similar oxygen fluctuations and fed in both hypoxia and normoxia (Paper I). Together, the results from Paper I, II and III, emphasize that reductions in DO to levels as low as 50% O₂ at 16 °C represents a physiological challenge, and reduce the ability of post-smolts to maintain optimal feed intake, regardless of feeding strategy, when hypoxia occur in tidal cycles. An oxygen saturation of 50% O₂ is close to the hypoxia tolerance threshold (47% O₂ at 16 °C) (Paper III), and the anaerobic metabolism observed at such levels (Paper I) suggest that energy-demanding recovery processes (Svendsen et al., 2012) may have restricted the aerobic scope for feeding also in normoxic periods. Reductions to 60% O₂ in tidal cycles induced similar responses, but to a lesser extent (Paper I, II, III).

6.2.2. Effects on feed utilization

Cyclic hypoxia was not found to compromise the digestion of ingested nutrients, even when oxygen was reduced to levels as low as 50% O₂ in cyclic, hypoxic periods. This agrees with Glencross (2009) and Pouliot and De La Noüe (1989), who found that nutrient utilization was maintained in rainbow trout subjected to similar, continuous levels of hypoxia (40 and 56% O₂). It has been proposed that the ability of fish to maintain digestion in hypoxia may be related to a compensatory increase in the passage time of food through the intestine, which delays, but maintains the digestion of ingested nutrients (reviewed by Wang et al., 2009). For fish subjected to moderate hypoxia, such down-regulation of the digestion rate, together with the reduced feed intake, may serve to reduce the oxygen demand. In Atlantic cod, this was indicated by the lower and prolonged increase in MO₂ after a meal, compared to fish kept in normoxic conditions (Jordan and Steffensen, 2007). Correspondingly, a reduction in the gastric evacuation rate was observed in Baltic cod (*Gadus morhua callarias* L.) subjected to hypoxia (Teschner et al., 2010). Results from Paper III substantiate that a down-regulation of the digestion rate in hypoxia may also occur in post-smolts, as the decreasing oxygen consumption rates with declining DO was more pronounced in fish with the highest feed intake during the fore-going feeding period. This resulted in similar MO₂ in all fish when DO reached the hypoxia tolerance threshold. It should be emphasized that the occurrence of hypoxia to levels below the hypoxia tolerance threshold (e.g. 47% O₂ at 16 °C) may negatively impact the digestion of ingested nutrients, due to the more severe physiological disturbance at such levels (see 6.1.2.). For example, Atlantic cod have been found to void their stomach when subjected to levels below the LOS (Claireaux et al., 2000).

Although the digestion of ingested nutrient is not necessarily compromised as a result of moderate oxygen reductions, the proportion of ingested energy used for routine metabolic demands increases as feed intake decreases, resulting in elevated feed conversion ratios (Thorarensen and Farrell, 2011). Results from Paper I and II agree with this, as FCR tended to increase in fish with the lowest feed intake (primarily in fish subjected to 40 and 50% O₂ at 16 °C).

6.2.3. Effects on growth

Generally, the reduction in the total feed intake during a period of cyclic hypoxia resulted in corresponding reductions in growth rates (Paper I, II). A strong correlation between feed intake and growth, as well as no effect on feed utilization, consent with previous observations in juvenile turbot (Pichavant, 2001), wolffish (*Anarhichas minor*) (Foss et al.,

2002) and European sea bass (Thetmeyer et al., 1999; Pichavant, 2001) subjected to moderate hypoxia. According to the effects of hypoxia on feed intake (Paper I), it can therefore be concluded that negative effects of hypoxia on growth performance is generally avoided if DO is kept above 70% O₂ at 16 °C. Further, the results from Paper I and II show that Atlantic salmon compensate quite effectively for the depressed feed intake in hypoxic periods by increasing feeding in normoxic periods, suggesting that some reductions in oxygen below 70% O₂ can be tolerated without negative effects on growth performance.

The “tidal hypoxia” investigated in Paper I and II (2 h of hypoxia every 6 h), was outside of the range of oxygen fluctuations that can be compensated for, as DO below 70% O₂ resulted in reduced growth, also when post-smolts were given the opportunity to acclimate and feed in normoxic conditions (Paper II). It can be assumed that a shorter duration of the hypoxic periods may enable Atlantic salmon to successfully compensate, as anaerobiosis will be less developed (Omlin and Weber, 2010; Vianen et al., 2001), and fish will have an increased opportunity to recover and compensate in normoxic conditions (Svendsen et al., 2012). As shown by Forsberg and Bergheim (1996), the production performance (FCR, SGR) of post-smolts was not negatively influenced by substantial, diurnal fluctuations in oxygen (42-137% O₂ at 8 °C), when the exposure to low levels of DO was short-term (only ~2% of measured DO was below 50% O₂). In comparison, the growth of post-smolts was reduced by approximately 40% when oxygen was kept continuously at 50% O₂ at 8 °C (Crampton et al., 2003). The exact threshold for limiting hypoxia frequency/ duration is not clear, yet less frequent/prolonged than 2 h every 6 h (Paper I, II).

