Regulation of appetite and growth of Atlantic salmon (*Salmo salar* L.) and effect of water oxygen, temperature and dietary energy

Vibeke Vikeså

Dissertation for the degree of philosophiae doctor (PhD)
at the University of Bergen

2017

Date of defense: June 27th

Year: 2017

Title: Regulation of appetite and growth of Atlantic salmon (*Salmo salar* L.) and effect of water oxygen, temperature and dietary energy.

Author: Vibeke Vikeså

Print: AiT Bjerch AS / University of Bergen

Scientific environment

This thesis was carried out at the National Institute of Nutrition and Seafood Research (NIFES) in collaboration with the Institute of Biology at the University of Bergen and Skretting Aquaculture Research Centre (ARC).

The PhD project was financed by two funding programs at Norges Forskningsråd (NFR) with the grant numbers 209655/O30 and 199683/S40 and by Skretting ARC.

Supervisors of the PhD have been Dr Leo Nankervis, Dr Ernst Morten Hevrøy and Prof. Rune Waagbø.

Acknowledgements

First of all, I thank the Norwegian Research Council (NFR) and my employer Skretting ARC for financing my PhD. I have had great support during my PhD work from all supervisors; Professor Rune Waagbø, Dr Leo Nankervis and Dr Ernst Morten Hevrøy. Your advice, support and input have been valuable and highly appreciated. I would also like to thank my boss, Dr. Wolfgang Koppe who saw this opportunity and encouraged me to start a PhD. All the best for your new business!

The analytical work that I conducted at NIFES would not be possible without the training and support of Eva Mykkeltvedt, Natalia Larsen, Jaap Wessels and Synne Wintertun. Thank you very much for your patience and for taking the time to guide me through the many rounds of real-time and RT-qPCR's.

I also extend my thanks to the helpful colleagues at Skretting ARC laboratory and Lerang Research station, who always tried to seek the best solutions to any issues relating to fish, samples and experimental quality.

My colleague, Dr. Saravanan Subramanian has been of invaluable help in writing of papers and my thesis. Thank you so much for your quick responses and constructive input on manuscripts. I would also like to thank my colleagues, Sophie Noonan and Nafiha Usman, for their superb help with illustrations and statistical support.

Support from family, friends and good colleagues has been essential for me to complete this PhD. To keep my spirits up while finalising the thesis, my colleague Dr Guido Riesen has treated me with Friday croissants for several months. Thanks, it was highly appreciated!

I would especially like to thank my good friends, Linda and Ellen for the support and great believe in me. I would also like to extend my appreciation to Ellen and her family for always welcoming me to stay at their house during the many visits to Bergen.

And last, but not least, a big thank you to my boyfriend Kjell Erik and son Bror, for happy times and lots of fun!

Abstract

High water temperature combined with low dissolved oxygen (LO or hypoxia) is one of the most challenging environmental conditions farmed fish experience. The oxygen requirement of fish increases in parallel to this, which limits the aerobic energy metabolism and consequently reduces feed intake and growth of fish. The global ocean warming followed by reduced oxygen availability, is expected to exacerbate associated physiological stress on fish in several areas where Atlantic salmon are currently farmed. Understanding the impact of temperature and limited oxygen on growth regulatory mechanisms and the energy metabolism, will be of significant relevance to both cultured and wild fish populations.

Conditions of high temperature and hypoxia are related with reduced feed intake and growth in fish. It is unclear whether the low oxygen availability directly affects growth regulatory mechanisms, and if low feed intake is the primary cause of depressed growth under LO conditions. Studies of appetite and growth regulation in salmon under such conditions are few, and considerations of the fluctuating character of endocrine signals and nutrient absorption are scarce. Limitation of the aerobic energy metabolism under reduced oxygen availability is further restricted by a thermal increase. It is therefore interesting to find out how high energy diets can potentially impact appetite and growth regulation under LO conditions.

This thesis therefore investigated mechanisms by which LO and high temperature conditions impact appetite and growth regulation in seawater adapted Atlantic salmon. Free amino acid (FAA) and endocrine dynamics in relation to meal time were also studied. Four fish trials were conducted, including the following variables; dissolved oxygen (DO; LO and high, HO), temperature and digestible energy (low and high, LE and HE). Endocrine appetite and growth signalling was investigated through analyses of plasma ghrelin and IGF-1 concentration, and mRNA levels of the growth hormone receptor (*ghr1*) and insulin like growth factor-1 (*igf1*) in liver and muscle tissue.
LO conditions demonstrated direct depressed effects on appetite and growth in salmon across temperatures. Reduced growth in salmon under LO was not caused only by a reduced

feed intake, but appeared to be a combined effect of impairment of growth regulation and increased metabolic costs, as demonstrated by a pair-feeding technique. Increased metabolic costs by LO were indicated by responses in oxyregulating mechanisms, such as increased haemoglobin, reduced blood pH and imbalanced osmoregulation. Reduced specific growth rate (SGR) and feed intake were also found for salmon under LO compared to HO groups, at both optimal and high temperatures. High temperature demonstrated a diminished growth potential in salmon compared to an optimal temperature. This was reflected in a faster 24 hour postprandial catabolism of absorbed FAA, and generally lower and faster declines of IGF-1 (plasma and mRNA) at 19°C compared to 13°C.

Ghrelin was found to signal feed anticipation in salmon, consistent with mammalian findings, and reflected by clear preprandial plasma ghrelin peaks at 12°C. Ghrelin and GH-IGF factors responded to LO at a high temperature, but further studies should focus on a postprandial perspective to confirm the preprandial peaks at 12°C.

Results from feeding HE diets to salmon, indicate that it is possible to stimulate growth, feed utilisation and the energy metabolism under LO conditions through DE level, regardless of temperature.

To summarise, the thesis shows that growth regulation in seawater adapted salmon is negatively affected by LO at optimal and high temperatures. Positive effects from feeding HE diets under LO, demonstrate possibilities to support energy metabolism through dietary means under challenging environmental conditions. Diet effects and environmental impact on growth regulation are of great relevance to salmon farming, as further knowledge can improve growth, welfare, health and future farming possibilities.

List of publications

Paper I

Vikeså V., Nankervis L., Remø S.C., Waagbø, R. & Hevrøy, E.M. (2015). Pre and postprandial regulation of ghrelin, amino acids and IGF-1 in Atlantic salmon (*Salmo salar* L.) at optimal and elevated seawater temperatures. Aquaculture 438, 159-169.

Paper II

Vikeså, V., Nankervis, L. & Hevrøy, E.M. (2017). High dietary energy level stimulates growth hormone receptor and feed utilization in large Atlantic salmon (*Salmo salar* L.) under hypoxic conditions. Aquaculture Nutrition In press.

Paper III

Vikeså, V., Nankervis, L. & Hevrøy, E.M. (2016). Appetite, metabolism and growth regulation in Atlantic salmon (*Salmo salar* L.) exposed to hypoxia at elevated seawater temperature. Aquaculture Research 12, 1-16.

Contents

Contents

1. Introduction

1.1 General introduction

Globally Atlantic salmon production exceeds 2.3 million tonnes per annum (FAO, 2014a). However, its farming is restricted to geographical areas with a suitable environment. Favourable farming conditions are characterised by deep waters with a high water exchange and invariable weather conditions, providing stable cool and oxygen-rich seawater, within optimal ranges for salmon growth. Since its beginning in Norway in the 1960's, salmon farming has grown to a great extent in both northern and southern hemispheres including regions in Scotland, Ireland, the Faroe Islands, North America, Chile and Tasmania (Australia) (FAO, 2014a). The world population increasingly relies on farmed fish to meet their requirement for long chain polyunsaturated (n-3) fatty acids and proteins, and the share of farmed fish in global fish consumption by humans is projected to exceed 53% by 2022 (FAO, 2014b). Forecasted global climate change revolves heavily around temperature change (Perry et al., 2005; Pörtner and Knust, 2007) and predicts negative impacts on wild fisheries, land-based food production (Ficke et al., 2007; IPCC, 2014) and coastal water quality, including increased prevalence of hypoxic conditions (Diaz, 2001; Diaz and Breitburg, 2009; Rabalais et al., 2009). This underlines the importance of knowledge generation relating to ensuring food production under suboptimal environmental conditions to facilitate a continued supply of farmed fish.

Global ocean oxygen content has decreased significantly due to warmer sea temperatures following the 1950's (Helm et al., 2011; IPCC, 2014), while increased river temperatures (Daufresne et al., 2009; Durance and Ormerod, 2007; 2009; Elliott and Elliott, 2010) indicate the same trend in freshwater. Climate models predict an increase in average surface temperatures of 1.0 to 5.0°C in Southern Hemisphere seawater and 1.5 to 11.0°C in Northern Hemisphere seawaters by 2100 (IPCC, 2014). This is expected to exacerbate associated physiological stress on fish in several areas where Atlantic salmon are currently farmed. In fact, the largest salmon farming countries, Norway and Chile, are considered the

top two marine production areas with highest vulnerability to associated climate changes in the world (Handisyde et al., 2016). Understanding the implications of climate change on growth regulatory mechanisms will have an increasing relevance to both cultured and wild fish populations globally.

1.2 Thermal tolerance and aerobic scope

Water oxygen and temperature are the major environmental factors affecting metabolic rate and growth potential of fish (Brett, 1979). While temperature directly controls metabolic rate, oxygen availability in water has a limiting role on energy metabolism, below a certain threshold level (Brett, 1979). The solubility of oxygen is reduced when temperature increases, and so warm conditions, such as summer and late autumn, is commonly accompanied with periods of low water oxygen.

1.3 Thermal tolerance

Thermal tolerance window is defined as a species specific temperature range in which the animal thrives and grows, and with additional upper and lower temperature ranges defined as lethal or critical. The width of the thermal window generally varies with size or life stage, starting with a narrow window which widens at later stages and then a narrowing, i.e. a reduced thermal tolerance, for larger specimens and during reproduction (Pörtner et al., 2006; Pörtner and Farrell, 2008) (Fig 1.1).

Conversion efficiency and rates of ingestion, growth and metabolism are accelerated by increasing temperature, peaking at different optimal temperatures before declining and eventually ceasing if peaking at the upper thermal limit (Brett, 1979; Jobling, 1997) (Fig 1.2).

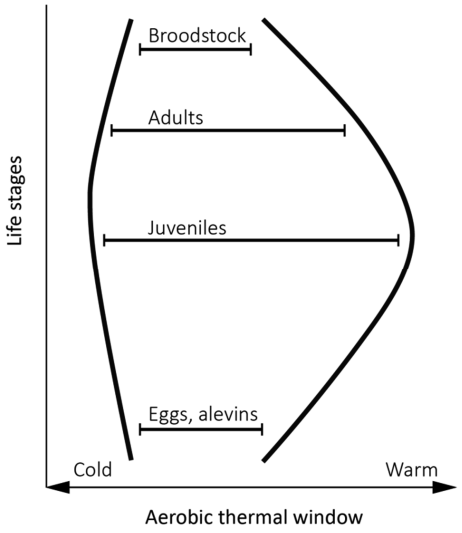

Figure 1.1. The aerobic thermal window across life stages for fish. Modified from Pörtner and Farrell (2008).

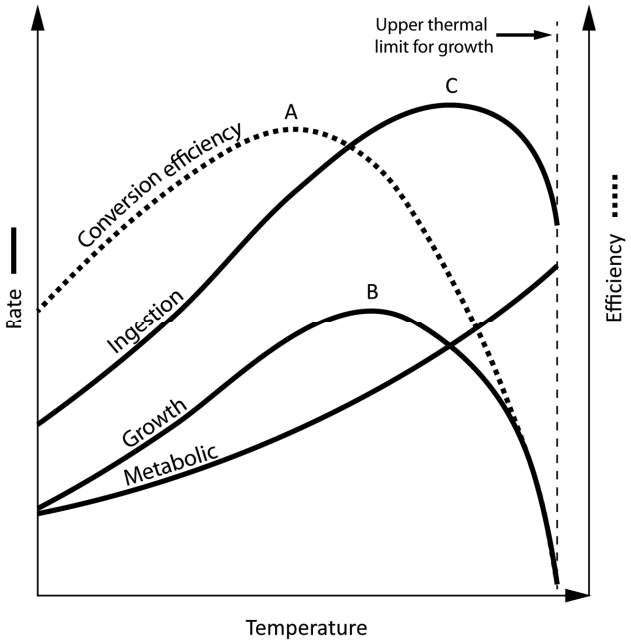

Figure 1.2. Effect of temperature on feed conversion efficiency (A) and rates of growth (B), ingestion (C) and metabolism. The letters (A, B, C) indicate respective maxima (optimum, peak of the curves) in relation to temperature. Modified from Jobling (1997).

Recommendations on how to optimise feeding and growth under different temperature and oxygen levels are lacking and would benefit both welfare and growth conditions of farmed fish. In this context, it is especially effects from fluctuating environmental conditions that should be investigated, as this is more representing the conditions fish are facing in the open sea.

Fish body temperatures rely on the surrounding water temperature, to which they are sensitive enough to detect changes as small as 0.03°C (Murray, 1971). Sensitivity to temperature changes can be an advantage, especially for wild fish, as they can respond by swimming away from waters of a suboptimal quality. Farmed salmon are more confined to the limit of their sea cages, and have restricted scope for migration to more favorable environments.

Large fish are generally considered more vulnerable to thermal stress than smaller fish (Fig 1.1), but this is debated (Clark et al., 2012; Clark et al., 2008; Daufresne et al., 2009; Elliott, 1981; Lefevre, 2016; Nilsson and Östlund-Nilsson, 2008; Pörtner and Knust, 2007).

Under short-term temperature fluctuations, large fish are claimed to have an advantage due to a smaller skin surface area in relation to body volume (Elliott, 1981). Internal temperature is exchanged mainly via the skin, and to a lesser degree through the gills (Elliott, 1981), and so a large body size requires longer time to adjust body temperature to ambient water temperature than smaller fish. This will then result in reduced effects on body temperature and metabolism compared to smaller fish (Elliott, 1981).

However, there is a faster reduction in growth and survival of large fish than small fish when thermal tolerance is limited by hypoxia (Pörtner and Knust, 2007). Large fish are therefore less robust when facing long term environmental changes and handling stress than smaller fish. This is supported by lower abundance and survival of large fish in wild fish populations exposed to prolonged water temperature increase (Daufresne et al., 2009; Pörtner and Knust, 2007). In fact, a reduced fish size is recognised as a common

consequence of global warming to aquatic animals, in addition to shifts in habitat and disorder in seasonal life cycle sequences (Daufresne et al., 2009).

Farming of salmon stands the biggest economic risk when large salmon, approaching the end of the production cycle, are exposed to suboptimally warm and hypoxic conditions. Future research on metabolic regulation of this size group is therefore of great interest. Acclimation to a wide temperature range can increase tolerance to critical temperatures, but has little effect on preferred or optimal temperature limits (Pörtner and Peck, 2010). This ability to adapt tolerance to thermal extremes can be an advantage at locations of frequent temperature changes and towards global warming.

Salmon farms experience changes in temperature due to stratification and oscillations occurring naturally across seasons in periods lasting from days to months. In parallel, salmon in cages are also exposed to significant variations in oxygen and light (Johansson et al., 2006). Within the water column of a cage, the temperature is generally higher closer to the surface during the warm season and the opposite in the cold season (Oppedal et al., 2007). Episodes of ice melting or heavy rain can alter this and create the opposite situation in fjords with high run off from land. Daily mean temperature in the fjords during a Norwegian summer or autumn can vary from 7-15°C (Aas-Hansen, 2010) with maximum reaching up to 20°C (Johansson et al., 2006; Oppedal et al., 2011), while Tasmanian salmon farming (Australia) holds mean summer temperatures of 17-21°C (Attard et al., 2012; Carter et al., 2008).

Temperatures optimal for growth are size and life stage and species dependent (Jobling, 1981). Literature on optimal temperatures varies, but the principles of the thermal window (Pörtner et al., 2006; Pörtner and Farrell, 2008) (Fig 1.1) generally applies to Atlantic salmon. Eggs have the narrowest thermal window (4-7°C and critical temperature at 7-8°C) (Elliott and Elliott, 2010), while a range of 16-20°C is reported optimal for the freshwater stages (typically 0-100 g), compared to 12.8-14°C for the postsmolt stage (50-300 g) (Coutant, 1977; Elliott and Elliott, 2010; Elliott and Hurley, 1997; Forseth et al., 2001; Handeland et al., 2008; Koskela et al., 1997; Peterson and Martin-Robichaud, 1989).

Optimal growth of large Atlantic salmon (1.6-2.9 kg) reared in tanks were found at 13°C compared to 15°C, 17°C and 19°C (Hevrøy et al., 2013).

Temperature effect on growth is highly dependent on feed intake (Jobling, 1997). A compromised feed intake (i.e. less ingested energy) can lower the fish's thermal tolerance (Elliott and Elliott, 2010; Jobling, 1997) and reduce the optimal temperature for growth (Elliott and Hurley, 2000a; b). A limitation in feed ration or a reduced feed intake also lower growth rate and so feed access must be unlimited to allow maximum growth. Elevated water temperature also reduces feed intake when temperature rises above preferred thermal optimum values (Brett, 1979). While optimal temperature for feed conversion (a) is slightly lower than that for maximum growth (b), the highest feed ingestion rate (c) is achieved at a little higher temperature than that for maximum growth (b) (Brett, 1979; Jobling, 1997) (Fig 1.2).

1.4 Oxygen

The impact of warm water on fish physiology cannot be evaluated without considering the water oxygen concentration, since the physicochemical characteristics of these parameters in water are closely connected. Oxygen solubility in water (DO) is reduced at higher temperatures (Brett, 1979), so raising temperature naturally lowers DO concentration (mg L^{-1}), even if it is maintained at 100 % oxygen saturation (Nilsson, 2010) (Fig 1.3). Oxygen is a limiting factor for fish metabolism due to its crucial role in aerobic synthesis of ATP and consequently regulates energy availability for metabolism.

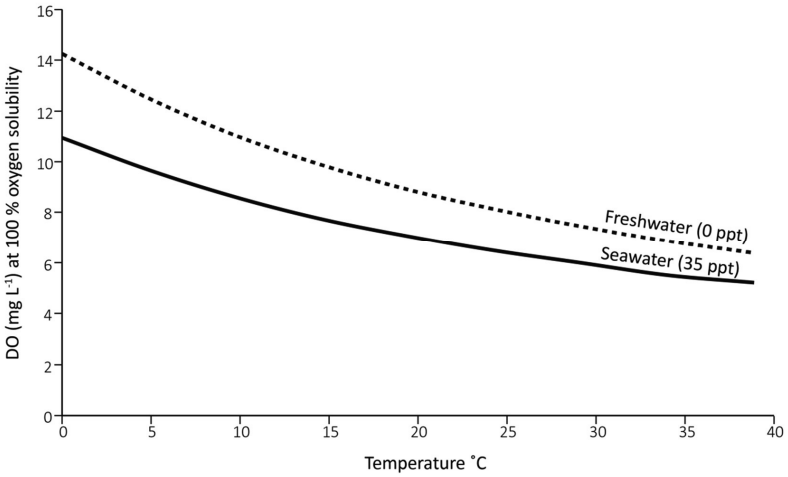

Figure 1.3. Dissolved oxygen concentration (DO; mg L^{-1}) at 100 % oxygen solubility (saturation) in freshwater and seawater in relation to temperature. Modified from source: Nilsson (2010).

Low dissolved water oxygen, also called environmental hypoxia, is recognised by a shortage of oxygen below the requirement for physiological functions of an organism (Farrell and Richards, 2009). Hypoxic water, normally followed by the build-up of excreted CO_2 and NH_3, impacts growth in fish at critical levels (Thorarensen and Farrell, 2011). Saturation level and duration of exposure decide when DO becomes critical for the fish (Nilsson and Randall, 2010). At the extremes, when oxygen levels are low (hypoxic) or absent (anoxic), fish metabolism goes from an anaerobic to anoxic state (Claireaux et al., 2000), and eventually death (Pörtner and Farrell, 2008; Pörtner and Knust, 2007).

An elevation in water temperature also increases oxygen consumption by fish (Barnes et al., 2011). The challenge for fish under warm conditions is therefore to meet an increasing oxygen demand under decreasing DO. Fish living in the marine environment are even more susceptible to low oxygen due to a lower DO in sea compared to freshwater (Fig 1.3).

As for thermal stress, also oxygen sensitivity according to fish size is debated. Large fish are considered to cope better than smaller fish under severe episodes of hypoxic and anaerobic conditions (Nilsson and Östlund-Nilsson, 2008). This is due to a lower metabolic rate and decreasing oxygen demand with increasing body mass, which means that easily accessible energy stores (glycogen) last longer and accumulation of unfavourable levels of lactic acid is slower than in smaller fish (Nilsson and Östlund-Nilsson, 2008). On the contrary, large body size is also claimed to be a disadvantage under hypoxic conditions due to a faster depletion of dissolved oxygen than in smaller fish (Pörtner and Peck, 2010). Blood oxygen levels are generally lower in larger fish (Clark et al., 2012; Clark et al., 2008), which require longer restoration time following hypoxia and stress handling (Clark et al., 2012).

However, in a review of body size and hypoxia tolerance, it was concluded that size did not matter in terms of oxygen uptake, since reduced gill surface in larger specimens is also followed by a reduced metabolic rate (Nilsson and Östlund-Nilsson, 2008) and a reduced oxygen consumption relative to body size (Gjedrem, 1993).

The aerobic scope (AS) is the capacity to consume oxygen from a calm state (standard metabolic rate, SMR) to a maximum (MMR), which indicates how much oxygen is available for swimming, behaviour, immune functions, reproduction and growth processes (Farrell and Richards, 2009; Pörtner and Peck, 2010; Rogers et al., 2016) (Fig 1.4). Optimal growth conditions are met at maximum AS within the species thermal window. As a consequence of decreasing oxygen availability and a reduced AS, the activity of vital functions are reduced or ceased, but how exactly this down regulation progress in fish is not known (Farrell and Richards, 2009). Water oxygen supply becomes critical (P_{crit}) when the

oxygen uptake cannot meet the requirement of SMR, which only supports very basic activities, and the metabolism shifts to anaerobic energy dependency (Pörtner, 2010).

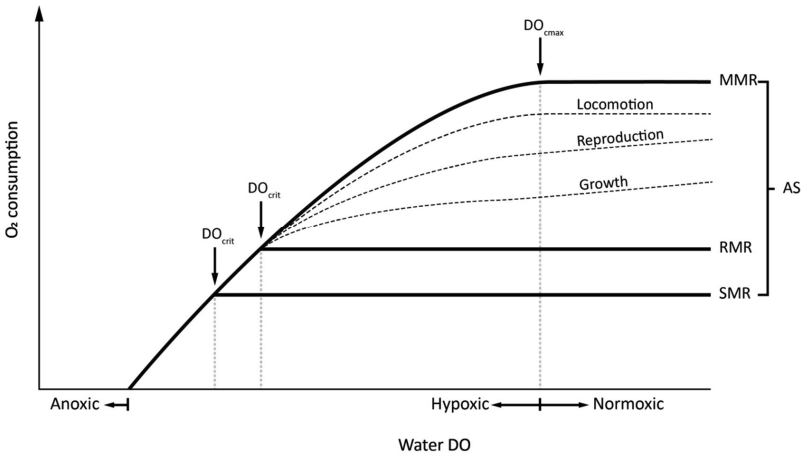

Figure 1.4: Diagram illustrating aerobic scope (AS) and metabolic responses of fish to dissolved oxygen (DO). Solid lines indicate O_2 consumption rates to support standard (SMR), routine (RMR), maximal metabolic rate (MMR) and AS of an oxyregulating fish. AS is the difference between MMR and SMR, which decreases with increasing hypoxia. Dashed lines show theoretical effects of decreases in DO level on physiological functions. Below critical dissolved oxygen (DO_{crit}) of RMR and SMR the metabolism becomes dependent on water O_2 and anaerobic energy. DO_{cmax} is defined as the critical DO level at which oxygen supply no longer meets the maximum demand for oxygen. Modified from Farrell and Richards (2009) and Pörtner (2010).

Recent studies on AS at a high temperature indicate that acclimation can prevent an acceleration of metabolic rate and maintain the AS in fish species (Gräns et al., 2014; Norin et al., 2014; Raby et al., 2016; Sandblom et al., 2014). A long term elevation in temperature may therefore maintain the growth potential better than under a sudden period of warming. It also highlights awareness of thermal adaptation time when interpreting growth results from high temperature fish studies.

The concept of oxygen and capacity-limited thermal tolerance (OCLT) describes how the aerobic scope and the thermal window interrelates (Pörtner and Peck, 2010) (Fig 1.5). Oxygen requirement is met within the thermal window, but as temperature increases the oxygen consumption also goes up (Barnes et al., 2011). When approaching the extremes of the thermal window, i.e. critically low or high temperature (T_{crit}), the aerobic scope limits

the performance and puts constraints on the metabolism due to reduced oxygen supply (Fig 1.5) from (Pörtner and Farrell, 2008; Pörtner and Knust, 2007). A raise in temperature increases P_{crit}, i.e. oxygen supply becomes limited at a higher level than P_{crit} at a lower temperature, which is demonstrated in Atlantic salmon (Barnes et al., 2011). Increasing hypoxia and carbon dioxide levels reduce both thermal window width and aerobic scope (Pörtner and Peck, 2010).

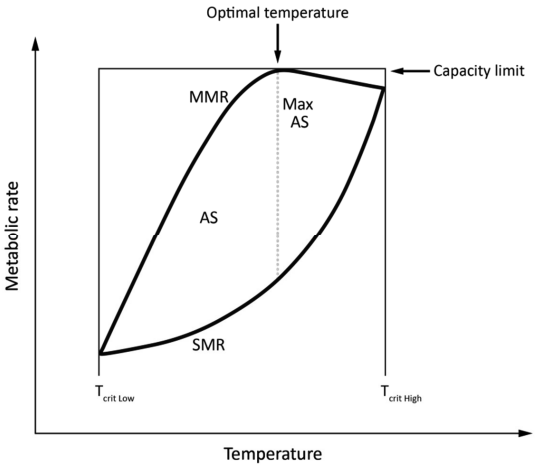

Figure 1.5. Model of how aerobic scope (AS) and metabolic rate are limited by thermal changes, i.e. the concept of oxygen and capacity-limited thermal tolerance (OCLT). AS is the difference between maximum metabolic rate (MMR) and standard metabolic rate (SMR), which is limited by critical low and high temperatures ($T_{crit\ Low}$ and $T_{crit\ High}$) and oxygen supply. AS is at maximum (Max) at an optimal temperature (grey dashed line). Source: Pörtner and Peck (2010).

The most predictable occurrence of hypoxia in commercial salmon farming is under conditions of high water temperature, but low and fluctuating oxygen also occurs in connection to shallow water, low tidal current, stratification, algae bloom, short day length, high fish biomass density and during feeding throughout the year.

Growth and feed utilisation of seawater adapted salmon improved when oxygen was raised from 50% to 100% saturation at 8-9°C (Bergheim et al., 2002) and reflects the importance of available oxygen for performance of this species. Growth of salmon is significantly

improved by raising oxygen saturation from 70-75% to 80-85% at 15°C (Bergheim et al., 2006) and oxygen level below 70% saturation reduces feed intake at 16°C (Remen et al., 2012). A review by Thorarensen and Farrell (2011) mentions that although recommended oxygen level for optimal growth of salmonids is minimum 70-80% saturation, it is close to 100% saturation to gain maximum growth of salmon. Salmon growth is found to increase even further at oxygen levels up to approximately 120 % (Hosfeld et al., 2008). A recent study of salmon postsmolt (Remen et al., 2016) demonstrates well how level of oxygen required to achieve maximum feed intake is strongly dependent on temperature. At a low temperature (7°C) maximum feed intake was found at 42 % DO, 11°C at 53% DO, 15°C at 66% and at 76% DO for the highest temperature (19°C) (Remen et al., 2016). These results are consistent with Atlantic salmon being increasingly sensitive to low oxygen water at high temperatures.

Fish can adapt to hypoxia by behavioural and physiological adaptations depending on species, size, habitat and degree of the hypoxia (Chapman and McKenzie, 2009). The behavioural responses to environmental changes by farmed fish are spatially limited in the sea cages. Positioning of fish in the water column is affected by temperature, oxygen and light, among other factors (Johansson et al., 2006; Oppedal et al., 2011; Oppedal et al., 2007; Oppedal et al., 2001). Fish energy metabolism has two coping mechanisms for declining oxygen availability depending on degree and duration; oxyregulation when oxygen is approaching, but is still above P_{crit}, and oxyconforming under anaerobic conditions below P_{crit} (Pörtner, 2010).

Oxyregulation is defined by behavioural, physiological or anatomical adaptations to maintain a steady oxygen supply to meet the demands of aerobic energy production without making any compromises in metabolic rate (Farrell and Richards, 2009; Perry et al., 2009). The most prominent physiological adaptation is the hypoxic ventilator response which is an immediate gill ventilation initiated by oxygen sensing cells (neuroepithelial) in gill filaments (Perry et al., 2009). Under long-term hypoxia, these cells can increase in density and change morphologically to facilitate oxygen uptake (Nilsson, 2007; Perry et al., 2009). Progression in hypoxia stimulates accompanying mechanisms supporting uptake and

transport of oxygen which can also relieve the degree of hyperventilation (Perry et al., 2009).

An increase of haemoglobin (Hb) concentration is the most common mechanism for fish to improve uptake and carrying capacity of oxygen and increase tolerance to hypoxia (Nilsson, 2007; Nilsson and Randall, 2010; Perry et al., 2009; Stevens et al., 1998). Additionally, fish can also increase mean red blood cell volume (MCV), red blood cell count (RBC) or haematocrit concentration (Hct) to support oxygen supply under low oxygen conditions (Perry and Gilmour, 2010). Hb's affinity for oxygen is reduced with higher temperatures, and so the ability to regulate its affinity to oxygen can be aiding delivery of oxygen to the tissues. Anaerobic metabolism reduces blood pH (Nilsson and Randall, 2010), which reduces oxygen affinity and consequently releases more oxygen to the tissue (Jensen et al., 1998). Changes in haematology and osmoregulation are known as secondary responses to environmental stress (Barton, 2002). Imbalances in ions may also impact the oxygen binding capacity of Hb, as an increased flow of Na^+ into red blood cells can inhibit binding of oxygen to Hb, releasing more oxygen to the metabolism (Ferguson and Boutilier, 1989; Ferguson et al., 1989).

Oxyconforming is a reduction of metabolic rate so that oxygen is used more sparingly (Nilsson and Randall, 2010; Perry et al., 2009; Ultsch et al., 1981). Reduced feed intake is perceived as a consequence of oxyconforming and is thought to be a major cause of depressed growth of fish under low DO conditions (Brett, 1979; Carter et al., 2008), while secondary mechanisms affecting growth under low DO conditions are yet to be well elucidated.

Atlantic cod (*Gadus morhua*) exposed to hypoxic conditions during feeding, immediately stopped eating when the declining oxygen uptake approached the critical oxygen level (P_{crit}, Fig 1.4), but resumed feeding activity when oxygen saturation reached normoxia again (Claireaux et al., 2000). Claireaux et al. (2000) suggest that this demonstrates that the behavioural adaption of reducing feed intake can support metabolism in maintaining its aerobic scope under increasingly energy demanding conditions. Another study with cod (Chabot and Dutil, 1999) showed that reduced feed intake was responsible for 97% of the

growth reduction going from a normoxic to a hypoxic condition. Reducing feed intake is no doubt an efficient energy saving mechanism since digestion of food in cod can require up to 90% of its aerobic scope (Soofiani and Hawkins, 1982).

Atlantic salmon are considered to have a poor tolerance to low oxygen, based on their naturally active life style in oxygen rich habitats, with limited capacity to oxyregulate under severe hypoxia. However, postsmolt demonstrated a high ability to oxyregulate under hypoxia at 14°C, while use of oxyconforming mechanisms dominated under hypoxia at higher temperatures (18-22°C) (Barnes et al., 2011). This indicates that there are still more to be elucidated in terms of hypoxia tolerance in Atlantic salmon.

1.5 Influence of nutritional factors under warm water conditions

Feed consists of mainly protein, fat, carbohydrates and moisture, but before the body can make use of ingested feed the nutrients are broken down to smaller units in the gut to enable absorption into the blood. In this way, the metabolism is provided with essential nutrients, where absorbed nutrients provide easy accessible energy for metabolism. Absorbed amino acids are transported via the circulatory system into the white muscle pool. Free amino acids in white muscle tissue indicate substrate availability for muscle growth (Espe et al., 1993). Post-digestive absorption of amino acids is also thought to stimulate endocrine growth promoting processes and signalling satiety (Mommsen, 2001; Planas et al., 2000).

Several studies report on postprandial concentrations of free amino acids (FAA) in plasma and tissues of Atlantic cod (*Gadus morhua*) (Lyndon et al., 1993), koi carp (*Cyprinus carpio*) (Kwasek et al., 2010; Ogata, 1986), rainbow trout (*Salmo gairdnerii* R./ *Oncorhynchus mykiss*) (Barrows et al., 2007; Carter et al., 1995; Kaushik and Luquet, 1979; Larsen et al., 2012; Schlisio and Nicolai, 1978; Yamada et al., 1981; Yamamoto et al., 2005) and Atlantic salmon (Carter et al., 2000; Espe et al., 1993; Mente et al., 2003; Sunde et al., 2003). However, data are lacking on amino acid flux in Atlantic salmon at elevated environmental temperatures.

Fat is the most energy-dense macronutrient and the energy yield is more than double that of proteins or carbohydrates (Schmidt-Nielsen, 1997a). Dietary digestible energy is therefore readily optimised by changing lipid inclusion and choice of raw materials. Dietary fat require less oxygen for growth (fat deposition) than protein or starch, which is shown for both Nile tilapia (*Oreochromis niloticus*) and rainbow trout (*Oncorhynchus mykiss*) (Saravanan et al., 2013a; Saravanan et al., 2012). This will result in a higher DE intake compared to DE supplied mainly as protein or starch in the diets (Saravanan et al., 2013a; Saravanan et al., 2012).

Dietary protein and fat recommendations are based on studies under normoxic conditions. However, the protein growth potential of salmon is reduced under limited oxygen availability. Feeding salmon with high protein under hypoxia, will then require fish to

catabolise access protein above requirement, and convert it into fat. This will demand more oxygen than utilising a diet that meets the requirement. Therefore, it is possible that a reduction in dietary protein and increase in fat, might support the oxygen budget under hypoxia, since fat can be deposited with less oxygen use than protein and starch. This could be an interesting diet to test under low oxygen conditions, especially for large salmon, due to the higher fat deposition than in smaller fish (Shearer et al., 1994). An additional thermal elevation puts an even further demanding challenge on salmon. It is therefore of particular interest to determine how dietary energy interacts with oxygen availability to regulate feed intake and growth under such conditions.

1.6 Endocrine regulation of appetite and growth

The endocrine system activates and maintains balance of important physiological functions like digestion, metabolic reactions, osmoregulation, appetite, growth, reproduction and behaviour.

Fish generally have the same endocrine organs as mammals and other vertebrates, except for the corpuscles of Stannius and urophysis, which are specific for fish species (Bone and Moore, 2008) (Fig 1.6).

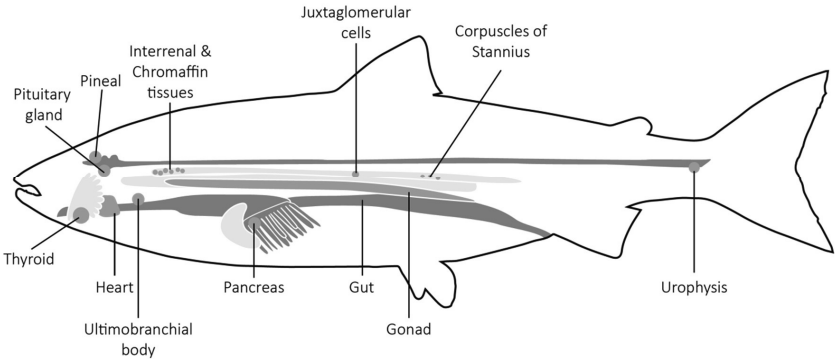

Figure 1.6. The endocrine organs of teleost fish. Modified from: Bone and Moore (2008).

Hormones, which are the chemical messengers of the endocrine system, are released from specialised glands in the brain (neuropeptides) or other organs into the blood stream. They are then transported to a peripheral target tissue (endocrine), a neighbouring cell (paracrine) or inside the cell it is secreted from (autocrine). Hormones are recognised by specific receptors on the target which signals the hormone to carry out its function or stimulate the release of other hormones (Schmidt-Nielsen, 1997b). Several target organs can be affected by a single hormone and also one target organ can be affected by several hormones. This makes it useful to study the interactions of hormones related to the response of interest.

The endocrine messengers can be categorised as fat-soluble (steroids) and water-soluble (peptides, proteins and tyrosine-derived hormones) based on chemical composition and characteristics. Endocrine regulation operates by interacting with the central nervous system, called neuroendocrine regulation.

The pituitary has a central role in hormonal regulation of diverse physiological functions, including growth, by controlling the secretion of selected hormones as well as producing its own. The gastrointestinal tract (GIT) is the largest endocrine organ and interacts with the brain (the brain-gut axis) to govern a number of endocrine reactions dealing with physiological functions like appetite, digestion, absorption and osmoregulation. One of the most profound neural influences on the endocrine signalling pathway is the hypothalamus' regulation of the pituitary gland (Bone and Moore, 2008). The hypothalamus is also the principle regulator of appetite in vertebrates including fish species (Kulczykowska and Sánchez Vázquez, 2010).

Endocrine function is vulnerable to stressors such as changes in environmental parameters (Pickering et al., 1991) making them relevant response indicators as well as functional descriptors of physiological processes. Sensitivity of fish to stressors also underlines the importance of having optimal adaptation conditions prior to sampling to rule out confounding factors.

1.7 Appetite regulation by ghrelin

Appetite is regulated by stimulating (orexigenic) and inhibiting (anorexigenic) hormones (Fig 1.7). Known orexigenic hormones in fish are apelin, galanin, ghrelin, growth hormone (GH1), neuropeptide Y (NPY) and orexins (Volkoff, 2016; Volkoff et al., 2010). The most studied anorexigenic factors include leptin, cocaine- and amphetamine-regulated transcript (CART), melanin-concentrating hormone (MCH) and cholecystokinin (CCK) (Volkoff et al., 2010).

While ghrelin studies within the Cyprinidae family are many and conclusive, the role of ghrelin in feeding regulation in salmonids is still not defined, according to a recent review by Volkoff (2016) (Fig 1.7).

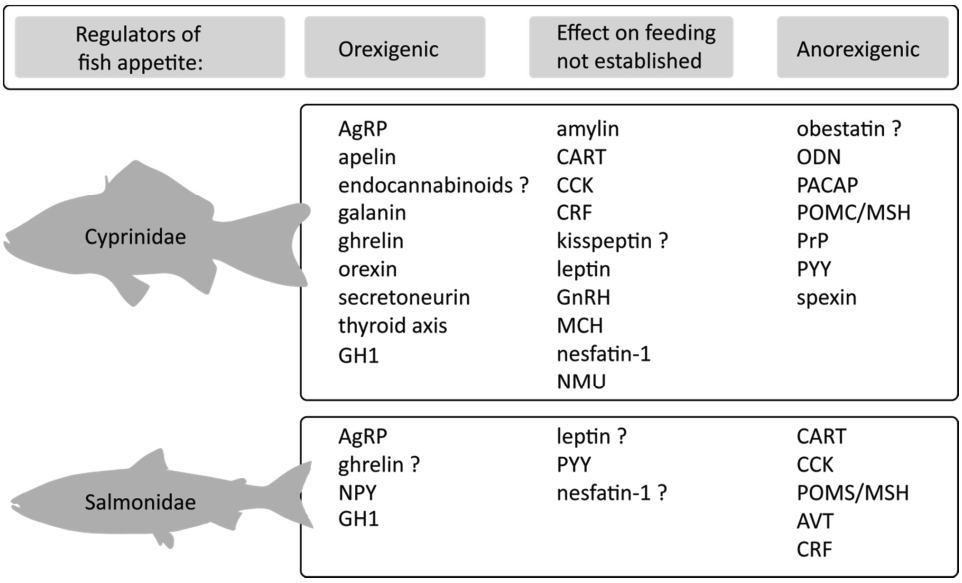

Figure 1.7. Key stimulating (orexigenic) and suppressing (anorexigenic) regulators of feeding in the most studied fish family Cyprinidae, and Salmonidae. Middle column lists factors where no effect on feeding is established. The symbol ? indicates uncertain role in regulation of feeding. Modified from Volkoff (2016) and Volkoff et al. (2010).

Ghrelin was first identified as a strong growth stimulant, hence the name, which is derived from the Proto-Indo-European word “ghre” meaning growth. Ghrelin was first isolated from rat stomach tissue, where it was identified as the natural ligand that specifically binds to the growth hormone secretagogue receptor (GHSR) in the pituitary (Kojima et al., 1999). This is also called the ghrelin receptor, which stimulates the secretion of pituitary GH1 (Fig 1.8). In the same study, human ghrelin was found to resemble rat ghrelin except for two amino acids (Kojima et al., 1999). This led to a cascade of identifications of ghrelin and its GH1 stimulating function in a wide range of vertebrates (Kojima and Kangawa, 2005), mammalian and non-mammalian, including a great number of fish species like goldfish (Unniappan et al., 2002), eel (Kaiya et al., 2003c), tilapia (Kaiya et al., 2003b), rainbow trout (Kaiya et al., 2003a), channel catfish (Kaiya et al., 2005), black sea bream (Yeung et al., 2006), cod (Xu and Volkoff, 2009) and Atlantic salmon (Hevrøy et al., 2011; Murashita et al., 2009).

Fish ghrelin varies in peptide size from 12-26 amino acids, while human ghrelin has 28 amino acids. Fish species can also have more than one molecular form of ghrelin including variations in the peptide structure (Kojima et al., 2008; Murashita et al., 2009). Activation of the biological function of ghrelin relies on a modification of the 3rd amino acid; usually serine, by acylation with a medium chained fatty acid (typically *n*-octanoic acid or *n*-decanoic) (Kojima et al., 2008).

As the only orexigenic hormone, ghrelin is synthesised in the gastrointestinal tract (GI), primarily in the stomach, but also in the brain (hypothalamus and pituitary), pancreas and heart tissue (Fig 1.8). Expression of ghrelin in teleost fish are also found in the spleen, liver, kidney and gills (Kaiya et al., 2008). In Atlantic salmon, expression of ghrelin has also been detected in adipose tissue, but its exact role is not yet described in this context (Murashita et al., 2009).

The role of ghrelin as a powerful appetite stimulant started out with the findings of elevated plasma ghrelin levels during fasting and a postprandial decline in humans (Cummings et al., 2001; Inui, 2001; Tschöp et al., 2001). Since then ghrelin has also received a lot of attention

in fish studies as the main hormone responsible for appetite stimulation (Frøiland et al., 2010; Hevrøy et al., 2012; Jönsson, 2013; Matsuda et al., 2006a; Matsuda et al., 2006b; Miura et al., 2006; 2007; Riley et al., 2005; Unniappan and Peter, 2005). A continuously growing number of ghrelin studies, show that this is a multifunctional peptide also in fish (Kaiya et al., 2008). Ghrelin interacts with other hormones, and stimulates synthesis of GH1, prolactin (PRL), somatolactin (SL), follicle stimulating hormone (FSH) and luteinising hormone (LH) (Kaiya et al., 2008). It is also involved in swimming and feeding behaviour and several physiological functions (Kaiya et al., 2008).

Ghrelin also holds a well-known role in energy balance in fish and higher vertebrates (Choi et al., 2003; Cummings, 2006; Cummings and Shannon, 2003; Frøiland et al., 2010; Hevrøy et al., 2012; Inui et al., 2004; Jönsson, 2013; Kaiya et al., 2008; Riley et al., 2005; Ueno et al., 2005; Unniappan and Peter, 2005). Correlations to body fat and increased feed intake and body weight following ghrelin treatment suggested an involvement of ghrelin in energy balance (Cummings, 2006; Tschöp et al., 2000).

Leptin is also involved in energy metabolism and in mammals has a counteracting function to ghrelin by imposing satiety (Zigman and Elmquist, 2003). Leptin in mammals is secreted from adipose tissue and signals body fat status to the brain via circulation to induce anorexia (Saladin et al., 1995; Zigman and Elmquist, 2003). In contrast, fish leptin seems to be of mainly hepatic origin (Huising et al., 2006; Kurokawa et al., 2005) and its functions in energy metabolism are not yet fully described (Rønnestad et al., 2010). Studies of leptin show a link to anorexia and responses to feed availability in various fish species (Kling et al., 2009; Moen and Finn, 2013; Murashita et al., 2011; Rønnestad et al., 2010; Won et al., 2013). This implies that leptin has a role in satiety signalling in fish as well as in mammals.

Appetite signalling to encourage fish to eat is mediated by circulating ghrelin signalling the brain via the central nervous system, by interacting or stimulating release of other appetite regulating peptides from the hypothalamus (Fig 1.8) (Hosoda et al., 2006; Kulczykowska and Sánchez Vázquez, 2010), or by working directly on tissue nearby its production site. Appetite and growth are integrally linked, with appetite providing stimulus to eat and

thereby the nutrients required for growth. This is exemplified by the central role of ghrelin in stimulating both appetite and GH1 release (Inui et al., 2004; Kling et al., 2012). Ghrelin is therefore a suitable candidate to study interactions between appetite regulation and the GH-IGF system (Kaiya et al., 2009). Concentration of ghrelin in plasma was analysed in this thesis (Fig 1.8).

Behaviour of swimming and feeding, and hormones and genes related to feeding and energy metabolism (ghrelin, GH secretagogue receptor, leptin, orexin, neuropeptide Y, melatonin) are shown to interact with the circadian system in many fish species (Betancor et al., 2014; Boujard and Leatherland, 1992; Feliciano et al., 2011; Hoskins, 2011; Kulczykowska and Sánchez Vázquez, 2010; Nisembaum et al., 2012; Rensing and Ruoff, 2002; Vera et al., 2013). This means that several functions display a daily and seasonal rhythm, and progression of hormones and their receptors over time can add valuable information to endocrine studies.

1.8 Growth regulation by the GH-IGF system

Fish growth regulation involves a range of hormones, with those receiving the most attention belonging to the GH-IGF system; specifically growth hormone (GH1 also called somatotropin) and insulin like growth factor-I (IGF-1) (Oksbjerg et al., 2004; Reinecke, 2006; Wood et al., 2005). The GH-IGF system is the main endocrine growth regulator (Reinecke, 2010) (Fig 1.8) and is strongly influenced by nutritional status and environmental factors, especially temperature and light (Beckman, 2011; Deane and Woo, 2009; Duan, 1998; MacKenzie et al., 1998; Pérez-Sánchez et al., 2002; Pérez-Sánchez et al., 1995; Pickering et al., 1991; Reindl and Sheridan, 2012; Reinecke, 2010). The influence of the GH-IGF system on growth regulation is further determined by the feed intake, diet composition and stress.

GH1 is well-documented as the most potent growth stimulant in vertebrates and reviewed by Björnsson et al. (2002). This is a multifunctional hormone that also plays significant roles in other essential functions like energy and protein metabolism (Kling et al., 2012; Mommsen, 2001; Sheridan, 1986), reproduction (Björnsson, 1997), appetite (Johnsson and Björnsson, 1994), behaviour (Björnsson, 1997; Jonsson and Bjornsson, 2002), osmoregulation, smoltification (Pelis and McCormick, 2001; Sakamoto and McCormick, 2006) and immune system (Chang and Wong, 2009; Verburg-Van Kemenade et al., 2009).

GH1 production takes place in somatotroph cells in the anterior pituitary (adenohypophysis) and is under a strong and complex hypothalamic control via nerve cells (Fig 1.8) (Ágústsson et al., 2000; Gahete et al., 2009; Gorbman, 1995). It is called the hypothalamic-pituitary-interrenal axis (HPI-axis) and is distinct from the mammalian organisation which uses the circulatory system as a signalling link between the two brain tissues (Gorbman, 1995).