The general oxygen threshold for maintained growth of Atlantic salmon of about 70% O₂ correspond to the threshold suggested by EFSA (2008), and is lower than the range of 85-120% O₂ suggested by Thorarensen and Farrell (2011). The usefulness of such general thresholds, or threshold ranges, is however rather limited if the goal is to assess whether Atlantic salmon are provided with sufficient DO to maintain optimal growth in sea cages. Both temperature and fish size influence the metabolic scope for feeding and the concurrent oxygen demand (Paper III; Brett and Glass, 1971; Brett and Groves, 1979; Neill and Bryan, 1991), and both vary to an extent in sea cages that can be expected to markedly influence the oxygen threshold for growth. As mentioned in 6.1.4., this may be particularly important to consider at high temperatures (>16 °C), due to the rapid increase in oxygen demand as temperature increases (Paper III; Brett and Groves, 1979; Farrell et al., 2009). While the oxygen threshold presented here is useful for a typical autumn temperature of 16 °C, the relationship between fish size, temperature and the threshold for maintained feeding must be

investigated further in order to assess whether Atlantic salmon are provided with sufficient DO to avoid negative effects on growth.

6.3. Welfare of Atlantic salmon subjected to fluctuating oxygen

As salmonids have a high oxygen demand, lack specialized mechanisms to deal with prolonged, severe hypoxia (Bickler and Buck, 2007; Nilsson and Nilsson, 2008), and because Atlantic salmon normally inhabit oxygen-rich waters, it is reasonable to assume that access to sufficient oxygen is a “welfare need” of Atlantic salmon (Bracke et al., 1999). Based on the description of the welfare concept and - assessment presented in the introduction, it is possible to use physiological and behavioral observations to assess the degree of need fulfillment, and thus the welfare, when Atlantic salmon experience oxygen fluctuations (see 2.5). The loss of appetite, exhaustion of the hypoxic ventilatory response, stress response, and the compromised survival at DO below the LOS, all together show that the sharp decline in post-smolt MO_2 below this threshold induced a struggle to maintain basic functions and to survive (Paper I, III). The need for oxygen was therefore not fulfilled, and such severe hypoxia can be considered to result in poor welfare. However, fish did in fact survive the frequent hypoxic periods, the stress response was down-regulated (within 16-24 repetitions of hypoxic periods), and fish started to utilize normoxic periods for compensatory feeding (Fig. 6.4; Paper I). As the effect of hypoxic periods on metabolism seemed to persist over time (Paper I, II, III), this may imply that the perception of oxygen shortage changed, and that this may explain the down-regulation of stress. For example, it has been shown that the response of Atlantic cod to a stressor (dipping of net into the fish tank), changed from fright to anticipation over time (~20 repetitions), when the fish were taught that the stressor is followed by a reward (food) (Nilsson et al., 2012). Thus, it is possible that post-smolts over time learned that hypoxic periods were not life-threatening, and/or that keeping calm (avoid increased oxygen demand) was the most “rewarding” response to this stressor (reduced lactate accumulation and acidosis).

For reductions in oxygen within the zone of tolerance, the increased ventilation rate and the reduced feeding (Paper I, III) may be considered to be among the frequently used, compensatory responses that serve to maximize the performance capacity when environmental conditions fluctuate (Claireaux et al., 2000; Korte et al., 2007). As seen in Paper I, the reduced feed intake in hypoxic periods, was to a large extent compensated for by increasing feed intake in normoxia. It is therefore possible that reductions in oxygen within

the zone of tolerance do not reduced the overall “quality of life”, unless the hypoxic periods are of a severity, duration or frequency that induces hunger or a reduction of the metabolic capacity to fulfill other welfare needs (Bracke et al., 1999). When oxygen was reduced to 50% O₂ for 2 hours in tidal cycles, it was clear that post-smolts were not able to maintain optimal feed intake, even though the feed was exclusively offered when oxygen levels were high (80% O₂). This resulted in an overall reduction in growth rates (13% reduction) (Paper III), and show that the nutrient demand was not completely fulfilled. For salmonids, which have high growth-rates in the seawater phase, unrestricted feeding can be considered a welfare need. The moderate reduction of growth does however indicate that the negative effect on welfare was also moderate. It is suggested here that reduced growth can be used as a sign of undue environmental challenge when oxygen fluctuates, and that welfare therefore decreases with the severity of growth depression. If oxygen is reduced to the lower end of the zone of tolerance continuously, this can be expected to induce starvation, weight loss, and poor welfare.