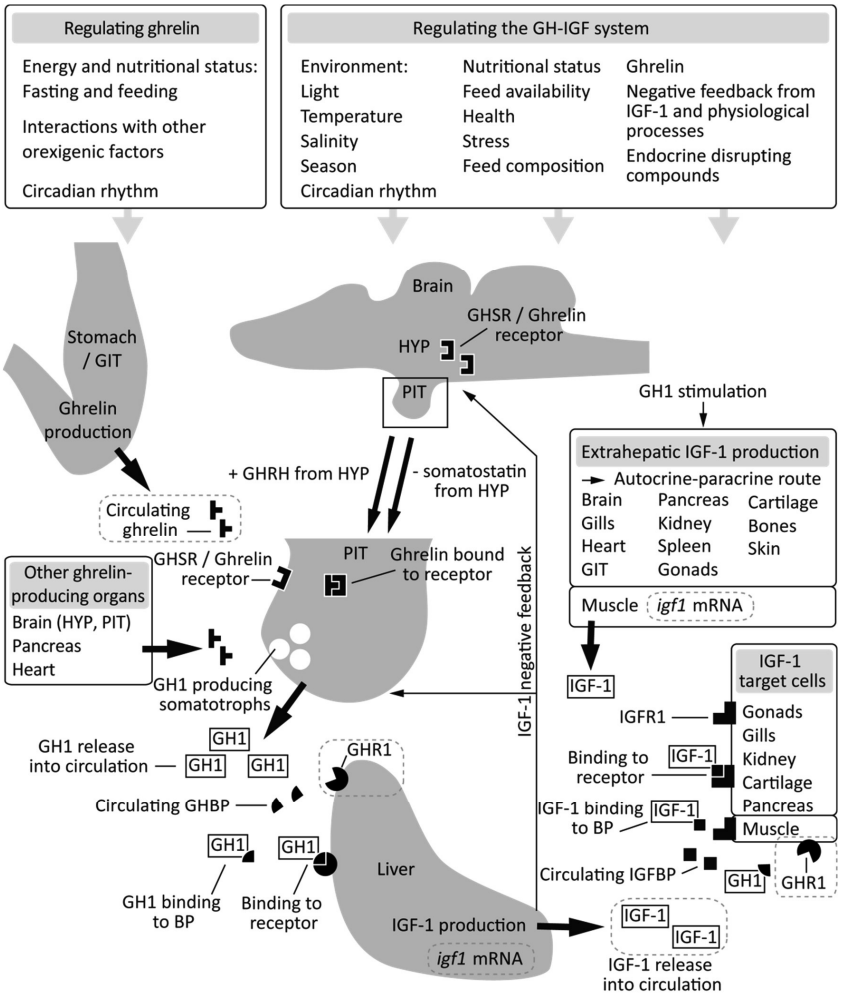
Regulating ghrelin
Energy and nutritional status: Fasting and feeding
Interactions with other orexigenic factors
Circadian rhythm
Regulating the GH-IGF system
Environment:
Light
Temperature
Salinity
Season
Circadian rhythm
Nutritional status
Feed availability
Health
Stress
Feed composition
Ghrelin
Negative feedback from IGF-1 and physiological processes
Endocrine disrupting compounds
Brain
GHSR / Ghrelin receptor
HYP
PIT
Stomach / GIT
Ghrelin production
GH1 stimulation
Extrahepatic IGF-1 production
Autocrine-paracrine route
Brain
Pancreas
Cartilage
Gills
Kidney
Bones
Heart
Spleen
Skin
GIT
Gonads
+ GHRH from HYP
- somatostatin from HYP
Circulating ghrelin
Other ghrelin-producing organs
Brain (HYP, PIT)
Pancreas
Heart
GHSR / Ghrelin receptor
PIT
Ghrelin bound to receptor
IGF-1 negative feedback
Muscle igf1 mRNA
IGF-1
IGF-1 target cells
GH1 producing somatotrophs
IGFR1
Gonads
Gills
Kidney
Cartilage
Pancreas
Muscle
Binding to receptor
IGF-1 binding to BP
GH1 release into circulation
GH1
GHR1
Circulating GHBP
Liver
Circulating IGFBP
GH1 binding to BP
Binding to receptor
IGF-1 production
igf1 mRNA
IGF-1 release into circulation
Receptor binding mediates biological functions of hormones
Ghrelin
Appetite stimulation
Growth stimulation
Hormone secretion and interactions
GH1 and IGF-1
Growth (muscle, skeleton, proliferation, regeneration)
Hormone interactions
GH1: hormone secretion (IGF-1)
IGF-1: negative feedback control of GH release
Other ghrelin roles
Energy metabolism
Locomotion
Physiology
Other GH1 roles
Energy metabolism
Protein metabolism
Appetite
Behaviour
Immune system
Osmoregulation
Reproduction
Smoltification
Other IGF-1 roles
Development
Osmoregulation
Reproduction

Figure 1.8. Regulation of appetite and growth in fish by ghrelin and the GH-IGF system. GH1 is produced in somatotroph cells in PIT (pituitary). GH1 release from PIT is stimulated (+) by GH releasing hormone (GHRH) and inhibited (-) by somatostatin from the hypothalamus (HYP). Circulating GH1 binds to binding proteins (GHBP) which control their availability to the receptor (GHR1) in mainly liver and muscle. Production of IGF-1 is mainly in liver and muscle. IGF-1 released to circulation also binds to binding proteins (IGFBP) before binding to receptor (IGFR1) in target organs. Binding to receptors mediates biological functions of ghrelin, GH1 and IGF-1. Main roles of ghrelin, GH1 and IGF-1 are also given. Circulating ghrelin, mainly from the stomach and gastrointestinal tract (GIT), binds to the GH secretagogue receptor (GHSR, called the ghrelin receptor) in PIT and HYP to stimulate GH1 production. The availability of GH1 and IGF-1 to their receptors is controlled by binding to circulating binding proteins (BP). GH1 also has a possible stimulating role in extrahepatic IGF-1 production. Factors analysed in thesis are enclosed with dashed lines. Modified from sources: Chang and Wong (2009); Kaiya et al. (2008); Reinecke (2010); Unniappan and Peter (2005).

Stimulatory or inhibitory signals from the hypothalamus are influenced by endogenous cues, such as nutritional state and humoral factors, and exogenous cues, such as temperature and photoperiod (Björnsson et al., 2002; Canosa et al., 2007). The hypothalamus produces the main GH1 stimulator; the GH-releasing hormone (GHRH, also called GHR factor) (Fig 1.8) and other stimulating peptides including the pituitary adenylate cyclase-activating peptide (PACAP), corticotropin-releasing hormone (CRH), neuropeptide Y (NPY), thyrotropin releasing hormone (TRH) and gonadotropin releasing hormone (GnRH) (Gahete et al., 2009).

The identification of a peripheral GH1 stimulant; ghrelin (Inui et al., 2004; Kaiya et al., 2003a; Kojima et al., 1999; Ueno et al., 2005), gave a new perspective to the GH signalling pathway and the established hypothalamic regulation (Fig 1.8). There are also indications that leptin can act as a GH secretagogue in mammals (Giusti et al., 2002) and recently in fish (Won et al., 2013), suggesting further endocrine factors possibly interacting with the GH-IGF system that should be elucidated to increase the understanding of fish growth regulation.

The main inhibition of pituitary GH1 release is caused by the somatotropin release-inhibiting factor (SRIF, or somatostatin) which is also of hypothalamic origin (Fig 1.8) (Gahete et al., 2009; Reinecke, 2010). Other inhibiting factors include the neuropeptide serotonin and the neurotransmitter norepinephrine (NE) (Gahete et al., 2009). Plasma IGF-1 also plays an important inhibiting role in regulating GH1 secretion under catabolic conditions through a negative feedback function (Duan, 1998; Reinecke, 2010). Besides,

GH1 itself is reported to have a direct negative feedback function on GH1 pituitary release in rainbow trout (Ágústsson and Björnsson, 2000). The diverse role of GH1 is reflected in the many factors involved in GH-IGF regulation and wide expression of GH1 in extra-pituitary tissue and receptors in extra-hepatic tissues (Canosa et al., 2007), which also supports a paracrine/ autocrine function of GH1 (Waters et al., 1999).

Under anabolic conditions GH1 is released into the circulatory system and can stimulate tissue growth either directly or indirectly by binding to GH receptors (GHR1 and GHR2) and stimulate synthesis and secretion of IGF-1 mainly from the liver, but also from a wide range of other tissues (Beckman, 2011; Pérez-Sánchez, 2000; Pérez-Sánchez et al., 2002; Reinecke, 2010). The relative growth stimulating potency of IGF-1 originating from either liver or muscle is still not fully described in fish, although both promote muscle growth (Fuentes et al., 2013).

Two types of GHR exist; GHR1 and GHR2, both of which are expressed in various body tissues and fish species (Saera-Vila et al., 2007). The functions of GHR's are still not fully understood (Saera-Vila et al., 2007), but reported results indicate that function and expression seem to be dependent on type of tissue and fish species and that there are some similarities between the GHRs (Hevrøy et al., 2015; Reindl and Sheridan, 2012). In Atlantic salmon few functional differences were found between the two GHRs, and mRNA levels in both liver and muscle tissue reacted similarly to temperature treatments of 13°C and 19°C (Hevrøy et al., 2015). In trout, liver *ghr2* mRNA levels were significantly lower at 19°C compared to 13°C, while liver *ghr1* mRNA was unaffected by temperature treatments (Hevrøy et al., 2015).

The availability of GH1 and IGF-1 to their receptors is controlled by binding to circulating binding proteins (BP); called GHBP and IGFBP, respectively (Fig 1.8) (Baumann et al., 1988; Clemmons et al., 1998; Duan and Xu, 2005; Kopchick and Andry, 2000; Mannor et al., 1991; Wood et al., 2005). Hence, the BPs act as a reservoir and can create a disagreement between secretion and circulating levels of GH1 as shown recently in Atlantic salmon (Einarsdottir et al., 2014). Variations in GHR1 and levels of circulating BPs can also

create an imbalance between GH1, IGF-1 and growth (Duan, 1998; Wood et al., 2005). The role of BPs in the GH-IGF system is less known and the majority of our understanding of their functions and regulation is described for mammals. In fish, BPs role is in the early phase of research and their influence on the GH-IGF actions is still poorly understood, especially for GHBP (Reindl and Sheridan, 2012). However, in recent studies of zebra fish (Kajimura et al., 2005; Kamei et al., 2008), it was found that two genes of *igfbp1* (*a* and *b*) influence growth under hypoxia by binding up free IGF-1.

IGFs promote growth by stimulating growth of muscle and skeleton (bones and cartilage) and preventing breakdown of protein and cells (Duan et al., 2010; Oksbjerg et al., 2004; Wood et al., 2005). IGF-1 is also reported to have a stimulatory effect in development, osmoregulation and reproduction (Duan, 1997; Reinecke et al., 2005).

As the name IGF-1 indicates, this endocrine growth stimulant has similar functions and structures to insulin (Duan et al., 2010). However, in terms of food availability the IGF-1 responds more to long-term changes than insulin, which reacts to short and abrupt changes (Gabillard et al., 2006; Shimizu et al., 2009). The GH-IGF system consists of both IGF-1 and IGF-2. This thesis focussed on IGF-1 (gene expressions in liver and muscle, and plasma concentration) (Fig 1.8) as it is well known to be the principle stimulant of muscle growth in fish, and the endocrine role and biological actions of IGF-2 are less understood (Reinecke, 2006). Other factors of the GH-IGF system investigated in this thesis were gene expression analyses of the receptors; GHR1 and IGFR1 in liver and muscle tissue (Fig 1.8).

Growth and IGF-1 often respond similarly to external factors, thus IGF-1 is also considered a useful growth indicator in fish in addition to GH1 (Beckman, 2011; Beckman et al., 2004; De-Santis and Jerry, 2007; Dyer et al., 2004; Pérez-Sánchez and Le Bail, 1999; Picha et al., 2008; Wilkinson et al., 2006). Like GH1 (Chang and Wong, 2009), IGF-1 has an immune related function in fish (Segner et al., 2006; Yada, 2007; Yada, 2009), but its exact role is not yet well defined (Franz et al., 2016).

The GH-IGF regulation in fish is responsive to stressors like high temperature, fasting, handling, overcrowding and salinity (Pickering, 1993; Pickering et al., 1991; Reinecke, 2010). Generally growth reduction in relation to environmental stressors are caused by elevation of secreted stress hormones (catecholamines and corticosteroids) (Pickering et al., 1991). Reduced growth in gilthead seabream following confinement stress is thought to be caused by modifications in secretion and availability of circulating GH-IGF hormones (Rotllant et al., 2001). Also salmonids respond to handling stress by changes in circulating levels of hormones related to the GH-IGF system (Wilkinson et al., 2006), but discrepancies in results from various studies can be complicated due to different environmental conditions and sampling time regimes. When elevated levels of GH1 do not correlate with increased growth under warm conditions (Handeland et al., 2000), this may indicate an impaired growth regulation due to thermal stress (Deane and Woo, 2009; Pickering, 1993). However, due to GH1's multifunctional role, several factors need to be considered when attempting to identify GH1 responses in growth regulation.

Atlantic salmon exposed to sudden stress episodes resulted in elevated levels of both plasma GH1 and IGF-1 along with reduced growth (McCormick et al., 1998). However, elevated plasma GH1 is also found in crowded and uncrowded rainbow trout exposed to low oxygen conditions, while crowding itself resulted in reduced plasma GH1 levels (Pickering et al., 1991).

Nutritional status and temperature can interact to stimulate the GH-IGF signalling pathway in different ways. Rainbow trout exposed to high temperature (16°C) had higher circulating GH1 levels than those at 8°C and 12°C irrespective of feeding regimes (Gabillard et al., 2003c), while plasma IGF-1 was elevated by a combination of high temperature and higher feed ration (Gabillard et al., 2003b). Postprandial peaks of both GH1 and IGF-1 are reported for various fish species (Ayson et al., 2007; Fox et al., 2009; Shimizu et al., 2009; Valente et al., 2012), and varies with factors like species, fish size, nutritional status, temperature (Reinecke, 2010). Starvation or restricted feeding generally results in increased plasma GH1, and reduced IGF-1 concentrations and hepatic mRNA levels of *ghr1* and *igf1*, while refeeding or high feed ration reduce plasma GH1 and elevates plasma IGF-1 and hepatic

ghr1 and *igf1* mRNA levels (Reinecke, 2010). Contradicting responses indicate that there may be species specific differences in how the GH-IGF system responds to fasting and refeeding or nutritional status (Reindl and Sheridan, 2012).

GHR1 declines in fasted fish, implying a reduced stimulation of IGF-1 synthesis and growth (Gray et al., 1992; Pérez Sánchez et al., 1994), but also lack of negative feedback on GH1 synthesis (Duan, 1998; Pérez Sánchez et al., 1994) leading to GH1 accumulation (Pérez-Sánchez, 2000). GH1 also has a role in metabolic use of energy from fat, which can be influenced by nutritional status and dietary energy level (Company et al., 1999; Deane and Woo, 2009; Pérez-Sánchez, 2000). Feeding a high fat diet increases the GH1 level in several fish species (Cameron et al., 2002; Company et al., 1999; Pérez-Sánchez, 2000) possibly caused by a shift in use of dietary fat as metabolic energy source rather than protein (a protein sparing effect) (Company et al., 1999; Pérez-Sánchez, 2000) or by an enhanced lipolytic activity of GH1 (Björnsson, 1997; Hevrøy et al., 2013).

The direct and interrelated actions of stimulating and inhibiting factors of the GH-IGF system are essential to describe how growth mechanisms are regulated. The tight relationship between appetite and growth regulation underlines the importance of studying both ghrelin and the GH-IGF system in order to elucidate more on these functions in fish under warm hypoxic conditions. Studies on endocrine regulation of appetite and growth are increasing in fish (Kang, 2011), but very few studies report on the interactions of the integrally linked mechanisms of the GH-IGF system and ghrelin in Atlantic salmon (Hevrøy et al., 2011; Hevrøy et al., 2012; Kullgren et al., 2013; Moen et al., 2010). Many useful parallels can be drawn from extensive mammalian studies, but variations in endocrine regulation of fish and vertebrates in general, have been reported for several functions, like appetite, growth and stress (Gahete et al., 2009; Jönsson, 2013; Jönsson et al., 2007; Kaiya et al., 2008; Pickering et al., 1991; Volkoff et al., 2010). This suggests significant species differences and a need for more endocrine studies on target fish species.

2. Aims of the thesis

The main goal of the present PhD work is to increase knowledge on appetite and growth regulation in seawater adapted Atlantic salmon in relation to high water temperature, low oxygen conditions and dietary energy concentration.

This topic is divided into three specific aims:

1. Determine pre- and postprandial nutrient dynamics and endocrine regulation of appetite and growth
2. Investigate the mechanisms by which low oxygen and high temperature impact appetite and growth
3. Determine how dietary energy concentration affects growth regulation under hypoxia and high temperature

3. Abstract of papers

3.1 Paper I

Atlantic salmon farmed in sea cage facilities are exposed to changing environmental conditions. While growth decline under lower water temperatures are reasonably well understood, the mechanisms behind production decline under high water temperatures are yet to be well elucidated and have been hampered by a lack of data describing pre- and postprandial patterns of endocrine fluctuation. The present research therefore aims to determine whether peak nutrient flux into the blood plasma is the most appropriate time point to investigate the endocrine regulation of growth and appetite under conditions of normal and high temperature, and to investigate the interrelationship between appetite and growth on a pre- and postprandial time scale. Two experiments are presented which examine ghrelin (GHRL) as an indicator of appetite stimulation, the GH-IGF (growth hormone-insulin-like growth factor) system to describe growth-regulating processes and free amino acids (FAA) to indicate postprandial nutrient influx and link appetite and anabolic processes. Postprandial sampling of plasma and white muscle tissue from short-term adapted postsmolt was conducted at 13°C and 19°C at 4, 8, 12, 16, 20 and 24 hours (h). The same samples were taken from long-term adapted big salmon at 12°C, -4, -2, -1, 0 h pre-prandially and 2, 3, 4 and 6 h post-prandially. While limited relationship between plasma ghrelin concentration and meal times was found for short term adapted postsmolt, clear ghrelin peaks were described for long term adapted salmon prior to the timing of anticipated meals. Possible explanations and consequences to experimental design in this area will be discussed. Postprandial FAA in plasma and white muscle from postsmolt were reduced at 19°C compared to 13°C and plasma levels peaked 8 h post-prandially. Muscle *igf1* mRNA expression levels were consistently higher at 13°C than 19°C, with no clear postprandial patterns. In contrast, plasma IGF-1 concentration was relatively constant over time at 12°C and 13°C, but significantly declined from 20 h postprandially at 19°C. GH receptor (*ghr1*) mRNA expression in muscle was unaffected by temperature, peaking 4 h post-prandially at both temperatures. This paper describes growth and appetite-regulating

processes under conditions of normal and elevated temperature for Atlantic salmon, which is fundamental to our understanding of growth limitations inherent to high water temperature situations.

3.2 Paper II

This study examines how appetite and growth regulation of Atlantic salmon are affected by low dissolved oxygen (LO) and dietary digestible energy levels (DE: high [HE] vs. low [LE]). Long-term exposure to LO resulted in a reduced feed intake, growth, digestible protein and fat retention efficiencies and increased feed conversation ratio and plasma ghrelin concentrations ($p < 0.05$) compared to high dissolved oxygen (HO). Pair-feeding of rations based on the feed intake of the LO groups, but fed at HO, resulted in a 50% growth improvement in HE diet groups. This suggests that the poor growth under LO was not entirely caused by the reduced feed intake. Salmon adapted to LO by increased haemoglobin concentrations, while osmoregulation was affected by increased plasma chloride concentrations ($p < 0.05$). Plasma ghrelin concentration was unaffected by DE ($p > 0.05$). Growth regulation was affected by the HE diet, with increased liver and muscle growth hormone receptor *ghr1* mRNA ($p < 0.05$), regardless of oxygen level. The growth depression due to low oxygen appears to be related to higher metabolic costs, while higher DE upregulates the GH-IGF system at the *ghr1* level and found to be beneficial for growth, feed intake, oxyregulation and osmoregulation under hypoxia.

3.3 Paper III

High temperature combined with low dissolved oxygen (DO) is one of the most challenging environmental conditions farmed fish experience, thus understanding their impact on growth regulation is of relevance to cultured and wild populations. This study examines appetite and growth regulating mechanisms in Atlantic salmon postsmolt exposed to either high (HO) or low oxygen (LO) at a suboptimally high temperature (17°C). Additionally, the

effects of high (HE) and low (LE) dietary energy (DE) were examined. After a month of treatment, analyses of hormones regulating appetite (ghrelin) and growth (growth hormone receptor *ghr1* and insulin-like growth factor IGF-1), and free amino acids (FAA) were measured pre- and postprandially at -4, -2, 0, 2, 4 and 6 hours (h). No pre-prandial ghrelin peaks were detected despite a significant reduction of feed intake and growth under hypoxia compared to normoxia. LO treatment also had an overall negative effect on survival compared to HO, while nutrient retention, FCR and plasma FAA concentrations were unaffected ($p > 0.05$). Feeding HE diet resulted in increased growth (+17%) and improved FCR (-14%) and energy retention efficiency (+26%) independent of DO. Plasma FAA concentrations were unaffected by LO treatment and DE ($p > 0.05$). Growth regulatory gene expressions possibly reflect an overall lower growth at a high temperature overriding the impacts of DO and DE. This study also indicates that optimal adaptation time to environmental conditions and feeding regime is crucial for establishing a regular hormonal appetite signalling that reflects real feeding anticipation in salmon.

4. General discussion

The present thesis investigates appetite and growth regulation in seawater adapted Atlantic salmon in relation to high water temperature, variable low oxygen conditions and dietary energy. This chapter compares and discusses the main findings from four fish trials which are published in three scientific papers: I (Vikeså et al., 2015), II (Vikeså et al., 2017) and III (Vikeså et al., 2016). Further implications for salmon farming and forecasted environmental challenges are also discussed.

To determine pre- and postprandial nutrient dynamics and endocrine regulation of appetite and growth, two fish trials were conducting (Paper I). In the first trial, postsmolt (196 g) were exposed to an optimal and a sub-optimally high temperature (13°C and 19°C) for 35 days (short-term adaptation) before a 24 h postprandial sampling of blood, and liver and muscle tissue were carried out. This was followed up by a long term adaptation (four months) of large salmon (3.7 kg) to feeding regimes at 12°C. The sampling set-up for large salmon was changed to separate pre- and postprandial sampling groups, to ensure postprandial sampled fish were not disturbed by the preprandial sampling, and therefore had normal feeding behaviour. Sampling range was also changed to be closer around mealtime; from -4 h pre-prandially to 6 h post-prandially. This was to determine whether any plasma FAA or endocrine responses were rather peaking closer to mealtime, than detected by the 4-24 h postprandial sampling regime. Pre- and postprandial analyses (-4 h to 6 h) were also conducted with short-term adapted postsmolt at a high temperature (17°C, Paper III).

To investigate the mechanisms by which low dissolved oxygen (LO, hypoxia) and high temperature regulate appetite and growth, different LO regimes were tested against groups at stable high oxygen conditions (HO, normoxia) in two fish trials (Paper II and III). In the first LO trial (Paper II), large salmon (1.3 kg) were held under a fluctuating LO regime, to mimic a possible natural situation, for 4 months at an optimal temperature for growth (12°C). A pair feeding technique was applied in order to separate the influences of oxygen

from feed intake. The second trial was conducted at a high temperature (17°C) (Paper III) with postsmolt (264 g) under stable LO and HO regimes, for 30 days.

Finally, to determine how dietary energy concentration (DE) affects growth regulation under hypoxia and high temperature, diets with high and low energy (HE and LE) were tested in both LO studies (Paper II and III).

4.1 High temperature

An elevation in water temperature has a well-known stimulatory effect on both appetite and growth within an optimal temperature range. Temperature is the main driver of metabolic rate (Brett, 1979; Jobling, 1997) and, together with feed availability, it greatly defines the potential for growth in fish. Feed intake is also important for growth as it initiates a cascade of metabolic reactions including the digestion process and secretion of regulatory factors. However, less is known about the mechanisms behind feed intake depression and growth regulation following a further thermal elevation beyond optimum, which has been the focus of this thesis.

The signalling pathways of appetite and growth regulating hormones fluctuate and interact in response to important drivers of growth, such as feed availability and temperature. Despite this, these dynamics have not been systematically studied in Atlantic salmon. The majority of studies investigating endocrine growth regulation at high temperature, have sampled fish at an undefined period or a single, fixed sampling point after a feeding. This approach assumes that either the endocrine or nutrient factors under investigation are similar among the experimental groups over time, or that the time point chosen yields the most informative data. In order to test these assumptions and better understand the interaction of feed intake, temperature and regulation of appetite and growth, a study on postprandial dynamics was first conducted. Fish trials at optimal and high temperatures (Paper I and III) with up to 24 h postprandial sampling regimes were therefore designed to detect peak times for circulating FAA and endocrine growth factor levels in relation to meal time. The aim was to define the most appropriate time point to investigate regulation of growth and appetite and to get a basic understanding of how these mechanisms are regulated and interact in relation to temperature in Atlantic salmon (Paper I).

Postprandial FAA regulation

Several studies have been conducted to describe postprandial absorption and storage of ingested FAA in Atlantic salmon (Carter et al., 1995; Carter et al., 2000; Espe et al., 1993; Espe et al., 1999; Mente et al., 2003; Torrisen et al., 1994). However, postprandial FAA data comparing high temperature with optimal conditions is lacking.

A 24 hour postprandial sampling regime revealed that plasma FAA in postsmolt (Paper I) peaked 4-8 h postprandially at 13°C, while the high temperature treatment group at 19°C had a generally lower level and less pronounced peak for the same time interval. There was a general decrease in amino acid pools in both plasma and muscle associated with high water temperature (Paper I, Fig 2ab and 3ab). The greatest difference in FAA concentrations between temperature treatments did not occur at the time of peak nutrient absorption, but rather at 12-24 h postprandially when FAA levels were descending. Fish were fed regularly for a month prior to the postprandial sampling, and the consistently depressed FAA levels in muscle tissue indicate that the potential for growth at a high temperature can be reduced even under conditions of unlimited feed access.

A narrowed pre- and postprandial time sampling of large salmon at 12°C and postsmolt at 17°C did not reveal any distinct plasma FAA peaks from -4 h to 6 h (Paper I; Fig 7, Paper III; Fig 3ab), and supports the postprandial peak time at 4-8 h found for postsmolt at 13°C and 19°C (Paper I, Fig 2ab and 3ab). The sampling at 17°C (Paper III) showed similar plasma FAA levels at 4 h and 6 h postprandially, which were significantly higher than just before meal time (0 h), and demonstrates that protein digestion and absorption are rapid mechanisms.

A significantly reduced feed intake was not found in postsmolt held at 19°C compared to 13°C, which could be due to the short experimental period. In retrospect, it would have been interesting to monitor daily feed intake to look for trends throughout the trial period. Feed intake and growth were similar between treatments, while FAA pools were decreased at 19°C, indicating a moderately elevated amino acid catabolism at higher temperature. This is also consistent with the known increase in metabolic rate and maintenance costs due to high

temperatures, which alters many metabolic functions like growth and ingestion rates (Brett, 1979; Jobling, 1997).

The 24 h postprandial investigation presents data on the FAA dynamics in salmon postsmolt exposed to both optimal and a high temperature treatment (13°C v. 19°C).
It is in contrast to other FAA studies in salmon by not applying fasting prior to the postprandial sampling (Carter et al., 1995; Carter et al., 2000; Espe et al., 1993; Espe et al., 1999; Mente et al., 2003; Torrisen et al., 1994). Nutrient status at time of sampling should therefore represent that from continuous feeding.

Several characteristics to salmon life cycle are size dependent, and so the effect of high temperature on the FAA dynamics in large salmon should be followed up. From the study with postsmolt (Paper I), the use of a wide postprandial sampling regime and inclusion of both an optimal and a high temperature treatment made it possible to describe how temperature affects the postprandial absorption and storage of FAA. These parameters can serve as a useful template for future studies on FAA dynamics.

Growth regulation

Study of the GH-IGF system in relation to high temperature is in its infancy, and current knowledge is hampered by frequent use of single postprandial sampling points. In the high temperature study (Paper I), exposure of postsmolt to 19°C resulted in declining plasma IGF-1 concentration and lower muscle *igf1* mRNA levels postprandially compared to 13°C treatment groups (Paper I, Fig 4 and 5b).

High temperatures are frequently reported to increase plasma levels of both GH1 (Deane and Woo, 2009; Gabillard et al., 2003a; Handeland et al., 2000; Kullgren et al., 2013; Ricordel et al., 1995) and IGF-1 (Beckman et al., 1998; Davis and McEntire, 2011; Imsland et al., 2007) in a variety of fish species, including salmon. Recent studies with both large and small salmon showed no differences in plasma IGF-1 levels in the temperature range 13-19°C sampled at 4 h postprandially (Hevrøy et al., 2013; Hevrøy et al., 2015). This is somewhat inconsistent with the plasma IGF-1 levels reported in Paper I (Fig 4). Although a significant high temperature effect was not detected in the present study at 4 h, the decline in plasma IGF-1 levels from 12 h postprandially and the continued significant decline from 20 to 24 h postprandially indicates a down-regulated growth potential at 19°C in contrast to the stable levels at 13°C. This demonstrates how temperature also controls growth through endocrine regulation and that IGF-1 plays a key role, and importantly that this was only detected by way of a wide postprandial sampling range of 24 h.

High temperatures (15-19°C vs 13°C) have previously been reported to reduce muscle, but not liver, *igf1* mRNA levels, 4 h post-prandially in large salmon (Hevrøy et al., 2013; Hevrøy et al., 2015). The postprandial sampling regime in Paper I also revealed that this depression is continued up to 24 h with a tendency to decline faster in fish at a high temperature compared to 13°C (Fig 5b).

A high temperature effect was not detected for muscle *ghr1* mRNA levels (Paper I, Fig 5a), which for both temperature groups showed a clear drop after 4 h postprandially down to stable values thereafter. This implies that *ghr1* activity is more closely coupled to meal times, feed intake or appetite rather than temperature. Similar studies with salmon are few

and results from 4 h postprandial samples are variable. Large salmon exposed to 15-19°C had increased muscle *ghr1* mRNA compared to 13°C and was related to lipolytic activity by GH1 (Hevrøy et al., 2013). In the same study, liver *ghr1* mRNA was not affected by temperatures. In postsmolt at 19°C, a temperature effect was not found for muscle *ghr1* mRNA, but liver *ghr1* mRNA was reduced at a high temperature compared to 13°C (Hevrøy et al., 2015).

Overall, the contradicting effect of high temperature on growth regulating factors demonstrates that existing knowledge on this subject is still at an early stage. The GH-IGF system is influenced by many factors such as fish size, stress, different sampling regimes, adaptation time and dietary differences, which should be the focus of further studies. A range of endocrine growth factors have been identified in fish, but analyses of GHR1 and IGF-1 were chosen due to the key role of the GH-IGF system as a growth mediator and its sensitivity to nutritional state and temperature changes. Both plasma IGF-1 concentration and *igf1* gene expression were analysed to see if the real circulating levels confirmed the mRNA expression levels. Since gene expression precedes the IGF-1 protein, this can also indicate what to expect from the hormone for which it encodes. However, there are more factors involved in endocrine growth regulation, e.g. the binding proteins (BPs) of both GH1 and IGF-1. Little is still known about BPs in fish and further research should focus on defining their role in growth regulation mediated by the GH-IGF system.

Analyses of endocrine growth factors in postsmolt exposed to 17°C (Paper III) corresponded poorly with previous reported studies and observations suggest experimental conditions were suboptimal for detecting endocrine signals. Preprandial levels of plasma IGF-1 and muscle *igf* mRNA were significantly higher close to meal time (0 h) compared to postprandial levels (4-6 h) (Paper III, Fig 3 and 4ab). These results are not consistent with the postprandial findings at 19°C, nor at optimal temperatures (12°C and 13°C) as reported in Paper I (Fig 4, 5ab, 9 and 10ab). Whether this actually represents the GH-IGF regulation at high temperature needs to followed up, and should preferably include an optimal temperature treatment for better interpretation of responses to temperature and DO.

Stressors like environmental changes or handling are known to increase secretion of stress related hormones (Pickering et al., 1991), but also to alter circulating levels of GH-IGF hormones in fish, including salmonids (Pickering, 1993; Pickering et al., 1991; Reinecke, 2010; Rotllant et al., 2001; Wilkinson et al., 2006). The overall high mortality (~15%) in the trial at 17°C (Paper III) could be a reaction to such stressors and it is possible that the adaptation time going from 12°C to 17°C during the initial phase of the study was insufficient duration.

The high temperature study at 19°C (Paper I) shows a clearly diminished growth potential for salmon at a suboptimally high temperature, which was demonstrated by a faster postprandial catabolism of FAA and generally lower levels and faster declines of endocrine growth stimulating factors. Applying a wide postprandial sampling range made it possible to determine how salmon absorption and growth are affected by high temperature on a broader scale. This approach revealed that several important trends and differences between temperature treatments occurred after 8 h post-prandially. Future studies on nutrient and endocrine dynamics would gain from including more than one sampling point as this can strengthen interpretation of results.

4.2 Appetite regulation

Ghrelin and meal anticipation in salmon

Since its identification as a GH secretagogue (Kojima et al., 1999), ghrelin has received a lot of attention in human studies related to obesity and eating disorders due to its central role in metabolic energy balance, appetite and food intake regulation (Cummings and Shannon, 2003; Klok et al., 2007; Zigman and Elmquist, 2003). This thesis deals with the same subjects, but from the opposite perspective; attempting to elucidate mechanisms behind growth depression under adverse environmental conditions. The multiple roles of ghrelin in energy metabolism, appetite stimulation and regulation of feed intake and growth, links several central paradigms associated with fish farming productivity. Understanding the mechanisms behind ghrelin signalling is therefore of great interest, as feed intake optimisation is crucial for salmon to utilise its growth potential.

Ghrelin is often referred to as the "hunger hormone", referring to its role in initiating feed intake. This supports the metabolism, maintaining a positive energy balance and increasing body weight or adiposity (Cummings, 2006). Circulating levels of ghrelin peak close to start of a meal, followed by a postprandial depression in humans and other mammals (Bagnasco et al., 2002; Cummings et al., 2001; Cummings and Shannon, 2003; Muller et al., 2002; Tschöp et al., 2001). Plasma ghrelin concentration also showed clear preprandial peaks in large salmon, which were long term adapted to 12°C and two daily meals (Paper I, Fig 6). The peaks appeared within the space of one to two hours before each meal was presented. This is consistent with mammalian findings, but data from fish studies are scarce and contradictory.

For Atlantic salmon, no other reports exist on pre- and postprandial investigations of circulating ghrelin. However, an up-regulation of *ghrelin* mRNA expression in salmon larvae preceding first feeding (Moen et al., 2010) also supports that ghrelin is an appetite signal in this species. A postprandial investigation of several other appetite regulating hormones (*npy, cart, cck, peptide yy; pyy, agouti-related protein; agrp, cholecystokinin; pomc*) in Atlantic salmon showed that brain mRNA expression levels of these hormones react to feeding, but analyses of ghrelin was unfortunately not included (Valen et al., 2011).

Ghrelin responses in relation to meal time have been reported in rainbow trout (*Oncorhynchus mykiss*) and Atlantic cod (*Gadus morhua*). Blood analyses of rainbow trout showed that plasma ghrelin increased at the end of a 24 h postprandial sampling range (Pankhurst et al., 2008a). As the fish were long-term adapted (three months) to only one daily meal, this can be considered a preprandial plasma ghrelin peak in rainbow trout (Pankhurst et al., 2008a). This resembles the present finding in large salmon (Paper I) where each of two long-term adapted meal times had a corresponding preprandial ghrelin peak.

In Atlantic cod, stomach *ghrelin* mRNA levels were upregulated during meal time compared to 2 h before and after the meal (Xu and Volkoff, 2009). In goldfish (*Carassius auratis*) a postprandial decline was found for both serum ghrelin and *preproghrelin* mRNA levels (precursor of ghrelin) in brain and gut tissue, although sampling range was limited to -3h to 3 h (Unniappan et al., 2004). In contrast to the above findings from rainbow trout, Atlantic cod and goldfish (Pankhurst et al., 2008a; Unniappan et al., 2004; Xu and Volkoff, 2009), no significant postprandial changes in plasma ghrelin were found in another study with rainbow trout (Jönsson et al., 2007) nor in Mozambique tilapia (*Oreochromis mossambicus*) (Fox et al., 2009).

Ghrelin response to feed deprivation shows how this hormone is linked to energy status, but also here the reported findings in fish vary. Fasting of salmon for two days increased plasma ghrelin levels, but declined to the level of fed groups after 14 days (Hevrøy et al., 2011). Similar responses of ghrelin (plasma or mRNA) to fasting is also found in plasma from goldfish (Unniappan et al., 2004) and Mozambique tilapia (Fox et al., 2009), and *ghrelin* mRNA expression in stomach tissue from sea bass (*Dicentrarchus labrax*) (Terova et al., 2008). In contrast, studies of rainbow trout (Jönsson et al., 2007), Nile tilapia (Riley et al., 2008) and Atlantic cod (Xu and Volkoff, 2009) found no effect of fasting on plasma ghrelin concentration nor the mRNA expression levels. However, small fish has a lower protein to fat ratio than adults with a higher fat deposition (Shearer et al., 1994). This should be kept in mind when interpreting ghrelin studies, as the use of different fish sizes may produce different ghrelin signalling and responses.

Possible species specific ghrelin regulation is also demonstrated in the varied effects reported from ghrelin injection, although precautions must be taken regarding possible effects from using different ghrelin sources (e.g. ghrelin from rainbow trout and goldfish vs. mammalian). Both central and peripheral administration (*intracerebroventricular, icv* and *intraperitoneal, ip*) of ghrelin peptides resulted in depressed feed intake in trout (839 g) held at 11°C (Jönsson et al., 2010), while there was no effect of an *ip* injection in study with small trout (133 g) held at 12-14°C (Jönsson et al., 2007). On the contrary, ghrelin injections (*icv and ip*) in goldfish increased feed intake (Unniappan et al., 2004; Unniappan et al., 2002), which is consistent with the results of central injections in rats (Nakazato et al., 2001). A study with rainbow trout also revealed different responses to ghrelin treatment compared to mammal studies in terms of glucose homeostasis (Polakof et al., 2011), and suggested that even injection method (*icv* or *ip*) and different brain regions can impact the detected ghrelin responses.

The present pre- and postprandial analyses (Paper I) is the first study to detect preprandial plasma ghrelin peaks in Atlantic salmon, and demonstrates that ghrelin has a role in appetite regulation in this species.

Adaptation time and endogenous feeding rhythm

Contradictory results were also found in the present studies in relation to meal time, but also temperature. In short term adapted postsmolt held at optimal and high temperatures (Paper I and III) plasma ghrelin measurements failed to detect significant preprandial peaks despite a wide time sampling range. The ghrelin levels in these trials (Paper I; Fig 1, Paper III; Fig 2) were similar to the lowest levels found in the study with clear preprandial peaks in long term adapted large salmon (Paper I, Fig 6).

Although it is well known that energy metabolism is dependent on temperature, the effect of temperature on ghrelin regulation in fish is not clear. In the 24 h postprandial sampling of postsmolt, a temperature effect (19°C vs. 13°C) on plasma ghrelin was absent (Paper I, Fig 1). However, the work by Nieminen et al. (2003) and Hevrøy et al. (2012) suggest that ghrelin regulation is affected by temperature. Two weeks fasting of burbot (*Lota lota*) reduced the levels of both plasma ghrelin and leptin at a cold temperature (2°C vs. 10°C) (Nieminen et al., 2003), while large salmon displayed reduced plasma ghrelin, and gene expressions of ghrelin and the GH secretagogue receptor when held at a high temperature (19°C vs. 14°C) (Hevrøy et al., 2012).

Hormones related to appetite and energy metabolism, as well as behaviour of swimming and feeding interact with the circadian system in salmon and several other fish species (Boujard and Leatherland, 1992; Feliciano et al., 2011; Kulczykowska and Sánchez Vázquez, 2010; Nisembaum et al., 2012). However, the understanding of how this is regulated and how an internal feeding rhythm is established is still not clear. The circadian system is highly influenced by light regime, feeding frequency and temperature in fish (Boujard and Leatherland, 1992; Nisembaum et al., 2012; Rensing and Ruoff, 2002). In Atlantic salmon, handling can suppress plasma ghrelin in addition to appetite (Pankhurst et al., 2008b) indicating that stress-induced appetite depression can be ghrelin-mediated.

Plasma ghrelin peaks were not detected before meal times in short term adapted postsmolt held at 17°C (Paper III, Fig 2). It is possible that the experimental conditions and adaptation period to feeding regimes (only 11 days) in the high temperature study (Paper III) were

insufficient to develop and establish a regular feeding anticipation regime, as was demonstrated in plasma from long-term adapted large salmon (Paper I, Fig 6).

There were several experimental differences, such as pre- and postprandial sampling methods, temperature, light regimes, fish size and adaptation time to feeding regimes in the three studies of pre- and postprandial plasma ghrelin patterns. When comparing with external studies of ghrelin in fish, there is also a variation in similar experimental factors and reports of contradictory results, as well as different ways of administering ghrelin treatments, sampling frequency and sample tissue. Based on this, it seems the lack of a consistent ghrelin correlation to meal times need not necessarily represent the long-term endocrine regulation of appetite. It is possible that ghrelin under stable long-term conditions produces a stronger feed anticipation and therefore more likely to stimulate growth due to the GH secretagogue effect. From a farming management perspective, this means that deviations to regular feeding times can alter this long-term appetite stimulation. It could also be that the contradictory ghrelin results reported from fish studies, in general, are caused by the variation in experimental conditions and sampling regimes, but also by species differences.

It is clear that ghrelin is a sign of appetite in Atlantic salmon, as demonstrated by rapid plasma peaks before anticipated meals. It is also evident that a sufficient adaptation to feeding regime and a wide sampling range are essential to develop and detect an endogenous feeding rhythm that correlates ghrelin levels to feed anticipation and endocrine appetite regulation. Effects of fish size, high temperature and oxygen on ghrelin regulation in salmon remains to be elucidated. Light manipulation is a common farming practice in optimising fish growth and controlling maturation. Since light has a strong impact on circadian rhythms, this factor should not be ignored in future studies on ghrelin and endocrine appetite regulation. Reports from fish studies are also contradictory and indicate species specific responses of ghrelin. Further studies of ghrelin in Atlantic salmon are therefore critical to reach a better understanding of how ghrelin fits in the cues of appetite regulation in this species.

A growing number of ghrelin studies in humans and mammals keep revealing more functions of ghrelin related to behaviour, gut physiology, cardiovascular performance, circadian rhythms and glucose metabolism (Müller et al., 2015). It is therefore important to increase the knowledge on the multiple functions of ghrelin in fish species as well, since awareness of mechanisms interacting with appetite regulation also increases the understanding of how growth works in fish.

4.3 Low oxygen availability

Decreasing oxygen has a limiting effect on the energy metabolism by directly suppressing aerobic energy synthesis (Brett, 1979) which leads to compromises of both welfare and growth of farmed fish. Low oxygen availability, or hypoxia, is most commonly found at warm water conditions, but naturally occurs at cooler temperatures as well (Chabot and Dutil, 1999). A range as wide as 60-100 % saturated oxygen is commonly reported for sea cages along the Norwegian coast and occasionally as low as 30-50%, which can be caused by periods of feeding and altered swimming densities in aquaculture (Johansson et al., 2006; Oppedal et al., 2011; Vigen, 2008).

It is generally believed that depressed growth in fish, including salmon, is caused by a reduced feed intake related to low oxygen conditions (Brett, 1979; Buentello et al., 2000; Carter et al., 2008; Chabot and Claireaux, 2008; Foss et al., 2002; Pichavant et al., 2001; Thetmeyer et al., 1999; Tran-Duy et al., 2008). This thesis aimed at finding out whether other mechanisms for growth regulation in Atlantic salmon were altered under hypoxia.

Low oxygen (LO) treatment resulted in a significantly lower feed intake and a depressed growth of large salmon held at 12°C and postsmolt at 17°C, compared to salmon held under normoxia (high oxygen, HO) (Paper II; Table 3, Paper III; Table 2). This is in line with several reported studies with fish (Bergheim et al., 2002; Buentello et al., 2000; Carter et al., 2008; Dam and Pauly, 1995; Davis, 1975; Evans, 2007; Glencross, 2009; Mallekh and Lagardère, 2002; Pichavant et al., 2001; Saravanan et al., 2013b; Thetmeyer et al., 1999).

A study with postsmolt clearly shows how growth and feed intake are strongly correlated to water oxygen saturation level ranging from 50 to 100 % (Bergheim et al., 2002) (Fig 4.1).

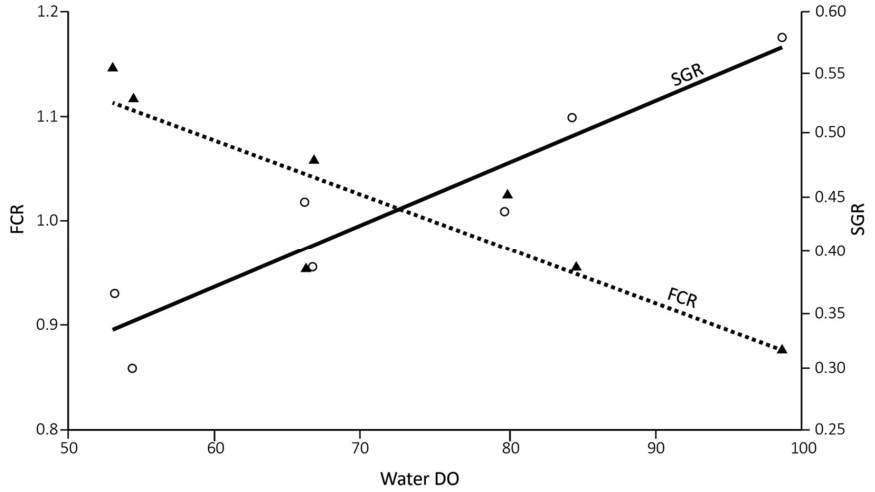

Figure 4.1. Feed utilisation (FCR) and growth (SGR) in postsmolt Atlantic salmon exposed to four levels of DO concentrations at 8-9°C sea water. FCR y= -0.0051x + 1.3881, R^2= 0.7795. SGR y= 0.0052x + 0.0573, R^2= 0.8819. Modified from Bergheim et al. (2002).

To find out if other growth regulating mechanisms are affected under low oxygen, the effect from the feed intake needed to be separated from the low oxygen effect by applying a pair feeding technique (Paper II). This revealed that when large salmon were pair fed with the same amount of feed eaten by salmon groups under low oxygen, it resulted in a better growth when given under normoxic conditions (HO), at the same temperature (12°C) (Paper II, Table 3). This suggests that there must be other mechanisms as well as the reduced feed intake affecting growth regulation in salmon under low oxygen conditions.

Significant effects from pair feeding were detected for high energy diet groups (HE), while low energy groups (LE) only showed the same tendency ($p > 0.05$). Effects of DE under hypoxia and high temperature will be discussed in the next chapter (4.4).

The LO treatment at 12°C (Paper II) also resulted in increased plasma ghrelin levels compared to the HO groups across DE level at 4 h postprandially. This indicates that salmon appetite is not suppressed under low oxygen availability, but rather up-regulated (Paper II, Fig 2). From this, it is safe to say that DO can alter appetite signalling. However, it is not

possible to conclude whether this up-regulation under LO conditions is consistent postprandially or only valid for this specific single time point. This makes it an interesting result to follow up in future postprandial studies on salmon appetite under low dissolved oxygen conditions.

Fish possess a range of adaptation mechanisms to maintain oxygen supply under hypoxic conditions including physiological (oxyregulating and oxyconforming), morphological and behavioral changes (Chapman and McKenzie, 2009). Analyses of blood were therefore conducted as an attempt to detect any adjustments in maintaining oxygen supply to the metabolism, i.e. oxyregulating mechanisms.

After the four month treatment, the blood analyses reflected a disrupted homeostasis in large salmon treated with low oxygen compared to normoxic groups, all at the same temperature (12°C) (Paper II, Table 3). A clear oxyregulating adaptation was demonstrated by increased levels of blood haemoglobin in low oxygen treated salmon, which is one of the most common mechanisms for vertebrates to support a sufficient oxygen supply when available oxygen decreases (Nilsson and Randall, 2010). This was supported by a reduced pH, a documented reaction to an increased anaerobic metabolism in fish exposed to hypoxia (Nilsson and Randall, 2010), which reduces the bloods affinity to oxygen and releases more oxygen into the tissues (Jensen et al., 1998). An elevation in Cl^- concentration indicated an osmotic imbalance from the low oxygen treatment. Since the blood responses were similar between pair-fed groups and fish fed to satiety under normoxia, the detected differences to low oxygen groups can be ascribed to water oxygen level rather than differences in feed intake.

However, other oxyregulating mechanisms like increased mean cell volume (MCV), red blood cell count (RBC) or haematocrit (Hct) (Perry and Gilmour, 2010) were not significantly affected by low water oxygen conditions (Paper II, Table 3). The results confirm that salmon can oxyregulate, and apparently a few oxyregulating mechanisms were sufficient to maintain aerobic scope (AS, Fig 1.4) for salmon under the fluctuating oxygen treatment at 12°C. Whether this is due to the daily episodes of normoxia and full AS, or that

the DO drop down to 57-63% oxygen saturation represents a mild hypoxia for large Atlantic salmon at 12°C, requires further experiments to conclude on. If the low oxygen treatment was severe one would expect the AS to approach or go below critical oxygen levels (P_{crit}, Fig 1.4) which shifts the metabolic balance from oxyregulating to oxyxonforming dependency, of which was not part of this study. However, the reduction in feed intake under hypoxia may be interpreted as a down-regulation of metabolic rate. This is explained by the fact that a reduced feed intake is perceived as consequence of the oxyconforming strategy to lower the need for oxygen and energy (Nilsson and Randall, 2010; Perry et al., 2009).

Although a small selection of blood parameters were analysed in the present study (Paper II, Table 3), they support that depressed growth under hypoxia is partly due to a combination of increased metabolic costs and a reduced feed intake, as shown by the pair feeding technique.

Maximum growth of fish requires a high feed intake and a certain level of available oxygen. The oxygen level which allows for a maximum feed intake (DO_{maxFI}) has recently been described for salmon postsmolt in relation to a wide temperature range (Remen et al., 2016) (Fig 4.2). DO_{maxFI} was determined as the threshold DO below which feed intake started to decline. The work by Remen et al. (2016) showed clearly how oxygen requirement increase with increasing temperature, which is consistent with literature. Further, the DO_{maxFI} were as low as 42% up to 76% saturation for 11°C to 19°C, and feed intake started to decline below these break points. This is also in line with Remen et al. (2012), where DO_{maxFI} was found at 70% saturation for postsmolt at 16°C.