7. CONCLUSIONS AND FUTURE PERSPECTIVES

Based on the results from the present studies, it can be expected that the oxygen reductions previously observed in Atlantic salmon sea cages in autumn (down to 30% O₂ in extreme cases, and frequent reductions below 60% O₂), negatively influence the production performance and welfare. The initial limitation of appetite occurs around 70% O₂ in Atlantic salmon post-smolts at a typical autumn temperature of 16 °C (Paper I). Feed intake, and presumably also the digestion rate, decreases with increasing hypoxia severity, and reaches low levels when DO approaches the hypoxia tolerance threshold (47% O₂ at 16 °C) (Paper I, II, III). At DO around/below this threshold, ATP must be produced anaerobically, a stress response is induced and survival is time-limited (Paper I). An inhibition of appetite caused by hypoxic stress continues also after restoration of normoxic conditions, until fish become habituated, and start to utilize normoxic periods for feeding (< 1 week) (Paper I). As production performance can be expected to be zero, and welfare can be considered to be poor at DO below the LOS, this threshold is suggested to be a lower limit for acceptable drops in oxygen in Atlantic salmon sea cages. This threshold increases with temperature and the activity level of the fish (e.g. feed intake, swimming speed and stress level) (Paper III). For post-smolts with low swimming speed and relatively low growth rates, LOS range from 29 to 55% O₂ with temperatures ranging from 6-18 °C, while it may increase by at least 40% in fish with activity levels closer to that of fish in sea cages (Paper III). It should be emphasized that individual variation in LOS may be substantial, and that higher levels of DO is required to prevent all individuals from reaching their LOS.

When DO fluctuates within the zone of tolerance, the severity, frequency and duration of hypoxia determine whether, and to what extent production performance and welfare is compromised, depending on the overall feed intake. When hypoxia occur in “tidal” cycles (2 h every 6 h), any reduction in DO below 70% O₂ at 16 °C reduces growth, also when feed is offered in normoxia only (Paper II). This is a result of reduced feed intake, and not impaired digestion (Paper II). By feeding in normoxia exclusively, negative effects can be minimized (Paper I, II). The response to hypoxic periods, in terms of reduced feed intake and anaerobiosis, is relatively stable over time, and the acclimation to cyclic hypoxia does not lower the hypoxia tolerance threshold of post-smolts (Paper I, II, III). What duration and frequency of moderate hypoxia that can be tolerated without negative effects on performance and welfare needs further investigation.

In order to provide salmon farmers with a more complete knowledge base for decision-making, it is also necessary to further investigate the relationship between temperature and the oxygen threshold for maintained feed intake, for the entire temperature range that is experienced in salmon sea cages. Further, the change in these thresholds with increasing fish size should be determined (only post-smolts <1 kg were studied here). When such knowledge is available, a model can be presented, which predicts feed intake (and thus, the growth rate) in response to environmental temperature and oxygen. This can be used for development of more sophisticated feeding systems which respond to real-time changes in the sea cage environment. Further, environmental data from previous production cycles can be used to assess whether, when, and what kind of measures that are necessary to avoid detrimental effects of hypoxic periods on growth and welfare at a specific farm site. Examples of possible measures that may reduce or prevent negative effects of hypoxic periods may be to stop feeding if hypoxia is short-term and severe (reduce the LOS), to make sure that feed is provided when DO is at its highest if oxygen fluctuates within the zone of tolerance, to maximize water exchange rates (e.g. remove biofouling, or avoid short distance between sea cages), to reduce oxygen consumption (lower the fish stocking density) and to oxygenate or lower the water temperatures when temperatures are above the thermal optimum (collect water from deeper levels, or lower the sea cages). Based on the prospective model that predicts feed intake in response to environmental oxygen and temperature, a cost-benefit analysis may be performed, in order to decide what measures that are necessary and feasible for the environmental fluctuations experienced at the specific farm site. This model may also be used to assess whether oxygen levels were sufficient to fulfill the needs of Atlantic salmon during the production cycle, as part of a “welfare certificate”.

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