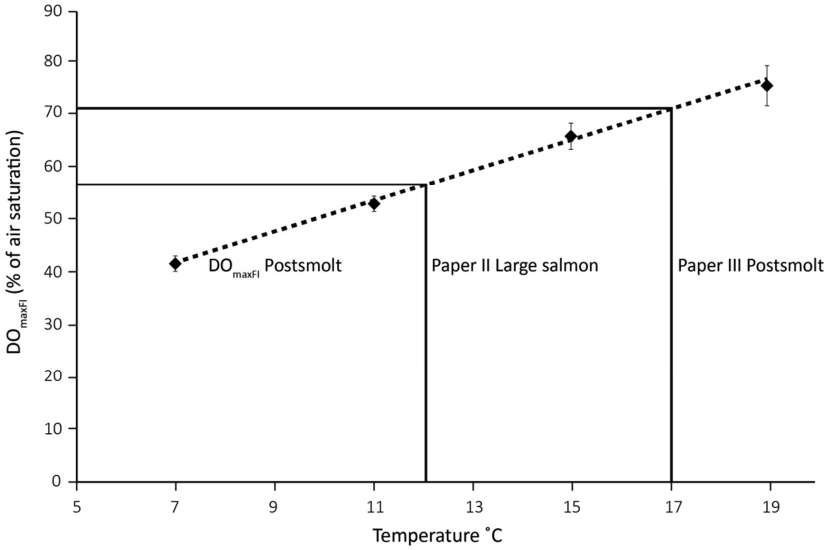

Figure 4.2. The effect of temperature on the minimum DO required for maximal feed intake (DO_{maxFI}, % of air saturation, SE of estimate, dashed line) of Atlantic salmon postsmolt kept at different temperatures. DO_{maxFI} was determined as the threshold DO below which feed intake started to decline. The DFImax represents mean feed intake on days with DO levels higher than the DO_{maxFI}. DO_{maxFI} y= 2.8675T + 21.848, R^2= 0.997. Solid lines indicate treatments in Paper II and III. Modified from Remen et al. (2016).

When adding the experimental temperatures (12°C and 17°C) from the low oxygen trials into the equation in Fig 4.2 (Remen et al., 2016), the oxygen (DO_{maxFI}) is given as above approximately 55% (solid line, Paper II) and 70% (solid line, Paper III) to allow a maximum feed intake. The applied low oxygen treatment varied around 57-63% when oxygen was low at 12°C (Paper II, Fig 1), but was stable and close to 70% at 17°C (Paper III, Fig 1). As feed intake and growth were both reduced by the low oxygen treatments in these two studies, the results do not seem to correlate that well with the curve in Fig 4.2 (Remen et al., 2016). This is just an indication, as a direct comparison of these studies is not possible due to differences in experimental factors, like fish size, temperature and oxygen regimes.

An inconsistency with previous findings on DO needed to maximise growth in salmon (Bergheim et al., 2002; Bergheim et al., 2006; Hosfeld et al., 2008) is also mentioned by Remen et al. (2016). Several reasons can explain this inconsistency and possibly alter the

DO_{maxFI} model (Fig 4.2). Feed intake and oxygen consumption are also influenced by diet composition (like high or low protein or fat and fat source) (Saravanan et al., 2013c) and a dietary amino acid imbalance can increase the oxygen consumption (Saravanan et al., 2013b), or feed quality in general. There is also a genetic variation in tolerance of Atlantic salmon to temperature and hypoxia (Anttila et al., 2013; Barnes et al., 2011). Under fluctuating hypoxia, salmon adjusted by eating more during the normoxic periods and daily feed intake and growth potential were thereby maximized (Remen et al., 2012). Even a salinity change is found to impact oxygen consumption in fish (Altinok and Grizzle, 2003).

Fish size is a factor expected to have a significant effect on the DO_{maxFI} model (Fig 4.2) and is probably why the present results from the hypoxia trial at 12°C did not fit the model, which is based on postsmolt measurements (Remen et al., 2016). Larger fish differ from smaller specimens by having a lower temperature tolerance, lower metabolic rate, higher oxygen requirement and a lower oxygen consumption capacity due to a decreased gill surface area relative to body weight. The oxygen level allowing a maximum feed intake is therefore likely to be different for large fish than small, possibly higher. This is consistent for large salmon (> 1.7-1.9 kg) found to require 82% oxygen saturation to maximize growth at 15°C (Bergheim et al., 2006), and salmon (600-800 g) showing a significantly poorer growth and FCR even at 85% saturation compared to a 100% treatment at a cold temperature (8-9°C) (Bergheim et al., 2002). The result of these two studies with large salmon would both show a requirement above the DO_{maxFI} model (Fig 4.2) by Remen et al. (2016). In a study with barramundi, the effect of fish size was even stronger on oxygen consumption (mg O_2 h^{-1}) than that from water temperature with small fish consuming less than larger individuals (Glencross and Felsing, 2006).

In the study by Remen et al. (2016) it was also concluded that 16.3°C is an optimal temperature for growth of postsmolt. This is high compared to other studies on optimal temperature for growth of the same size (12.8-14°C) (Coutant, 1977; Elliott and Elliott, 2010; Elliott and Hurley, 1997; Forseth et al., 2001; Handeland et al., 2008; Koskela et al., 1997; Peterson and Martin-Robichaud, 1989), but also above the optimal range reported for adult salmon (13°C) (Hevrøy et al., 2013). However, the low oxygen study at 17°C was

conducted with postsmolt (Paper III), and these results do not correlate well with DO_{maxFI} model (Fig 4.2), as feed intake was significantly reduced at 70% compared to 100 % saturation. The study in Paper III was recognized by relatively high mortality, short adaptation time to experimental conditions and inconsistent endocrine results, indicating suboptimal experimental conditions. Also other studies with pre- and postsmolt deviate from the DO_{maxFI} model, but for unknown reasons. As any factor causing stress, such as low oxygen availability, high temperature or handling, creates increased metabolic costs, which is likely to alter the DO_{maxFI} found by Remen et al. (2016).

When comparing performance by feed intake, SGR and FCR with the postsmolt trial at 13°C and 19°C (Paper I) and other studies (Carter et al., 2008; Handeland et al., 2008; Remen et al., 2012), it seems the postsmolt behind the DO_{maxFI} model (Fig 4.2) is underperforming (Remen et al., 2016). For this kind of model to predict and plan good appetite, growth and welfare under changing temperature and oxygen, the data behind should represent high performing and healthy fish, which after all is the main goal of producing fish. Although the present low oxygen studies (Paper II and III) were not performed under the same experimental conditions or parameters as Remen et al. (2016), the simple comparison indicate that it is also possible to strengthen the DO_{maxFI} model by relating it to size of salmon.

From the two studies on low oxygen availability it is clear that oxygen has a strong negative influence on feed intake and growth of Atlantic salmon at both optimal and sub optimally high temperatures (Paper II and III). Under low and fluctuating saturation levels, the ability of salmon to adjust to hypoxia was illustrated by changes in blood parameters facilitating oxygen uptake and supply (Paper II). Analyses of blood can therefore be useful indicators of metabolic status of Atlantic salmon under changing and suboptimal environmental conditions.

Other mechanisms important to appetite and growth are also influenced by reduced oxygen availability, such as feeding, absorption and digestion of ingested nutrients. In terms of the nutrient utilisation, both protein and energy retention efficiencies were reduced under low

oxygen conditions compared to normoxic groups, but only at 12°C (Paper II, data not shown before). At 17°C, there was no significant effect from LO on retention efficiencies (Paper III, Table 2). This can be explained by accelerated metabolic rates at high temperatures, which means that the energy demand, i.e. oxygen is also increased. A metabolic restraint by high temperature would affect both oxygen treatments in Paper III, possibly overriding the effect from oxygen, hence the lack of significant effect from oxygen at 17°C on retention efficiencies. In hindsight, the study at 17°C lacked control treatments at an optimal temperature (Paper III), which would have made comparison of high temperature effects more correct, but the study at 12°C still serves as a suitable base to interpret the results from 17°C.

Absorption and digestion of food is oxygen demanding, which is demonstrated by an increased oxygen consumption postprandially in Tilapia (Saravanan et al., 2013c). Oxygen consumption was not recorded in any studies in this thesis, but indirectly an increased postprandial oxygen use is reflected in the oxygen profiles of tank water from the hypoxia study at 12°C (Paper II, Fig 1). More knowledge on the mechanisms of pre- and postprandial oxygen requirement could increase the understanding of growth regulation and is an interesting topic for future studies. This might be of extra value to the growing business of salmon smolt production in recirculation aquaculture systems (RAS), as oxygenation could be adjusted according to feeding operations. Postprandial oxygen requirement is also an interesting area for possible diet effects, as raw materials differ in degree of digestibility which is likely to impact the metabolic energy budget.

Another important factor to growth regulation in farmed salmon, is exposure to natural temperature fluctuations, which cannot be avoided by any easy practical means. Present high temperature studies (Paper I and III) were short (about 30 days), while in a farming situation this can last for months and at different layers of the water column. How long-term temperature fluctuations above optimal temperatures affect feed utilisation and growth, and if that can be influenced by dietary factors, are interesting questions for new research and future salmon farming.

In a farming situation, the present findings mean that large salmon are especially vulnerable to hypoxia and that even small drops from 100% saturation may have detrimental effects on welfare and productivity. Adding oxygen to sea cages with salmon under hypoxic periods has proven positive both on a performance and economic perspective (Bergheim et al., 2006) and could be a solution to prevent hypoxia. The current plans in Norway of changing the traditional open sea cage farming into enclosed farming units in the sea (Aale Hægermark, 2013; Kvamme, 2016; Nodland, 2016), is possibly even more promising in terms of maintaining stable oxygen levels as close as possible to 100% saturation.

4.4 Can dietary factors impact performance under hypoxia and high temperature?

Farmed salmon have few possibilities to avoid unfavourable environmental conditions other than migrating within the water column of a cage. Efforts to impact the environmental factors defining water quality in open sea cages are also restricted. One way to make an impact and possibly support welfare and growth of fish under challenging environmental conditions is through feed and feeding changes. The diet provides fish with essential nutrients, but also energy by combustion of ingested nutrients.

Low water oxygen is related with higher energetic costs than under unlimited oxygen availability (Brett, 1979). The energy requirement needed for maximum feed intake and growth is compromised (reduced aerobic scope, Pörtner and Knust (2007)) and the metabolism must make adaptations and reallocate energy expenditure to maintain vital functions. Since oxygen availability regulates energy metabolism and the metabolic rate is controlled by temperature, it was relevant to look at how energy from ingested feed can affect growth regulatory mechanisms under suboptimal low oxygen and high temperature conditions.

Experimental diets were made to differ in digestible energy (DE) by varying oil and starch inclusions, while protein levels were kept similar (iso-nitrogenous). Two different sets of diets with high and low DE (HE and LE) were fed to large salmon at 12°C (Paper II, Table 2) and to postsmolt at 17°C (Paper III, Table 1). Energy from fat was chosen because of its higher energy yield and lower oxygen requirement in metabolisation compared to protein and carbohydrates. The idea behind the present dietary study was to find out if salmon metabolism can be impacted by dietary energy under challenging metabolic conditions.

Feeding HE diets resulted in a significantly improved feed utilisation at both at 12°C and 17°C (Paper II and III), which was reflected in lower FCR and increased energy retention efficiencies (Fig 4.3 a and b).

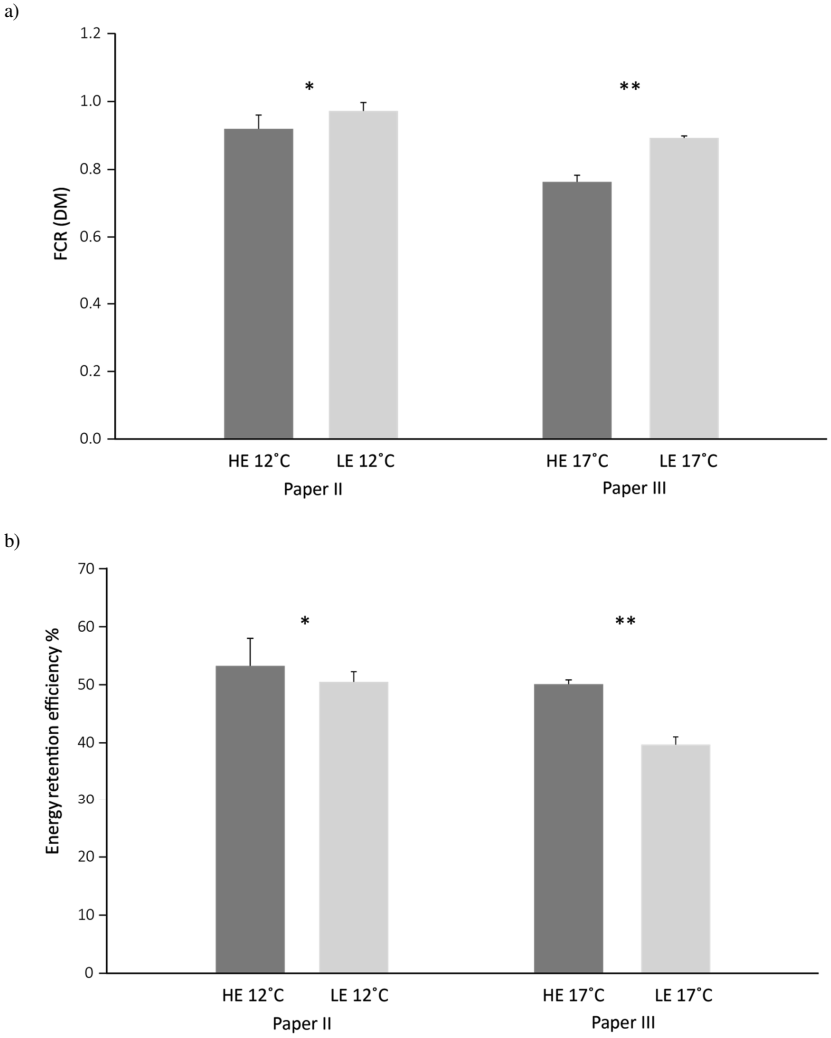

Figure 4.3. Effect of dietary energy (high; HE and low; LE) on FCR (a) and energy retention efficiency % (b) in Atlantic salmon at 12°C and 17°C (Paper II and III). The symbol * and ** indicate significant differences ($p < 0.05$) between two dietary treatments (HE vs LE).

Postsmolt at 17°C also had an increased growth performance from feeding HE diets (Paper III, Table 2), consistent with previous studies on increased feed utilisation in salmon by feeding high fat diets (Bendiksen et al., 2003; Hevrøy et al., 2012). Both the LE diet and

low oxygen treatment resulted in lower growth of postsmolt at 17°C (Paper III, Table 2) (LO effects discussed in Chapter 4.3).

Effect of DE level on growth was limited for large salmon at 12°C with no significant effect on SGR (Paper II, Table 3), but an increased GH1 receptor activity in HE diet groups (Paper II, Fig 3a and 4), whereas no DE effects were found for IGF-1 (plasma and mRNA) (Paper II; Fig 3b). GH1 and IGF-1 both bind to specific binding proteins (GHBP and IGFBP) to make them available for their receptors, and a better understanding of the regulating roles of BPs and receptors could have helped explain the inconsistencies in present endocrine responses.

High energy diets have become standard practice in salmon farming due to the recognised benefits linked to dietary fat such as stimulation of feed intake, growth, feed utilisation, and the sparing effect on dietary proteins. DE levels in commercial diets are commonly in the range of 21-23 MJ kg^{-1} in diets for large salmon (>500 g) and 18-20 MJ kg^{-1} for postsmolt (100-300 g) (pers. comm. Sissel Tjøstheim Susort, Global Product Manager, Skretting). In comparison, present experimental diets named HE were at 21.1 MJ kg^{-1} (large salmon) and 20.0 MJ kg^{-1} (postsmolt), of which both were about 2 MJ kg^{-1} higher than the LE diet. It is possible that the indistinct effects from HE diet found in the present studies (Paper II and III) would have been different by having a higher HE level and a bigger DE difference between HE and LE diets. This is maybe why feeding HE vs LE diets did not produce any differences in growth, feed intake or nutrient utilisation under low oxygen conditions at either temperature. Energy requirement is also size dependent, and so ideally the studies should be with similar fish size for better interpretation of results. The pre- and postprandial analyses in Paper I also point at including more sampling points to detect treatment effects on endocrine growth signals.

Feed intake has also been linked to limitation in oxygen consumption, regardless of water oxygen availability in studies with tilapia and rainbow trout (Saravanan et al., 2013a; Saravanan et al., 2012). The dietary oxygen demand (DOD, mg O_2 kg^{-1} or kJ feed) varies with nutrient composition of the diet where a high energy diet derived from fat gives a

reduced DOD. Studies with tilapia and rainbow trout showed that increased dietary oxygen consumption limited the DE intake and the use of fat as source of energy increased growth significantly (Saravanan et al., 2013a; Saravanan et al., 2012). This means when oxygen is limited (under hypoxia) a high DOD will have a greater negative impact on feed intake and metabolism than under high oxygen availability.

It is well known that nutrient imbalanced diets limit appetite and growth performance at normoxia, but less is known about the impact of nutrient composition in fish under hypoxia. By feeding diets low in essential amino acids to rainbow trout, the feed intake was significantly reduced under both hypoxic and normoxic conditions compared with a balanced diet, and the difference was largest under normoxia (Saravanan et al., 2013b). Not only did an amino acid imbalanced diet require more oxygen per digestible protein compared to a balanced diet, but trout also consumed more oxygen per digestible protein when fed the imbalanced diet under hypoxia compared to normoxia (Saravanan et al., 2013b). The impact from nutrient composition on DOD and feed intake underlines the importance of formulating diets according to the specific nutrient requirement of a species and size. As energy metabolism and feed intake is affected by both DOD and oxygen availability, it also suggests a need to redefine well established nutritional criteria in relation to critical environmental conditions.

The present findings (Paper II and III) are also in support of using dietary energy from fat when the energy metabolism is challenged by hypoxia across temperatures. However, exact requirement for dietary DE at varying level of oxygen and temperature for salmon still remains to be elucidated. The thesis by Saravanan (2013) indicate that feed intake is not all about digestible energy, but also how much oxygen is required to make use of the energy; the DOD. This area of possible dietary impact makes it interesting to investigate further in farmed salmon, as DOD could be a critical factor for feed intake under low oxygen conditions.

Another dietary factor of potential interest for the salmon energy metabolism under challenging conditions, is type of raw materials and their combinations. Raw materials are

commonly combined at various inclusion levels to meet nutritional requirement of digestible protein and energy, essential amino acids and fatty acids, and at a certain production cost. Oils and protein meals are derived from a wide range of fish and land animal species and from a variety of plant origins, which generally differ in essential fatty acid and amino acids profiles. These differences require different metabolic costs to metabolise for growth and energy. So even though essential nutrient requirement is met, the DOD will depend on the raw material combination as this varies the balance between essential and non-essential nutrients. Future studies on this topic should consider DOD in diet formulation and find out how this could possibly affect feed intake and growth regulation in salmon under environmentally challenging conditions.

Stress responses and diseases are also physiologically challenging for the energy metabolism and several nutrients are recognized as having an impact the immune system in fish (Pohlenz and Gatlin, 2014). This makes it relevant to look into how DE and DOD possibly could affect survival and growth regulation in fish with a poor health condition, like recovering from infectious diseases.

Although the detected effects from feeding HE diets on salmon performance were limited to stimulation of growth regulation and feed utilisation (Paper II and III), it shows that dietary energy has a potential to alter salmon metabolism under limiting oxygen conditions across temperatures. However, the role of DE in salmon growth regulation still needs to be further described and should be followed up with a systematic approach over a wide range of temperatures and oxygen levels in relation to fish size and oxygen consumption. Fish size is an important factor to consider due to the differences in protein and fat deposition between small and large salmon, with a relative higher protein than fat growth in small fish compared to large salmon (Shearer et al., 1994). Fish of different size can also differ in energy status (fat storage), which can impact short- and long-term feed intake regulation. This needs to be clarified in future studies on digestible energy source (protein, fat or starch) and feed intake regulation.

5. Conclusions

The current PhD work demonstrates how changes in temperature, water oxygen and dietary energy concentration can impact appetite and growth regulation in sea water adapted Atlantic salmon.

The following conclusions can be drawn from the experimental results of this thesis:

Ghrelin: a "hunger hormone" also in Atlantic salmon

Ghrelin signals feed anticipation in salmon, consistent with mammalian findings.

- Plasma ghrelin concentration showed clear preprandial peaks in large salmon, after long term feeding, adapted at 12°C.
- A sufficient adaptation to feeding regime and a wide sampling range are essential to develop and detect an endogenous feeding rhythm that correlates ghrelin levels to feed anticipation and endocrine appetite regulation in salmon.

Pre- and postprandial nutrient dynamics and growth regulation

A high water temperature represents a clear diminished growth potential for salmon, and was reflected in both FAA and endocrine growth dynamics.

Reduced aerobic scope under LO conditions

Growth depression in salmon following low oxygen conditions appears to be a combined effect of limited growth regulation and increased metabolic costs, and not just by a lower feed intake, as often assumed.

- Atlantic salmon can adapt, to a certain extent, to long term low oxygen availability by various oxyregulating mechanisms.
- Increased metabolic costs under LO, i.e. limitation in aerobic scope, was also indicated by impairment of functions important for growth, such as reduced SGR and feed intake, at both 12°C and 17°C compared to HO groups.

- Endocrine analyses showed alterations in ghrelin and GH-IGF factors by high temperature and low oxygen availability.

High energy diets

High digestible energy diets can support growth regulation, feed utilisation and the energy metabolism when fed to salmon under hypoxia and high temperature.

- Salmon fed HE diets had increased SGR and feed utilisation at 12°C and 17°C, and increased GH1 receptor activity at 12°C, regardless of DO level.
- DE concentration also caused alterations in survival, oxyregulation and osmoregulation under LO and high temperature.

6. Future perspectives

Feed anticipation by way of preprandial plasma ghrelin peaks represents baseline knowledge for salmon appetite regulation. A high feed intake is critical for maximising growth, and studies should continue focusing on ghrelin signalling and interactions with high temperature and hypoxia. Present ghrelin peaks were detected after long-term adaptation to feeding regimes and experimental conditions. This indicates that adaptation is important for developing an endogenous feeding rhythm that reflects appetite signalling in salmon. Adaptation time to feeding regime should therefore be part of future studies on ghrelin as an appetite stimulant in fish.

Little data exist relating environment to endocrine growth regulation in salmon, and this thesis studied selected key factors belonging to the GH-IGF system. A wider approach, i.e. including factors taking part in each of the endocrine steps, leading to growth stimulation, such as binding proteins, receptor activity and ghrelin, could identify interesting knowledge gaps.

Present investigations indicate size and species specific effects on endocrine appetite and growth factors, and should be stressed in future studies.

The results show that dietary energy concentration can make an impact on appetite and growth regulating factors, including energy metabolism. Raw materials and combinations of these do not just provide digestible energy, but making use of the energy requires oxygen. This indicates potential diet effects on aerobic scope by looking into specific raw materials and how they impact the metabolic energy budget.

New studies of DE effects on feed intake regulation should also investigate differences in fish size and short- and long-term effects, due to the difference in protein and fat deposition in small vs. large fish, and impact of nutrient status.

References

Aale Hægermark, W., 2013. Mer kontroll i lukkede oppdrettsanlegg. in: Kristiansen, N. (Ed.). Forskning.no.

Aas-Hansen, Ø., 2010. Ny teknologi for overvåking av oppdrettsmiljø og fiskevelferd i oppdrettsmerder: sluttrapport for FHF-prosjekt 900085. Nofima marin, Tromsø.

Ágústsson, T., Björnsson, B.T., 2000. Growth hormone inhibits growth hormone secretion from the rainbow trout pituitary *in vitro*. Comparative Biochemistry and Physiology. Part C: Comparative Pharmacology and Toxicology. 126, 299-303.

Ágústsson, T., Ebbesson, L.O.E., Björnsson, B.T., 2000. Dopaminergic innervation of the rainbow trout pituitary and stimulatory effect of dopamine on growth hormone secretion *in vitro*. Comparative Biochemistry and Physiology, Part A. 127, 355-364.

Altinok, I., Grizzle, J.M., 2003. Effects of Low Salinities on Oxygen Consumption of Selected Euryhaline and Stenohaline Freshwater Fish. J. World Aquacult. Soc. 34, 113-117.

Anttila, K., Dhillon, R.S., Boulding, E.G., Farrell, A.P., Glebe, B.D., Elliott, J.A.K., Wolters, W.R., Schulte, P.M., 2013. Variation in temperature tolerance among families of Atlantic salmon (*Salmo salar*) is associated with hypoxia tolerance, ventricle size and myoglobin level. J. Exp. Biol. 216, 1183-1190.

Attard, M., Thompson, M., Kelly, R., Locatelli, A., 2012. Tamar estuary monitoring report. Natural Resource Management in Northern Tasmania, http://www.nrmnorth.org.au/, pp. 96.

Ayson, F.G., de Jesus-Ayson, E.G.T., Takemura, A., 2007. mRNA expression patterns for GH, PRL, SL, IGF-I and IGF-II during altered feeding status in rabbitfish, *Siganus guttatus*. Gen. Comp. Endocrinol. 150, 196-204.

Bagnasco, M., Kalra, P.S., Kalra, S.P., 2002. Ghrelin and leptin pulse discharge in fed and fasted rats. Endocrinology. 143, 726.

Barnes, R., King, H., Carter, C.G., 2011. Hypoxia tolerance and oxygen regulation in Atlantic salmon, *Salmo salar* from a Tasmanian population. Aquaculture. 318, 397-401.

Barrows, F.T., Gaylord, T.G., Stone, D.A.J., Smith, C.E., 2007. Effect of protein source and nutrient density on growth efficiency, histology and plasma amino acid concentration of rainbow trout (*Oncorhynchus mykiss* Walbaum). Aquacult. Res. 38, 1747-1758.

Barton, B.A., 2002. Stress in Fishes: A Diversity of Responses with Particular Reference to Changes in Circulating Corticosteroids 1. Integr. Comp. Biol. 42, 517-525.

Baumann, G., Amburn, K., Shaw, M.A., 1988. Circulating growth hormone (GH)-binding protein complex: a major constituent of plasma GH in man. Endocrinology. 122, 976-984.

Beckman, B.R., 2011. Perspectives on concordant and discordant relations between insulin-like growth factor 1 (IGF1) and growth in fishes. Gen. Comp. Endocrinol. 170, 233-252.

Beckman, B.R., Larsen, D.A., Moriyama, S., Lee-Pawlak, B., Dickhoff, W.W., 1998. Insulin-like growth factor-I and environmental modulation of growth during smoltification of spring chinook salmon (*Oncorhynchus tshawystscha*). Gen Comp Endocrinol. 109, 325-335.

Beckman, B.R., Shimizu, M., Gadberry, B.A., Parkins, P.J., Cooper, K.A., 2004. The effect of temperature change on the relations among plasma IGF-I, 41-kDa IGFBP, and growth rate in postsmolt coho salmon. Aquaculture. 241, 601-619.

Bendiksen, E.Å., Berg, O.K., Jobling, M., Arnesen, A.M., Måsøval, K., 2003. Digestibility, growth and nutrient utilisation of Atlantic salmon parr (*Salmo salar* L.) in relation to temperature, feed fat content and oil source. Aquaculture. 224, 283-299.

Bergheim, A., Gausen, M., Næss, A., Fjermedal, A.B., Hølland, P.M., Molversmyr, Å., 2002. Effects of oxygen deficit on post-smolt Atlantic salmon. Trial I., *Report RF- Rogaland Research*. Rogaland Research, pp. 25.

Bergheim, A., Gausen, M., Næss, A., Hølland, P.M., Krogedal, P., Crampton, V., 2006. A newly developed oxygen injection system for cage farms. Aquacult. Eng. 34, 40-46.

Betancor, M., McStay, E., Minghetti, M., Migaud, H., Tocher, D.R., Davie, A., 2014. Daily Rhythms in Expression of Genes of Hepatic Lipid Metabolism in Atlantic Salmon (*Salmo salar* L.). PLoS ONE. 9, 1-9.

Björnsson, B.T., 1997. The biology of salmon growth hormone: from daylight to dominance. Fish Physiol. Biochem. 17, 9-24.

Björnsson, B.T., Johansson, V., Benedet, S., Einarsdottir, I.E., Hildahl, J., Agustsson, T., Jönsson, E., 2002. Growth Hormone Endocrinology of Salmonids: Regulatory Mechanisms and Mode of Action. Fish Physiol. Biochem. 27, 227-242.

Bone, Q., Moore, R.H., 2008. Endocrinology. in: Owen, E. (Ed.), Biology of fishes. Taylor & Francis, New York, pp. 255-288.

Boujard, T., Leatherland, J., 1992. Circadian rhythms and feeding time in fishes. Environ. Biol. Fishes. 35, 109-131.

Brett, J.R., 1979. Environmental factors and growth. in: Hoar, W.S., Randall, C.F., Brett, J.R. (Eds.), Fish Physiology. Academic Press, Inc., London, pp. 599-675.

Buentello, J.A., Gatlin, D.M., Neill, W.H., 2000. Effects of water temperature and dissolved oxygen on daily feed consumption, feed utilization and growth of channel catfish (*Ictalurus punctatus*). Aquaculture. 182, 339-352.

Cameron, C., Gurure, R., Reddy, K., Moccia, R., Leatherland, J., 2002. Correlation between dietary lipid:protein ratios and plasma growth and thyroid hormone levels in juvenile Arctic charr, *Salvelinus alpinus* (Linnaeus). Aquacult. Res. 33, 383-394.

Canosa, L.F., Chang, J.P., Peter, R.E., 2007. Neuroendocrine control of growth hormone in fish. Gen. Comp. Endocrinol. 151, 1-26.

Carter, C.G., He, Z.Y., Houlihan, D.F., McCarthy, I.D., Davidson, I., 1995. Effect of feeding on the tissue free amino acid concentrations in rainbow trout (*Oncorhynchus mykiss* Walbaum). Fish Physiol. Biochem. 14, 153-164.

Carter, C.G., Houlihan, D.F., He, Z.Y., 2000. Changes in tissue free amino acid concentrations in Atlantic salmon, *Salmo salar* L., after consumption of a low ration. Fish Physiol. Biochem. 23, 295-306.

Carter, C.G., Katersky, R.S., Barnes, J.C., Bridle, A.R., Hauler, R.C., 2008. Assessment of fish growth performance under limiting environmental conditions: aquaculture nutrition subprogram, FRDC Final Report. Tasmanian Aquaculture and Fisheries Institute, Tasmania, Australia, pp. 147.

Chabot, D., Claireaux, G., 2008. Environmental hypoxia as a metabolic constraint on fish: The case of Atlantic cod, *Gadus morhua*. Mar. Pollut. Bull. 57, 287-294.

Chabot, D., Dutil, J.-D., 1999. Reduced growth of Atlantic cod in non-lethal hypoxic conditions. J. Fish Biol. 55, 472-491.

Chang, J.P., Wong, A.O.L., 2009. Chapter 4 Growth Hormone Regulation in Fish: A Multifactorial Model with Hypothalamic, Peripheral and Local Autocrine/Paracrine Signals, Fish Physiology. Academic Press, pp. 151-195.

Chapman, L.J., McKenzie, D.J., 2009. Behavioral responses and ecological consequences. in: Richards, J.G., Farrell, A.P., Brauner, C.J. (Eds.), Hypoxia. Academic press, Oxford, pp. 25-78.

Choi, K., Roh, S.G., Hong, Y.H., Shrestha, Y.B., Hishikawa, D., Chen, C., Kojima, M., Kangawa, K., Sasaki, S., 2003. The role of ghrelin and growth hormone secretagogues receptor on rat adipogenesis. Endocrinology. Mar 144, 754-759.

Claireaux, G., Webber, D.M., Lagardère, J.P., Kerr, S.R., 2000. Influence of water temperature and oxygenation on the aerobic metabolic scope of Atlantic cod (*Gadus morhua*). J. Sea Res. 44, 257-265.

Clark, T., Donaldson, M., Pieperhoff, S., Drenner, S., Lotto, A., Cooke, S., Hinch, S., Patterson, D., Farrell, A., 2012. Physiological Benefits of Being Small in a Changing World: Responses of

Coho Salmon (*Oncorhynchus kisutch*) to an Acute Thermal Challenge and a Simulated Capture Event. PLoS ONE. 7.

Clark, T.D., Sandblom, E., Cox, G.K., Hinch, S.G., Farrell, A.P., 2008. Circulatory limits to oxygen supply during an acute temperature increase in the Chinook salmon (*Oncorhynchus tshawytscha*). Am. J. Physiol. Regul. Integr. Comp. Physiol. 295, 1631-1639.

Clemmons, D.R., Busby, W., Clarke, J.B., Parker, A., Duan, C., Nam, T.J., 1998. Modifications of insulin-like growth factor binding proteins and their role in controlling IGF actions. Endocr. J. 45 Suppl, S1.

Company, R., Calduch-Giner, J.A., Kaushik, S., Pérez-Sánchez, J., 1999. Growth performance and adiposity in gilthead sea bream (*Sparus aurata*): risks and benefits of high energy diets. Aquaculture. 171, 279-292.

Coutant, C.C., 1977. Compilation of Temperature Preference Data. Journal of the Fisheries Board of Canada. 34, 739-745.

Cummings, D.E., 2006. Ghrelin and the short- and long-term regulation of appetite and body weight. Physiol Behav. 89, 71-84.

Cummings, D.E., Purnell, J.Q., Frayo, R.S., Schmidova, K., Wisse, B.E., Weigle, D.S., 2001. A Preprandial Rise in Plasma Ghrelin Levels Suggests a Role in Meal Initiation in Humans. Diabetes. 50, 1714-1719.

Cummings, D.E., Shannon, M.H., 2003. Roles for ghrelin in the regulation of appetite and body weight. Archives of surgery (Chicago, Ill. : 1960). 138, 389-396.

Dam, A.A., Pauly, D., 1995. Simulation of the effects of oxygen on food consumption and growth of Nile tilapia, *Oreochromis niloticus* (L.). Aquac Res. 26, 427-440.

Daufresne, M., Lengfellner, K., Sommer, U., 2009. Global warming benefits the small in aquatic ecosystems. Proceedings of the National Academy of Sciences of the United States of America. 106, 12788-12793.

Davis, J.C., 1975. Minimal Dissolved Oxygen Requirements of Aquatic Life with Emphasis on Canadian Species: a Review. Journal of the Fisheries Board of Canada. 32, 2295-2332.

Davis, K.B., McEntire, M., 2011. Influence of reproductive status, sex hormones and temperature on plasma IGF-I concentrations in sunshine bass (*Morone chrysops* × *Morone saxatilis*). Comp Biochem and Physiol, Part A. 158, 13-16.

De-Santis, C., Jerry, D.R., 2007. Candidate growth genes in finfish -- Where should we be looking? Aquaculture. 272, 22-38.

Deane, E.E., Woo, N.Y.S., 2009. Modulation of fish growth hormone levels by salinity, temperature, pollutants and aquaculture related stress: a review. Rev. Fish Biol. Fish. 19, 97-120.

Diaz, R.J., 2001. Overview of hypoxia around the world. J. Environ. Qual. 30, 275-281.

Diaz, R.J., Breitburg, D.L., 2009. The hypoxic environment. in: Jeffrey, G.R., Anthony, P.F., Colin, J.B. (Eds.), Hypoxia. Academic press, Oxford, pp. 2-25.

Duan, C., 1997. The Insulin-like Growth Factor System and Its Biological Actions in Fish. 37, 491-503.

Duan, C., 1998. Nutritional and Developmental Regulation of Insulin-like Growth Factors in Fish. J. Nutr. 128, 306S-314S.

Duan, C., Ren, H., Gao, S., 2010. Insulin-like growth factors (IGFs), IGF receptors, and IGF-binding proteins: Roles in skeletal muscle growth and differentiation. Gen. Comp. Endocrinol. 167, 344-351.

Duan, C., Xu, Q., 2005. Roles of insulin-like growth factor (IGF) binding proteins in regulating IGF actions. Gen. Comp. Endocrinol. 142, 44-52.

Durance, I., Ormerod, S.J., 2007. Climate change effects on upland stream macroinvertebrates over a 25-year period. Global Change Biol. 13, 942-957.

Durance, I., Ormerod, S.J., 2009. Trends in water quality and discharge confound long-term warming effects on river macroinvertebrates. Freshwat. Biol. 54, 388-405.

Dyer, A.R., Barlow, C.G., Bransden, M.P., Carter, C.G., Glencross, B.D., Richardson, N., Thomas, P.M., Williams, K.C., Carragher, J.F., 2004. Correlation of plasma IGF-I concentrations and growth rate in aquacultured finfish: a tool for assessing the potential of new diets. Aquaculture. 236, 583-592.

Einarsdottir, I.E., Gong, N., Jonsson, E., Sundh, H., Hasselberg-Frank, L., Nilsen, T.O., Stefansson, S.O., Sundell, K., Bjornsson, B.T., 2014. Plasma growth hormone-binding protein levels in Atlantic salmon *Salmo salar* during smoltification and seawater transfer, pp. 1279.

Elliott, J.M., 1981. Some aspects of thermal stress on freshwater teleosts. in: Pickering, A.D. (Ed.), Stress and fish. Academic Press, London, pp. 209-245.

Elliott, J.M., Elliott, J.A., 2010. Temperature requirements of Atlantic salmon *Salmo salar*, brown trout *Salmo trutta* and Arctic charr *Salvelinus alpinus*: predicting the effects of climate change. J. Fish Biol. 77, 1793-1817.

Elliott, J.M., Hurley, M.A., 1997. A functional model for maximum growth of Atlantic salmon parr, *Salmo salar*, from two populations in northwest England. Funct. Ecol. 11, 592-603.

Elliott, J.M., Hurley, M.A., 2000a. Daily energy intake and growth of piscivorous brown trout, *Salmo trutta*. Freshwat. Biol. 44, 237-245.

Elliott, J.M., Hurley, M.A., 2000b. Optimum energy intake and gross efficiency of energy conversion for brown trout, *Salmo trutta* , feeding on invertebrates or fish. Freshwat. Biol. 44, 605-615.

Espe, M., Lied, E., Torrissen, K.R., 1993. Changes in plasma and muscle free amino acids in atlantic salmon (*Salmo salar*) during absorption of diets containing different amounts of hydrolysed cod muscle protein. Comp Biochem and Physiol, Part A. 105, 555-562.

Espe, M., Sveier, H., Høgøy, I., Lied, E., 1999. Nutrient absorption and growth of Atlantic salmon (*Salmo salar* L.) fed fish protein concentrate. Aquaculture. 174, 119-137.

Evans, D.O., 2007. Effects of hypoxia on scope-for-activity and power capacity of lake trout (*Salvelinus namaycush*). Can. J. Fish. Aquat. Sci. 64, 345-361.

FAO, 2014a. Global Aquaculture Production 1950-2014.

FAO, 2014b. The State of the World Fisheries and Aquaculture. FAO (Food and Agriculture Organization of the United Nations), Rome, Italy, pp. 223.

Farrell, A.P., Richards, J.G., 2009. Chapter 11 Defining Hypoxia: An Integrative Synthesis of the Responses of Fish to Hypoxia. in: Jeffrey G. Richards, A.P.F., Colin, J.B. (Eds.), Fish Physiology. Academic Press, pp. 487-503.

Feliciano, A., Vivas, Y., de Pedro, N., Delgado, M.J., Velarde, E., Isorna, E., 2011. Feeding Time Synchronizes Clock Gene Rhythmic Expression in Brain and Liver of Goldfish (*Carassius auratus*). J. Biol. Rhythms. 26, 24-33.

Ferguson, R.A., Boutilier, R.G., 1989. Metabolic-membrane coupling in red blood cells of trout: the effects of anoxia and adrenergic stimulation. J Exp Biol. 143, 149.

Ferguson, R.A., Tufts, B.L., Boutilier, R.G., 1989. Energy metabolism in trout red cells: consequences of adrenergic stimulation *in vivo* and *in vitro*. J Exp Biol. 143, 133.

Ficke, A., Myrick, C., Hansen, L., 2007. Potential impacts of global climate change on freshwater fisheries. Rev. Fish Biol. Fish. 17, 581-613.

Forseth, T., Hurley, M.A., Jensen, A.J., Elliott, J.M., 2001. Functional models for growth and food consumption of Atlantic salmon parr, *Salmo salar*, from a Norwegian river. Freshwat. Biol. 46, 173-186.

Foss, A., Evensen, T.H., Øiestad, V., 2002. Effects of hypoxia and hyperoxia on growth and food conversion efficiency in the spotted wolffish *Anarhichas minor* (Olafsen). Aquac Res. 33, 437-444.

Fox, B.K., Breves, J.P., Hirano, T., Grau, E.G., 2009. Effects of short- and long-term fasting on plasma and stomach ghrelin, and the growth hormone/insulin-like growth factor I axis in the tilapia, Oreochromis mossambicus. Domest. Anim. Endocrinol. 37, 1-11.

Franz, A.-C., Faass, O., Köllner, B., Shved, N., Link, K., Casanova, A., Wenger, M., D'Cotta, H., Baroiller, J.-F., Ullrich, O., Reinecke, M., Eppler, E., 2016. Endocrine and Local IGF-I in the Bony Fish Immune System. Biology. 5, 9, 1-13.

Frøiland, E., Murashita, K., Jørgensen, E.H., Kurokawa, T., 2010. Leptin and ghrelin in anadromous Arctic charr: cloning and change in expressions during a seasonal feeding cycle. Gen. Comp. Endo. 165, 136-143.

Fuentes, E.N., Valdés, J.A., Molina, A., Björnsson, B.T., 2013. Regulation of skeletal muscle growth in fish by the growth hormone – Insulin-like growth factor system. Gen. Comp. Endocrinol. 192, 136-148.

Gabillard, J.C., Kamanger, B.B., Montserrat, N., 2006. Coordinated regulation of the GH/IGF system genes during refeeding in rainbow trout (*Oncorhynchus mykiss*). J. Endocrinol. 191, 15-24.

Gabillard, J.C., Rescan, P.Y., Fauconneau, B., Weil, C., Le Bail, P.Y., 2003a. Effect of temperature on gene expression of the Gh/Igf system during embryonic development in rainbow trout (*Oncorhynchus mykiss*). J. Exp. Biol. Part A: Comp. Exp. Biol. 298, 134-142.

Gabillard, J.C., Weil, C., Rescan, P., Navarro, I., Gutiérrez, J., Le Bail, P.Y., 2003b. Effects of environmental temperature on IGF1, IGF2, and IGF type I receptor expression in rainbow trout (*Oncorhynchus mykiss*). Gen Comp Endocrinol. 133, 233-242.

Gabillard, J.C., Weil, C., Rescan, P.Y., Navarro, I., Gutiérrez, J., Le Bail, P.Y., 2003c. Environmental temperature increases plasma GH levels independently of nutritional status in rainbow trout (*Oncorhynchus mykiss*). Gen Comp Endocrinol. 133, 17-26.

Gahete, M.D., Durán-prado, M., Luque, R.M., Martínez-fuentes, A.J., Quintero, A., Gutiérrez-pascual, E., Córdoba-chacón, J., Malagón, M.M., Gracia-navarro, F., Castaño, J.P., 2009. Understanding the Multifactorial Control of Growth Hormone Release by Somatotropes. Ann. N. Y. Acad. Sci. 11631, 137-153.

Giusti, M., Bocca, L., Florio, T., Corsaro, A., Spaziante, R., Schettini, G., Minuto, F., 2002. In vitro effect of human recombinant leptin and expression of leptin receptors on growth hormone-secreting human pituitary adenomas. Clinical Endocrinology. 57, 449-455.

Gjedrem, T., 1993. Miljø for oppdrettsfisk. in: Gjedrem, T. (Ed.), Fiskeoppdrett. A/S Landbruksforlaget, Oslo, Norway, pp. 69-78.

Glencross, B.D., 2009. Reduced water oxygen levels affect maximal feed intake, but not protein or energy utilization efficiency of rainbow trout (*Oncorhynchus mykiss*). Aquacult. Nutr. 15, 1-8.

Glencross, B.D., Felsing, M., 2006. Influence of fish size and water temperature on the metabolic demand for oxygen by barramundi, *Lates calcarifer* (Bloch), in freshwater. Aquacult. Res. 37, 1055-1062.

Gorbman, A., 1995. Olfactory Origins and Evolution of the Brain-Pituitary Endocrine System: Facts and Speculation. Gen. Comp. Endocrinol. 97, 171-178.

Gray, E.S., Kelley, K.M., Law, S., Tsai, R., Young, G., Bern, H.A., 1992. Regulation of hepatic growth hormone receptors in coho salmon (*Oncorhynchus kisutch*). Gen. Comp. Endocrinol. 88, 243-252.

Gräns, A., Jutfelt, F., Sandblom, E., Jönsson, E., Wiklander, K., Seth, H., Olsson, C., Dupont, S., Ortega-Martinez, O., Einarsdottir, I., Björnsson, B.T., Sundell, K., Axelsson, M., 2014. Aerobic scope fails to explain the detrimental effects on growth resulting from warming and elevated CO2 in Atlantic halibut. The Journal of Experimental Biology. 217, 711.

Handeland, S.O., Berge, Å., Björnsson, B.T., Lie, Ø., Stefansson, S.O., 2000. Seawater adaptation by out-of-season Atlantic salmon (*Salmo salar* L.) smolts at different temperatures. Aquaculture. 181, 377-396.

Handeland, S.O., Imsland, A.K., Stefansson, S.O., 2008. The effect of temperature and fish size on growth, feed intake, food conversion efficiency and stomach evacuation rate of Atlantic salmon post-smolts. Aquaculture. 283, 36-42.

Handisyde, N., Telfer, T.C., Ross, L.G., 2016. Vulnerability of aquaculture-related livelihoods to changing climate at the global scale. Fish and Fisheries, 23.

Helm, K.P., Bindoff, N.L., Church, J.A., 2011. Observed decreases in oxygen content of the global ocean. Geophys. Res. Lett. 38, 6 p.

Hevrøy, E.M., Azpeleta, C., Shimizu, M., Lanzén, A., Kaiya, H., Espe, M., Olsvik, P.A., 2011. Effects of short-term starvation on ghrelin, GH-IGF system, and IGF-binding proteins in Atlantic salmon. Fish Physiol. Biochem. 37, 217-232.

Hevrøy, E.M., Hunskår, C., Gelder, S., Shimizu, M., Waagbø, R., Breck, O., Takle, H., Susort, S., Hansen, T., 2013. GH–IGF system regulation of attenuated muscle growth and lipolysis in Atlantic salmon reared at elevated sea temperatures. J Comp Physiol B. 183, 243-259.

Hevrøy, E.M., Tipsmark, C.K., Remø, S.C., Hansen, T., Fukuda, M., Torgersen, T., Vikeså, V., Olsvik, P.A., Waagbø, R., Shimizu, M., 2015. Role of the GH-IGF-1 system in Atlantic salmon and rainbow trout postsmolts at elevated water temperature. Comp Biochem Physiol A Mol Integr Physiol. 188, 127.

Hevrøy, E.M., Waagbø, R., Torstensen, B.E., Takle, H., Stubhaug, I., Jørgensen, S.M., Torgersen, T., Tvenning, L., Susort, S., Breck, O., Hansen, T., 2012. Ghrelin is involved in voluntary anorexia in Atlantic salmon raised at elevated sea temperatures. Gen. Comp. Endocrinol. 175, 118.

Hosfeld, C.D., Engevik, A., Mollan, T., Lunde, T.M., Waagbø, R., Olsen, A.B., Breck, O., Stefansson, S., Fivelstad, S., 2008. Long-term separate and combined effects of environmental hypercapnia and hyperoxia in Atlantic salmon (*Salmo salar* L.) smolts. Aquaculture. 280, 146-153.

Hoskins, L.J., 2011. Characterization of appetite-related neuropeptides in goldfish (*Carassius auratus*) and Atlantic cod (*Gadus morhua*): Daily rhythms in gene expression and effects of long-term administration, Biology. Memorial University of Newfoundland, St John's, Newfoundland and Labrador, Canada, pp. 175.

Hosoda, H., Kojima, M., Kangawa, K., 2006. Biological, physiological, and pharmacological aspects of ghrelin. Journal of pharmacological sciences. 100, 398.

Huising, M.O., Geven, E.J.W., Kruiswijk, C.P., Nabuurs, S.B., Stolte, E.H., Tom Spanings, F.A., Lidy Verburg-van Kemendate, B.M., Flik, G., 2006. Increased Leptin Expression in Common Carp (*Cyprinus carpio*) after Food Intake But Not after Fasting or Feeding to Satiation. Endocrinology. 147, 5786-5797.

Imsland, A.K., Bjornsson, B.T., Gunnarsson, S., Foss, A., Stefansson, S.O., 2007. Temperature and salinity effects on plasma insulin-like growth factor-I concentrations and growth in juvenile turbot (*Scophthalmus maximus*). Aquaculture. 271, 546-552.

Inui, A., 2001. Ghrelin: An orexigenic and somatotrophic signal from the stomach. Nat Rev Neurosci. 2, 551-560.

Inui, A., Asakawa, A., Bowers, C.Y., Mantovani, G., Laviano, A., Meguid, M.M., Fujimiya, M., 2004. Ghrelin, appetite, and gastric motility: the emerging role of the stomach as an endocrine organ. The FASEB Journal. 18, 439-456.

IPCC, 2014. Climate Change 2014: Synthesis Report. Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. in: Core Writing Team, R.K.P.a.L.A.M. (Ed.). IPCC, Geneva, Switzerland, pp. 151.

Jensen, F.B., Fago, A., Weber, R.E., 1998. 1 - Hemoglobin structure and function. in: Perry, S.F., Tufts, B.L. (Eds.), Fish Physiology. Academic press, San Diego, USA, pp. 1-40.

Jobling, M., 1981. Temperature tolerance and the final preferendum—rapid methods for the assessment of optimum growth temperatures. J. Fish Biol. 19, 439-455.

Jobling, M., 1997. Tempeture and growth: modulation of growth rate via temperature change. in: Wood, C.M., McDonald, D.G. (Eds.), Global warming : implications for freshwater and marine fish. Cambridge University Press, Cambridge, pp. 225-254.

Johansson, D., Ruohonen, K., Kiessling, A., Oppedal, F., Stiansen, J.-E., Kelly, M., Juell, J.-E., 2006. Effect of environmental factors on swimming depth preferences of Atlantic salmon (*Salmo salar* L.) and temporal and spatial variations in oxygen levels in sea cages at a fjord site. Aquaculture. 254, 594-605.

Johnsson, J.I., Björnsson, B.T., 1994. Growth hormone increases growth rate, appetite and dominance in juvenile rainbow trout, *Oncorhynchus mykiss*. Anim. Behav. 48, 177-186.

Jonsson, E., Bjornsson, B.T., 2002. Physiological functions of growth hormone in fish with special reference to its influence on behaviour. Fish. Sci. Res. 68, 742-748.

Jönsson, E., 2013. The role of ghrelin in energy balance regulation in fish. Gen Comp Endocrinol. 187, 79-85.

Jönsson, E., Forsman, A., Einarsdottir, I.E., Kaiya, H., Ruohonen, K., Björnsson, B.T., 2007. Plasma ghrelin levels in rainbow trout in response to fasting, feeding and food composition, and effects of ghrelin on voluntary food intake. Comp Biochem Physiol A Mol Integr Physiol. A 147, 1116-1124.

Jönsson, E., Kaiya, H., Björnsson, B.T., 2010. Ghrelin decreases food intake in juvenile rainbow trout (*Oncorhynchus mykiss*) through the central anorexigenic corticotropin-releasing factor system. Gen Comp Endocrinol. 166, 39-46.

Kaiya, H., Kojima, K., Hosoda, H., Moriyama, S., Takahashi, A., Kawauchi, H., Kangawa, K., 2003a. Peptide Purification, Complementary Deoxyribonucleic Acid (DNA) and Genomic DNA Cloning, and Functional Characterisation of Ghrelin in Rainbow Trout. Endocrinology. 144, 5215-5226.

Kaiya, H., Kojima, K., Hosoda, H., Riley, L.G., Hirano, T., Gordon Grau, E., Kangawa, K., 2003b. Identification of tilapia ghrelin and its effects on growth hormone and prolactin release in the tilapia, *Oreochromis mossambicus*. Comp Biochem Physiol, Part B. 135, 421-429.

Kaiya, H., Kojima, K., Hosoda, H., Riley, L.G., Hirano, T., Grau, E.G., Kangawa, K., 2003c. Amidated fish ghrelin: purification, cDNA cloning in the Japanese eel and its biological activity. J. Endocrinol. 176, 415-423.

Kaiya, H., Miyazato, M., Kangawa, K., Peter, R.E., Unniappan, S., 2008. Ghrelin: a multifunctional hormone in non-mammalian vertebrates. Comp Biochem and Physiol, Part A. 149, 109-128.

Kaiya, H., Mori, T., Miyazato, M., Kangawa, K., 2009. Ghrelin receptor (GHS-R)-like receptor and its genomic organisation in rainbow trout, *Oncorhynchus mykiss*. Comp Biochem Physiol A Mol Integr Physiol. 153, 438-450.

Kaiya, H., Small, B.C., Lelania Bilodeau, A., Shepherd, B.S., Kojima, K., Hosoda, H., Kangawa, K., 2005. Purification, cDNA cloning, and characterization of ghrelin in channel catfish, *Ictarulus punctatus*. Gen Comp Endocrinol. 143, 201-210.

Kajimura, S., Aida, K., Duan, C., Steiner, D.F., 2005. Insulin-Like Growth Factor-Binding Protein-1 (IGFBP-1) Mediates Hypoxia-Induced Embryonic Growth and Developmental Retardation. Proceedings of the National Academy of Sciences of the United States of America. 102, 1240-1245.

Kamei, H., Lu, L., Jiao, S., Li, Y., Gyrup, C., Laursen, L.S., Oxvig, C., Zhou, J., Duan, C., Callaerts, P., 2008. Duplication and Diversification of the Hypoxia-Inducible IGFBP-1 Gene in Zebrafish (Two Zebrafish IGFBP-1 Genes). PLoS ONE. 3, e3091.

Kang, K.S., Yahashia, S. and Matsuda, K., 2011. Central and peripheral effects of ghrelin on energy balance, food intake and lipid metabolism in teleost fish. Peptides. 32, 2242–2247.

Kaushik, S.J., Luquet, P., 1979. Influence of dietary amino acid patterns on the free amino acid contents of blood and muscle of rainbow trout (*Salmo gairdnerii* R.), Comparative Biochemistry and Physiology Part B: Comparative Biochemistry, pp. 175-180.

Kling, P., Jönsson, E., Nilsen, T.O., Einarsdottir, I.E., Rønnestad, I., Stefansson, S.O., Björnsson, B.T., 2012. The role of growth hormone in growth, lipid homeostasis, energy utilization and partitioning in rainbow trout: Interactions with leptin, ghrelin and insulin-like growth factor I. Gen. Comp. Endocrinol. 175, 153-162.

Kling, P., Rønnestad, I., Stefansson, S.O., Murashita, K., Kurokawa, T., Björnsson, B.T., 2009. A homologous salmonid leptin radioimmunoassay indicates elevated plasma leptin levels during fasting of rainbow trout. Gen. Comp. Endocrinol. 162, 307-312.

Klok, M.D., Jakobsdottir, S., Drent, M.L., 2007. The role of leptin and ghrelin in the regulation of food intake and body weight in humans: a review. Obesity Reviews. 8, 21-34.

Kojima, K., Takanori, I., Takahiro, S., 2008. Structure of mammalian and nonmammalian ghrelins. in: Litwack, G. (Ed.), Ghrelin. Academic press, pp. 31-46.

Kojima, M., Hosoda, H., Date, Y., Nakazato, M., Matsuo, H., Kangawa, K., 1999. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature. 402, 656-660.

Kojima, M., Kangawa, K., 2005. Ghrelin: structure and function. Physiol. Rev. 85, 495.

Kopchick, J.J., Andry, J.M., 2000. Growth Hormone (GH), GH Receptor, and Signal Transduction. Mol. Genet. Metab. 71, 293-314.

Koskela, J., Pirhonen, J., Jobling, M., 1997. Feed intake, growth rate and body composition of juvenile Baltic salmon exposed to different constant temperatures. Aquacult. Int. 5, 351-360.

Kulczykowska, E., Sánchez Vázquez, F.J., 2010. Neurohormonal regulation of feed intake and response to nutrients in fish: aspects of feeding rhythm and stress. Aquacult. Res. 41, 654-667.

Kullgren, A., Jutfelt, F., Fontanillas, R., Sundell, K., Samuelsson, L., Wiklander, K., Kling, P., Koppe, W., Larsson, D.G., Bjornsson, B.T., Jonsson, E., 2013. The impact of temperature on the metabolome and endocrine metabolic signals in Atlantic salmon (*Salmo salar*). Comp Biochem Physiol A Mol Integr Physiol. 164, 44-53.

Kurokawa, T., Uji, S., Suzuki, T., 2005. Identification of cDNA coding for a homologue to mammalian leptin from pufferfish, *Takifugu rubripes*. Peptides. 26, 745-750.

Kvamme, L., 2016. Skal bruke 600 mill. på lukkede oppdrettsegg. in: Helle, L., Hjertenes, Ø. (Ed.), Havbruk. Sysla, http://sysla.no/2016/02/11/havbruk/skal-bruke-600-mill-pa-lukkede-oppdrettsegg_77094/.

Kwasek, K., Zhang, Y., Dabrowski, K., 2010. Utilization of dipeptide/protein based diets in larval and juvenile Koi carp – post-prandial free amino acid levels. J. Anim. Physiol. Anim. Nutr. 94, 35-43.

Larsen, B.K., Dalsgaard, J., Pedersen, P.B., 2012. Effects of plant proteins on postprandial, free plasma amino acid concentrations in rainbow trout (*Oncorhynchus mykiss*). Aquaculture. 326–329, 90-98.

Lefevre, S., 2016. Are global warming and ocean acidification conspiring against marine ectotherms? A meta-analysis of the respiratory effects of elevated temperature, high CO2 and their interaction. Conservation Physiology. 4 (1).

Lyndon, A.R., Davidson, I., Houlihan, D.F., 1993. Changes in tissue and plasma free amino acid concentrations after feeding in Atlantic cod. Fish Physiol. Biochem. 10, 365-375.

MacKenzie, D.S., VanPutte, C.M., Leiner, K.A., 1998. Nutrient regulation of endocrine function in fish. Aquaculture. 161, 3-25.

Mallekh, R., Lagardère, J.P., 2002. Effect of temperature and dissolved oxygen concentration on the metabolic rate of the turbot and the relationship between metabolic scope and feeding demand. J. Fish Biol. 60, 1105-1115.

Mannor, D.A., Winer, L.M., Shaw, M.A., Baumann, G., 1991. Plasma Growth Hormone (GH)-Binding Proteins: Effect on GH Binding to Receptors and GH Action*. The Journal of Clinical Endocrinology & Metabolism. 73, 30-34.

Matsuda, K., Miura, T., Kaiya, H., Maruyama, K., Shimakura, S.-I., Uchiyama, M., Kangawa, K., Shioda, S., 2006a. Regulation of food intake by acyl and des-acyl ghrelins in the goldfish. Peptides. 27, 2321-2325.

Matsuda, K., Miura, T., Kaiya, H., Maruyama, K., Uchiyama, M., Kangawa, K., Shioda, S., 2006b. Stimulatory effect of n-octanoylated ghrelin on locomotor activity in the goldfish, *Carassius auratus*. Peptides. 27, 1335-1340.

McCormick, S.D., Shrimpton, J.M., Carey, J.B., O'Dea, M.F., Sloan, K.E., Moriyama, S., Björnsson, B.T., 1998. Repeated acute stress reduces growth rate of Atlantic salmon parr and alters plasma levels of growth hormone, insulin-like growth factor I and cortisol. Aquaculture. 168, 221-235.

Mente, E., Deguara, S., Santos, M.B., Houlihan, D., 2003. White muscle free amino acid concentrations following feeding a maize gluten dietary protein in Atlantic salmon (*Salmo salar* L.). Aquaculture. 225, 133-147.

Miura, T., Maruyama, K., Shimakura, S.-I., Kaiya, H., Uchiyama, M., Kangawa, K., Shioda, S., Matsuda, K., 2006. Neuropeptide Y mediates ghrelin-induced feeding in the goldfish, *Carassius auratus*. Neurosci. Lett. 407, 279-283.

Miura, T., Maruyama, K., Shimakura, S.-I., Kaiya, H., Uchiyama, M., Kangawa, K., Shioda, S., Matsuda, K., 2007. Regulation of food intake in the goldfish by interaction between ghrelin and orexin. Peptides. 28, 1207-1213.

Moen, A.-G.G., Finn, R.N., 2013. Short-term, but not long-term feed restriction causes differential expression of leptins in Atlantic salmon. Gen. Comp. Endocrinol. 183, 83-88.

Moen, A.G.G., Murashita, K., Finn, R.N., 2010. Ontogeny of energy homeostatic pathways via neuroendocrine signaling in Atlantic salmon. Developmental Neurobiology. 70, 649-658.

Mommsen, T.P., 2001. Paradigms of growth in fish. Comp Biochem Physiol, B. 129, 207-219.

Muller, A., Lamberts, S., Hofland, L., Bidlingmaier, M., Strasburger, C.J., Ghigo, E., Lely, A.-J., Janssen, J., Koetsveld, P., 2002. Ghrelin drives GH secretion during fasting in man. European Journal of Endocrinology. 146, 203-207.

Murashita, K., Jordal, A.-E.O., Nilsen, T.O., Stefansson, S.O., Kurokawa, T., Björnsson, B.T., Moen, A.-G.G., Rønnestad, I., 2011. Leptin reduces Atlantic salmon growth through the central pro-opiomelanocortin pathway. Comp Biochem and Physiol, Part A: Mol & Integr Physiol. 158, 79-86.

Murashita, K., Kurokawa, T., Nilsen, T.O., Rønnestad, I., 2009. Ghrelin, cholecystokinin, and peptide YY in Atlantic salmon (*Salmo salar*): Molecular cloning and tissue expression. Gen Comp Endocrinol. 160, 223-235.

Murray, R.W., 1971. 5 Temperature Receptors. in: Hoar, W.S., Randall, D.J. (Eds.), Fish Physiology. Academic Press, pp. 121-133.

Müller, T.D., Nogueiras, R., Andermann, M.L., Andrews, Z.B., Anker, S.D., Argente, J., Batterham, R.L., Benoit, S.C., Bowers, C.Y., Broglio, F., Casanueva, F.F., Amp, Apos, Alessio, D., Depoortere, I., Geliebter, A., Ghigo, E., Cole, P.A., Cowley, M., Cummings, D.E., Dagher, A., Diano, S., Dickson, S.L., Diéguez, C., Granata, R., Grill, H.J., Grove, K., Habegger, K.M., Heppner, K., Heiman, M.L., Holsen, L., Holst, B., Inui, A., Jansson, J.O., Kirchner, H., Korbonits, M., Laferrère, B., Leroux, C.W., Lopez, M., Morin, S., Nakazato, M., Nass, R., Perez-Tilve, D., Pfluger, P.T., Schwartz, T.W., Seeley, R.J., Sleeman, M., Sun, Y., Sussel, L., Tong, J., Thorner, M.O., van Der Lely, A.J., van Der Ploeg, L.H.T., Zigman, J.M., Kojima, M., Kangawa, K., Smith, R.G., Horvath, T., Tschöp, M.H., 2015. Ghrelin. Molecular Metabolism. 4, 437-460.

Nakazato, M., Murakami, N., Date, Y., Kojima, M., Matsuo, H., Kangawa, K., Matsukura, S., 2001. A role for ghrelin in the central regulation of feeding. Nature. 409, 194.

Nieminen, P., Mustonen, A.-M., Hyvärinen, H., 2003. Fasting Reduces Plasma Leptin- and Ghrelin-Immunoreactive Peptide Concentrations of the Burbot (*Lota lota*) at 2°C But Not at 10°C. UniBio Press. 20, 1109-1115.

Nilsson, G.E., 2007. Gill remodeling in fish - a new fashion or an ancient secret? J Exp Biol. 210, 2403-2409.

Nilsson, G.E., 2010. Introduction: why we need oxygen. in: Nilsson, G.E. (Ed.), Respiratory Physiology of Vertebrates. Cambridge University Press, Cambridge, UK, pp. 3-13.

Nilsson, G.E., Randall, D.J., 2010. Adaptations to hypoxia in fishes. in: Nilsson, G.E. (Ed.), Respiratory physoiology of vertebrates. Cambridge University Press, Cambridge, pp. 131-173.

Nilsson, G.E., Östlund-Nilsson, S., 2008. Does size matter for hypoxia tolerance in fish? Biological Reviews. 83, 173-189.

Nisembaum, L.G., Velarde, E., Tinoco, A.B., Azpeleta, C., De Pedro, N., Alonso-Gmez, A.L., Delgado, M.J., Isorna, E., 2012. Light-dark cycle and feeding time differentially entrains the gut molecular clock of the goldfish (*Carassius auratus*). Chronobiol. Int. 29, 665-673.

Nodland, E., 2016. Jeg mener fisken har det best i lukkede anlegg. in: Berge, A. (Ed.), Nyheter, iLaks, http://ilaks.no/jeg-mener-fisken-har-det-best-i-lukkede-anlegg/.

Norin, T., Malte, H., Clark, T.D., 2014. Aerobic scope does not predict the performance of a tropical eurythermal fish at elevated temperatures. The Journal of Experimental Biology. 217, 244.

Ogata, H., 1986. Correlations of essential amino acid patterns between the dietary protein and the blood, hepatopancreas, or skeletal muscle in carp [*Cyprinus carpio*]. Bull. Jap. Soc. Sci. Fish. 52, 307-312.

Oksbjerg, N., Gondret, F., Vestergaard, M., 2004. Basic principles of muscle development and growth in meat-producing mammals as affected by the insulin-like growth factor (IGF) system. Domest. Anim. Endocrinol. 27, 219-240.

Oppedal, F., Dempster, T., Stien, L.H., 2011. Environmental drivers of Atlantic salmon behaviour in sea-cages: A review. Aquaculture. 311, 1-18.

Oppedal, F., Juell, J.-E., Johansson, D., 2007. Thermo- and photoregulatory swimming behaviour of caged Atlantic salmon: Implications for photoperiod management and fish welfare. Aquaculture. 265, 70-81.

Oppedal, F., Juell, J.-E., Taranger, G.L., Hansen, T., 2001. Artificial light and season affects vertical distribution and swimming behaviour of post-smolt Atlantic salmon in sea cages. J. Fish Biol. 58, 1570-1584.

Pankhurst, N.W., King, H.R., Ludke, S.L., 2008a. Relationship between stress, feeding and plasma ghrelin levels in rainbow trout *Oncorhynchus mykiss*. Mar, Freshw, Behav, Phys. 41, 53-64.

Pankhurst, N.W., Ludke, S.L., King, H.R., Peter, R.E., 2008b. The relationship between acute stress, food intake, endocrine status and life history stage in juvenile farmed Atlantic salmon, *Salmo salar*. Aquaculture. 275, 311-318.

Pelis, R.M., McCormick, S.D., 2001. Effects of Growth Hormone and Cortisol on Na+–K+–2Cl− Cotransporter Localization and Abundance in the Gills of Atlantic Salmon. Gen. Comp. Endocrinol. 124, 134-143.

Pérez-Sánchez, J., 2000. The involvement of growth hormone in growth regulation, energy homeostasis and immune function in the gilthead sea bream (*Sparus aurata*): a short review. Fish Physiol. Biochem. 22, 135-144.

Pérez-Sánchez, J., Calduch-Giner, J.A., Mingarro, M., Vega-Rubín de Celis, S., Gómez-Requeni, P., Saera-Vila, A., Astola, A., Valdivia, M.M., 2002. Overview of Fish Growth Hormone Family. New Insights in Genomic Organization and Heterogeneity of Growth Hormone Receptors. Fish Physiol. Biochem. 27, 243-258.

Pérez-Sánchez, J., Le Bail, P.-Y., 1999. Growth hormone axis as marker of nutritional status and growth performance in fish. Aquaculture. 177, 117-128.

Pérez-Sánchez, J., Martí-Palanca, H., Kaushik, S.J., 1995. Ration size and protein intake affect circulating growth hormone concentration, hepatic growth hormone binding and plasma insulin-like growth factor-I immunoreactivity in a marine teleost, the gilthead sea bream (*Sparus aurata*). J Nutr. 125, 546.

Pérez Sánchez, J., Martí-Palanca, H., Le Bail, P.Y., 1994. Homologous growth hormone (GH) binding in gilthead sea bream (*Sparus aurata*). Effect of fasting and refeeding on hepatic GH-binding and plasma somatomedin-like immunoreactivity. J. Fish Biol. 44, 287-301.

Perry, A.L., Low, P.J., Ellis, J.R., Reynolds, J.D., 2005. Climate change and distribution shifts in marine fishes. Science (New York, N.Y.). 308, 1912.

Perry, S.F., Gilmour, K.M., 2010. Oxygen uptake and transport in water breathers. in: Nilsson, G.E. (Ed.), Respiratory physiology of vertebrates. Cambridge university press, Cambridge, pp. 49-94.

Perry, S.F., Jonz, M.G., Gilmour, K.M., 2009. Oxygen Sensing And The Hypoxic Ventilatory Response. in: Richards, J.G., Farrell, A.P., Brauner, C.J. (Eds.), Hypoxia. Academic Press, London, pp. 193-253.

Peterson, R.H., Martin-Robichaud, D.J., 1989. First feeding of Atlantic salmon (Salmo salar L.) fry as influenced by temperature regime. Aquaculture. 78, 35-53.

Picha, M., Turano, M., Beckman, B., Borski, R., 2008. Endocrine Biomarkers of Growth and Applications to Aquaculture: A Minireview of Growth Hormone, Insulin-Like Growth Factor (IGF)-I, and IGF-Binding Proteins as Potential Growth Indicators in Fish. N. Am. J. Aquacult. 70, 196-211.

Pichavant, K., Person-Le-Ruyet, J., Le Bayon, N., Severe, A., Le Roux, A., Boeuf, G., 2001. Comparative effects of long-term hypoxia on growth, feeding and oxygen consumption in juvenile turbot and European sea bass. J. Fish Biol. 59, 875-883.

Pickering, A.D., 1993. Growth and stress in fish production. Aquaculture. 111, 51-63.

Pickering, A.D., Pottinger, T.G., Sumpter, J.P., Carragher, J.F., Le Bail, P.Y., 1991. Effects of acute and chronic stress on the levels of circulating growth hormone in the rainbow trout, *Oncorhynchus mykiss*. Gen. Comp. Endocrinol. 83, 86-93.

Planas, J.V., Méndez, E., Baños, N., Capilla, E., Navarro, I., Gutiérrez, J., 2000. Insulin and IGF-I receptors in trout adipose tissue are physiologically regulated by circulating hormone levels. J. Exp. Biol. 203, 1153-1159.

Pohlenz, C., Gatlin, D., 2014. Interrelationships between fish nutrition and health. Aquaculture. 431, 111.

Polakof, S., Míguez, J.M., Soengas, J.L., 2011. Ghrelin effects on central glucosensing and energy homeostasis-related peptides in rainbow trout. Domest. Anim. Endocrinol. 41, 126-136.

Pörtner, H.O., 2010. Oxygen- and capacity-limitation of thermal tolerance: a matrix for integrating climate-related stressor effects in marine ecosystems. The Journal of Experimental Biology. 213, 881.

Pörtner, H.O., Bennett, A.F., Bozinovic, F., Clarke, A., Lardies, M.A., Lucassen, M., Pelster, B., Schiemer, F., Stillman, J.H., 2006. Trade-offs in thermal adaptation: the need for a molecular to ecological integration. Physiol. Biochem. Zool. 79, 295.

Pörtner, H.O., Farrell, A.P., 2008. Physiology and Climate Change. Science. 322, 690-692.

Pörtner, H.O., Knust, R., 2007. Climate change affects marine fishes through the oxygen limitation of thermal tolerance. Science (New York, N.Y.). 315, 95.

Pörtner, H.O., Peck, M.A., 2010. Climate change effects on fishes and fisheries: towards a cause-and-effect understanding. J. Fish Biol. 77, 1745-1779.

Rabalais, N.N., Turner, R.E., Díaz, R.J., Justic, D., 2009. Global change and eutrophication of coastal waters. ICES J. Mar. Sci. 66, 1528-1537.

Raby, G.D., Casselman, M.T., Cooke, S.J., Hinch, S.G., Farrell, A.P., Clark, T.D., 2016. Aerobic scope increases throughout an ecologically relevant temperature range in coho salmon. J. Exp. Biol. 219, 1922.

Reindl, K.M., Sheridan, M.A., 2012. Peripheral regulation of the growth hormone-insulin-like growth factor system in fish and other vertebrates. Comparative Biochemistry and Physiology, Part A. 163, 231-245.

Reinecke, M., 2006. Insulin-Like Growth Factor I and II in Fish. in: Reinecke, M., Zaccone, G., Kapoor, B. (Eds.), Fish Endocrinology. Science Publishers, Enfield, USA, pp. 87-130.

Reinecke, M., 2010. Influences of the environment on the endocrine and paracrine fish growth hormone–insulin-like growth factor-I system. J. Fish Biol. 76, 1233-1254.

Reinecke, M., Björnsson, B.T., Dickhoff, W.W., McCormick, S.D., Navarro, I., Power, D.M., Gutiérrez, J., 2005. Growth hormone and insulin-like growth factors in fish: Where we are and where to go. Gen. Comp. Endocrinol. 142, 20-24.

Remen, M., Oppedal, F., Torgersen, T., Imsland, A.K., Olsen, R.E., 2012. Effects of cyclic environmental hypoxia on physiology and feed intake of post-smolt Atlantic salmon: Initial responses and acclimation. Aquaculture. 326–329, 148-155.

Remen, M., Sievers, M., Torgersen, T., Oppedal, F., 2016. The oxygen treshold for maximal feed intake of Atlantic salmon poststmolt is highly temperature-dependent. Aquaculture. Accepted, not published yet.

Rensing, L., Ruoff, P., 2002. Temperature effect on entrainment, phase shifting, and amplitude of circadian clocks and its molecular bases. Chronobiol. Int. 19, 807-864.

Ricordel, M.J., Smal, J., Le Bail, P.Y., 1995. Application of a recombinant cichlid growth hormone radioimmunoassay to measure native GH in tilapia (Oreochromis niloticus) bred at different temperatures. Aquat. Living Resour. 8, 153-160.

Riley, L.G., Fox, B.K., Breves, J.P., Kaiya, H., Dorough, C.P., Hirano, T., Grau, E.G., 2008. Absence of effects of short-term fasting on plasma ghrelin and brain expression of ghrelin receptors in the tilapia, Oreochromis mossambicus. Zool. Sci. 25, 821.

Riley, L.G., Fox, B.K., Kaiya, H., Hirano, T., Grau, E.G., 2005. Long-term treatment of ghrelin stimulates feeding, fat deposition, and alters the GH/IGF-I axis in the tilapia, *Oreochromis mossambicus*. Gen Comp Endocrinol. 142, 234-240.

Rogers, N.J., Urbina, M.A., Reardon, E.E., McKenzie, D.J., Wilson, R.W., 2016. A new analysis of hypoxia tolerance in fishes using a database of critical oxygen level (Pcrit). Conservation Physiology. 4, 1-19.

Rotllant, J., Balm, P.H.M., Perez-Sanchez, J., Wendelaar Bonga, S.E., Tort, L., 2001. Pituitary and interrenal function in gilthead sea bream (Sparus aurata L., Teleostei) after handling and confinement stress. Gen. Comp. Endocrinol. 121, 333-342.

Rønnestad, I., Nilsen, T.O., Murashita, K., Angotzi, A.R., Gamst Moen, A.-G., Stefansson, S.O., Kling, P., Thrandur Björnsson, B., Kurokawa, T., 2010. Leptin and leptin receptor genes in Atlantic salmon: Cloning, phylogeny, tissue distribution and expression correlated to long-term feeding status. Gen. Comp. Endocrinol. 168, 55-70.

Saera-Vila, A., Calduch-Giner, J.A., Perez-Sanches, J., 2007. Co-expression of IGFs and GH receptors (GHRs) in gilthead sea bream (Sparus aurata L.): sequence analysis of the GHR-flanking region. J. Endocrinol. 194, 361-372.

Sakamoto, T., McCormick, S.D., 2006. Prolactin and growth hormone in fish osmoregulation. Gen. Comp. Endocrinol. 147, 24-30.

Saladin, R., Vos, P.d., Guerre-Millo, M., Leturque, A., Girard, J., Staels, B., Auwerx, J., 1995. Transient increase in obese gene expression after food intake or insulin administration. Nature. 377, 527-529.

Sandblom, E., Gräns, A., Axelsson, M., Seth, H., 2014. Temperature acclimation rate of aerobic scope and feeding metabolism in fishes: implications in a thermally extreme future. Proceedings. Biological sciences. 281, 20141490.

Saravanan, S., 2013. Feed intake and oxygen consumption in fish, Institute of Animal Science (WIAS). Wageningen University, Wageningen, NL, pp. 164.

Saravanan, S., Geurden, I., Figueiredo-Silva, A.C., Kaushik, S., Verreth, J., Schrama, J.W., 2013a. Voluntary Feed Intake in Rainbow Trout Is Regulated by Diet-Induced Differences in Oxygen Use. J. Nutr. 143, 781-787.

Saravanan, S., Geurden, I., Figueiredo-Silva, A.C., Kaushik, S.J., Haidar, M.N., Verreth, J.A.J., Schrama, J.W., 2012. Control of voluntary feed intake in fish: a role for dietary oxygen demand in Nile tilapia (*Oreochromis niloticus*) fed diets with different macronutrient profiles. Br J Nutr. FirstViewArticle, 1-11.

Saravanan, S., Geurden, I., Figueiredo-Silva, A.C., Nusantoro, S., Kaushik, S., Verreth, J., Schrama, J.W., 2013b. Oxygen Consumption Constrains Food Intake in Fish Fed Diets Varying in Essential Amino Acid Composition. PLoS ONE. 8, e72757.

Saravanan, S., Geurden, I., Kaushik, S., Verreth, J.A.J., Schrama, J.W., 2013c. Effect of dietary macronutrients on within-day variation in feed intake of Nile tilapia. in: Saravanan, S. (Ed.), Feed intake and oxygen consumption in fish, PhD thesis. Wageningen University, Wageningen, The Netherlands, pp. 113-127.

Schlisio, W., Nicolai, B., 1978. Kinetic investigations on the behaviour of free amino acids in the plasma and of two aminotransferases in the liver of rainbow trout (*Salmo gairdnerii* Richardson) after feeding on a synthetic composition containing pure amino acids. Comp Biochem and Physiol, Part B: Comp Biochem. 59, 373-379.

Schmidt-Nielsen, K., 1997a. Energy metabolism. in: Schmidt-Nielsen, K. (Ed.), Animal physiology, adaptation and environment. Cambridge university press, Cambridge, pp. 169-216.

Schmidt-Nielsen, K., 1997b. Hormonal control. in: Schmidt-Nielsen, K. (Ed.), Animal Physiology, adaptation and environment. Cambridge University Press, Cambridge, UK., pp. 497-531.

Segner, H., Eppler, E., Reinecke, M., 2006. The Impact of Environmental Hormonally Active Substances on the Endocrine and Immune Systems of Fish, Fish Endocrinology (2 Vols.). Science Publishers, pp. 809-866.

Shearer, K.D., Åsgård, T., Andorsdöttir, G., Aas, G.H., 1994. Whole body elemental and proximate composition of Atlantic salmon (*Salmo salar*) during the life cycle. J. Fish Biol. 44, 785-797.

Sheridan, M.A., 1986. Effects of thyroxin, cortisol, growth hormone, and prolactin on lipid metabolism of coho salmon, *Oncorhynchus kisutch*, during smoltification. Gen Comp Endocrinol. 64, 220-238.

Shimizu, M., Cooper, K.A., Dickhoff, W.W., Beckman, B.R., 2009. Postprandial changes in plasma growth hormone, insulin, insulin-like growth factor (IGF)-I, and IGF-binding proteins in coho salmon fasted for varying periods. Am J Physiol Regul Integr Comp Physiol. 297, R352-R361.

Soofiani, N.M., Hawkins, A.D., 1982. Energetic costs at different levels of feeding in juvenile cod, *Gadus morhua* L. J. Fish Biol. 21, 577-592.

Stevens, E.D., Sutterlin, A., Cook, T., 1998. Respiratory metabolism and swimming performance in growth hormone transgenic Atlantic salmon. Can. J. Fish. Aquat. Sci. 55, 2028-2035.

Sunde, J., Kiessling, A., Higgs, D., Opstvedt, J., Venturini, G., Rungruangsak-Torrissen, K., 2003. Evaluation of feed protein quality by measuring plasma free amino acids in Atlantic salmon (*Salmo salar* L.) after dorsal aorta cannulation. Aquacult. Nutr. 9, 351-360.

Terova, G., Rimoldi, S., Bernardini, G., Gornati, R., Saroglia, M., 2008. Sea bass ghrelin: Molecular cloning and mRNA quantification during fasting and refeeding. Gen. Comp. Endocrinol. 155, 341-351.

Thetmeyer, H., Waller, U., Black, K.D., Inselmann, S., Rosenthal, H., 1999. Growth of European sea bass (*Dicentrarchus labrax* L.) under hypoxic and oscillating oxygen conditions. Aquaculture. 174, 355-367.

Thorarensen, H., Farrell, A.P., 2011. The biological requirements for post-smolt Atlantic salmon in closed-containment systems. Aquaculture. 312, 1-14.

Torrisen, K.R., Lied, E., Espe, M., 1994. Differences in utilization of dietary proteins with varying degrees of partial pre-hydrolysis in Atlantic salmon (*Salmo salar* L.) with genetically different trypsin isozymes. in: Svasti, J., Rimphanitchayakit, V., Soontaros, S., Tassanakajorn, A., Limpaseni, T., Pongsawasdi, P., Wilairat, P., Sonthayanon, B., Boonjawat, J., Packdibamrung, K., Kamolsiripichaiporn, S. (Eds.), Biopolymers and Isozymes: Structure, Functions and Applications Samakkhisan (dokya) Public Company Limited, Marriot Royal Garden Riverside, Bangkok, Thailand, pp. 432-442.

Tran-Duy, A., Schrama, J.W., van Dam, A.A., Verreth, J.A.J., 2008. Effects of oxygen concentration and body weight on maximum feed intake, growth and hematological parameters of Nile tilapia, *Oreochromis niloticus*. Aquaculture. 275, 152-162.

Tschöp, M., Wawarta, R., Riepl, R., Friedrich, S., Bidlingmaier, M., Landgraf, R., Folwaczny, C., 2001. Post-prandial decrease of circulating human ghrelin levels. J. Endocrinol. Invest. 24, 19-21.

Tschöp, M.H., Smiley, D.L., Heiman, M.L., 2000. Ghrelin induces adiposity in rodents. Nature. 407, 908.

Ueno, H., Yamaguchi, H., Kangawa, K., Nakazato, M., 2005. Ghrelin: a gastric peptide that regulates food intake and energy homeostasis. Reg Pept. 126, 11-19.

Ultsch, G., Jackson, D., Moalli, R., 1981. Metabolic oxygen conformity among lower vertebrates: The toadfish revisited. Journal of comparative physiology. 142, 439-443.

Unniappan, S., Canosa, L.F., Peter, R.E., 2004. Orexigenic actions of ghrelin in goldfish: feeding-induced changes in brain and gut mRNA expression and serum levels, and responses to central and peripheral injections. Neuroendocrinology. 79, 100-108.

Unniappan, S., Lin, X., Cervini, L., Rivier, J., Kaiya, H., Kangawa, K., Peter, R.E., 2002. Goldfish ghrelin: molecular characterization of the complementary deoxyribonucleic acid, partial gene structure and evidence for its stimulatory role in food intake. Endocrinology. 143, 4143.

Unniappan, S., Peter, R.E., 2005. Structure, distribution and physiological functions of ghrelin in fish. Comp Biochem Physiol A Mol Integr Physiol. 140, 396-408.

Valen, R., Jordal, A.E.O., Murashita, K., Rønnestad, I., 2011. Postprandial effects on appetite-related neuropeptide expression in the brain of Atlantic salmon, *Salmo salar*. Gen. Comp. Endocrinol. 171, 359-366.

Valente, L.M.P., Bower, N.I., Johnston, I.A., 2012. Postprandial expression of growth-related genes in Atlantic salmon (Salmo salar L.) juveniles fasted for 1 week and fed a single meal to satiation. Br J Nutr. 108, 2148-2157.

Vera, L.M., Negrini, P., Zagatti, C., Frigato, E., Snchez-Vzquez, F.J., Bertolucci, C., 2013. Light and feeding entrainment of the molecular circadian clock in a marine teleost (*Sparus aurata*). Chronobiol. Int. 30, 649-661.

Verburg-Van Kemenade, B.M.L., Stolte, E.H., Metz, J.R., Chadzinska, M., 2009. Chapter 7 Neuroendocrine–Immune Interactions in Teleost Fish, Fish Physiology. Academic Press, pp. 313-364.

Vigen, J., 2008. Oxygen variation within a seacage, Department of Biology. University of Bergen, Bergen.

Vikeså, V., Nankervis, L., Hevrøy, E.M., 2016. Appetite, metabolism and growth regulation in Atlantic salmon (*Salmo salar L.*) exposed to hypoxia at elevated seawater temperature. Aquacult. Res., 1-16.

Vikeså, V., Nankervis, L., Hevrøy, E.M., 2017. High dietary energy level stimulates growth hormone receptor and feed utilization in large Atlantic salmon (*Salmo salar* L.) under hypoxic conditions. Aquacult. Nutr., 1-11.

Vikeså, V., Nankervis, L., Remø, S.C., Waagbø, R., Hevrøy, E.M., 2015. Pre and postprandial regulation of ghrelin, amino acids and IGF1 in Atlantic salmon (*Salmo salar* L.) at optimal and elevated seawater temperatures. Aquaculture. 438, 159-169.

Volkoff, H., 2016. The Neuroendocrine Regulation of Food Intake in Fish: A Review of Current Knowledge. Frontiers in Neuroscience. 10, 540, 31.

Volkoff, H., Hoskins, L.J., Tuziak, S.M., 2010. Influence of intrinsic signals and environmental cues on the endocrine control of feeding in fish: Potential application in aquaculture. Gen. Comp. Endocrinol. 167, 352-359.

Waters, M., Shang, C., Behncken, S., Tam, S.p., Li, H., Shen, B., Lobie, P., 1999. Growth hormone as a cytokine. Clin. Exp. Pharmacol. Physiol. 26, 760-764.

Wilkinson, R.J., Porter, M., Woolcott, H., Longland, R., Carragher, J.F., 2006. Effects of aquaculture related stressors and nutritional restriction on circulating growth factors (GH, IGF-I and IGF-II) in Atlantic salmon and rainbow trout. Comp Biochem and Physiol, Part A. 145, 214-224.

Won, E.T., Borski, R.J., Ferket, P., Patisaul, H., Sullivan, C., 2013. Cloning and Characterization of Leptin in a Teleost Fish and its Role in Mediating Appetite and Growth, Zoology. North Carolina State University, North Carolina, US, pp. 147.

Wood, A.W., Duan, C., Bern, H.A., 2005. Insulin-Like Growth Factor Signaling in Fish. Int. Rev. Cytol. 243, 215-285.

Xu, M., Volkoff, H., 2009. Molecular characterization of ghrelin and gastrin-releasing peptide in Atlantic cod (*Gadus morhua*): Cloning, localization, developmental profile and role in food intake regulation. Gen Comp Endocrinol. 160, 250-258.

Yada, T., 2007. Growth hormone and fish immune system. Gen. Comp. Endocrinol. 152, 353-358.

Yada, T., 2009. Effects of insulin-like growth factor-1 on non-specific immune functions in rainbow trout. Effects of insulin like growth factor 1 on non specific immune functions in rainbow trout. 26, 338-343.

Yamada, S., Simpson, K.L., Tanaka, Y., Katayama, T., 1981. Plasma amino acid changes in rainbow trout *Salmo gairdneri* force-fed Casein and a corresponding amino acid mixture. Bull. Jap. Soc. Sci. Fish. 47, 1035-1040.

Yamamoto, T., Sugita, T., Furuita, H., 2005. Essential amino acid supplementation to fish meal-based diets with low protein to energy ratios improves the protein utilization in juvenile rainbow trout *Oncorhynchus mykiss*. Aquaculture. 246, 379-391.

Yeung, C.M., Chan, C.B., Woo, N.Y., Cheng, C.H., 2006. Seabream ghrelin: cDNA cloning, genomic organization and promoter studies. J. Endocrinol. 189, 365-379.

Zigman, J.M., Elmquist, J.K., 2003. Minireview: From anorexia to obesity- the yin and yang of body weight control. Endocrinology. 144, 3749.

I

Aquaculture 438 (2015) 159–169

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

Pre and postprandial regulation of ghrelin, amino acids and IGF1 in Atlantic salmon (*Salmo salar* L.) at optimal and elevated seawater temperatures

V. Vikeså [a,b,c,*], L. Nankervis [a], S.C. Remø [b], R. Waagbø [b,c], E.M. Hevrøy [b]

[a] Skretting ARC (Aquaculture Research Centre), P.O. Box 148, 4016 Stavanger, Norway
[b] National Institute of Nutrition & Seafood Research (NIFES), P.O. Box 176 Sentrum, 5804 Bergen, Norway
[c] University of Bergen (UiB), 5020 Bergen, Norway

ARTICLE INFO

Article history:
Received 9 May 2014
Received in revised form 9 December 2014
Accepted 14 December 2014
Available online 13 January 2015

Keywords:
Elevated temperature
Free amino acids
Ghrelin
GH–IGF system

ABSTRACT

Atlantic salmon farmed in seacage facilities are exposed to changing environmental conditions. While growth decline under lower water temperatures are reasonably well understood, the mechanisms behind production decline under high water temperatures are yet to be well elucidated and have been hampered by a lack of data describing pre- and postprandial patterns of endocrine fluctuation. The present research therefore aims to determine whether peak nutrient flux into the blood plasma is the most appropriate time point to investigate the endocrine regulation of growth and appetite under conditions of normal and high temperature, and to investigate the interrelationship between appetite and growth on a pre- and post-prandial time scale. Two experiments are presented which examine ghrelin (GHRL) as an indicator of appetite stimulation, the GH–IGF (growth hormone–insulin-like growth factor) system to describe growth-regulating processes and free amino acids (FAA) to indicate postprandial nutrient influx and link appetite and anabolic processes. Postprandial sampling of plasma and white muscle tissue from short-term adapted postsmolt was conducted at 13 °C and 19 °C at 4, 8, 12, 16, 20 and 24 hours (h). The same samples were taken from long-term adapted big salmon at 12 °C, −4, −2, −1, 0 h pre-prandially and 2, 3, 4 and 6 h post-prandially. While limited relationship between plasma ghrelin concentration and meal times was found for short term adapted postsmolt, clear ghrelin peaks were described for long term adapted salmon prior to the timing of anticipated meals. Possible explanations and consequences to experimental design in this area will be discussed. Postprandial FAA in plasma and white muscle from postsmolt was reduced at 19 °C compared to 13 °C and plasma levels peaked 8 h post-prandially. Muscle *Igf1* mRNA expression levels were consistently higher at 13 °C than 19 °C, with no clear postprandial patters. In contrast, plasma IGF-1 concentration was relatively constant over time at 12 °C and 13 °C, but significantly declined from 20 h postprandially at 19 °C. GH receptor (*ghr1*) mRNA expression in muscle was unaffected by temperature, peaking 4 h post-prandially at both temperatures. This paper describes growth and appetite-regulating processes under conditions of normal and elevated temperature for Atlantic salmon, which is fundamental to our understanding of growth limitations inherent to high water temperature situations.

1. Introduction

Temperature and feed intake are the most prominent determinants of fish growth (Brett, 1979) making these two factors central to our understanding of growth-regulating processes. Most fish are poikilothermic ectotherms, with physiology particularly subject to changes in water temperature. Appetite increases with temperature to a thermal optimum, above which little data exists relating to factors controlling appetite and growth depression. Developments relating to appetite, feed intake, digestive processes and nutrient absorption kinetics are likely to provide important baseline information for strategies to cope with higher summer temperatures in commercial salmon aquaculture. While diurnal temperature fluctuation exists, it is common that commercial salmon farming sites experience periods of several weeks to months where water temperature exceeds 18 °C (Attard et al., 2012), which is well above generally accepted thermal optima for growth (Handeland et al., 2008; Hevrøy et al., 2012; Koskela et al., 1997; Kullgren et al., 2013; Peterson and Martin-Robichaud, 1989). Reduced production due to high water temperatures is expected to become an increasingly relevant problem due to climate change and concomitant seawater temperature elevation. Climate models for 2020 to 2099 predict an average increase of 0.5 to 3.5 °C in the Southern Hemisphere

Abbreviations: GHRL and IGF-1, concentration of the hormones ghrelin and insulin-like growth factor; ghr1 and igf1, gene expressions of the growth hormone receptor and insulin-like growth factor

* Corresponding author at: Skretting ARC, P.O. Box 148, 4016 Stavanger, Norway. Tel.: +47 48003603; fax: +47 51825501.
E-mail address: vibeke.vikesaa@skretting.com (V. Vikeså).

http://dx.doi.org/10.1016/j.aquaculture.2014.12.021
0044-8486/

waters and 0.5 to 7.5 °C in the Northern Hemisphere waters (Solomon et al., 2007). Understanding the implications of elevated temperatures is expected to have increasing relevance to both cultured and wild populations of fish globally. When determining the effects of suboptimal conditions on growth physiology it can be instructive to use endocrine signals to separate growth, appetite and nutrient circulation signals to identify the root causes of declining growth.

Growth is regulated by a range of hormones, with those receiving the most attention belonging to the GH–IGF system; specifically growth hormone (GH1) and insulin like growth factor-I (IGF-1) (Oksbjerg et al., 2004; Reinecke, 2006; Wood et al., 2005). GH1 is released from the anterior pituitary, depending on stimulatory or inhibitory signals from the hypothalamus (the hypothalamus–pituitary axis). The signals from the hypothalamus are influenced by endogenous cues, such as nutritional state and humoral factors, and exogenous cues, such as temperature and photoperiod (Björnsson et al., 2002; Canosa et al., 2007). Under anabolic conditions GH1 is released into the circulatory system and stimulates the synthesis and secretion of IGF-1 from a range of tissues.

Growth and appetite are integrally linked, with appetite providing stimulus to eat and thereby the nutrients required for growth. The peptide hormone ghrelin (GHRL) has received the most recent attention in fish studies as the main hormone responsible for immediate appetite stimulation (Frøiland et al., 2010; Hevrøy et al., 2012; Jönsson, 2013; Matsuda et al., 2006a, 2006b; Miura et al., 2006, 2007; Riley et al., 2005; Unniappan and Peter, 2005) and has a well-documented role in energy balance and promoting appetite for both fish and higher vertebrates (Choi et al., 2003; Cummings et al., 2001). In addition, GHRL can stimulate GH1 release directly (Inui et al., 2004), and is therefore suitable to study the interaction between appetite and growth regulation (Kaiya et al., 2009). Circulating GHRL concentration and *ghrl* mRNA expression are increased during fasting (Inui, 2001; Inui et al., 2004) in order to stimulate feed intake.

Post-digestive absorption of amino acids is thought to promote satiety and stimulate a number of growth promoting processes, including the IGF-1 response (Mommsen, 2001; Planas et al., 2000). Postprandial free amino acid levels (FAA) indicate substrate availability for muscle growth and provide a link between feed intake and anabolic processes. Several investigations have studied postprandial concentrations of FAA in plasma and tissues of Atlantic cod (*Gadus morhua*) (Lyndon et al., 1993), koi carp (*Cyprinus carpio*) (Kwasek et al., 2010; Ogata, 1986), rainbow trout (*Salmo gairdneri* R., *Oncorhynchus mykiss*) (Barrows et al., 2007; Carter et al., 1995; Kaushik and Luquet, 1979; Larsen et al., 2012; Schlisio and Nicolai, 1978; Yamada et al., 1981; Yamamoto et al., 2005) and Atlantic salmon (Carter et al., 2000; Espe et al., 1993; Mente et al., 2003; Sunde et al., 2003) however data are lacking relating amino acid flux to elevated environmental temperature.

Atlantic salmon seem to have higher temperature optima for early stages, with ranges of 15.6–20 °C reported for parr (typically 20–100 g), compared to 12.8–14 °C for postsmolt (typically 50–200 g) (Handeland et al., 2008; Koskela et al., 1997; Peterson and Martin-Robichaud, 1989). Large Atlantic salmon (1.6–2.9 kg) seem to grow optimally at 13 °C, with reduced growth after 45 days exposure to 15 °C, 17 °C and 19 °C (Hevrøy et al., 2013). Long term studies have been conducted at high temperatures up to 19 °C using large Atlantic salmon (2.2 kg) and postsmolt (175 g) (Hevrøy et al., 2012; Kullgren et al., 2013). These studies described, amongst other factors, reduced feed intake, somatic growth performance, feed utilisation and energy stores in fish at 19 °C and 18 °C, compared to fish at 14 °C and 12 °C, respectively. In large salmon these responses could be linked to lower plasma GHRL and reduced energy status in fish groups at 19 °C compared to 14 °C (Hevrøy et al., 2012). Smaller salmon (175 g) at 18 °C did not show differences in plasma GHRL concentration in relation to temperature, but a higher plasma concentration of leptin, a hormone known to inhibit food intake (Kullgren et al., 2013). While Hevrøy et al. (2013) did not find differences in circulating plasma IGF-1 concentration, they described profound local down-regulation of muscle *igf1* mRNA expression at elevated temperatures (15–19 °C) compared to 13 °C.

Common to the vast majority of work in this area is that sampling is done either at an undefined period or at a single, fixed sampling point after a feeding point. This approach assumes that either the endocrine or metabolite factors under investigation are unaffected by the meal time, or that the time point chosen yields the most informative data. The present study is designed to determine whether peak nutrient flux into the blood plasma is the most appropriate time point to investigate the endocrine regulation of growth and appetite under optimal and adverse environmental conditions, and to investigate the interrelationship between appetite and growth on a pre- and post-prandial time scale.

2. Materials and methods

Two experiments are presented in this paper; a postprandial study with short-term meal adapted postsmolt conducted at both normal (13 °C) and elevated temperatures (19 °C) and pre- and postprandial investigations with long-term meal adapted large salmon at 12 °C. The former of these studies was designed to evaluate differences in growth and appetite-related endocrinology under conditions of normal and elevated seawater temperatures in relation to meal timing and circulating amino acid levels after feeding. The latter experiment was optimised based on the former to better capture ghrelin dynamics before and after feeding periods, while also reporting on growth endocrinology in relation to meal time and circulating amino acid levels.

2.1. Experimental design (postsmolt 13 °C and 19 °C)

This study was conducted at Matre Havbruksstasjon (Institute of Marine Research) in Matredal, Norway. Atlantic salmon postsmolt (0+, AquaGen strain) with initial body weights (BW) of 101 ± 2.1 g (SE) were adapted to 13 °C for 3 weeks in 8 tanks (1 m^3) each containing 80 fish. The temperature was then increased by 1 °C/day in four of the eight tanks up to a desired temperature of 19 °C (days 1 to 6). Temperature profiles were then kept at 13 °C and 19 °C for four tanks each for the remaining experimental time (days 7 to 35). The salinity of the sea water was 28–30 g/l and oxygenated water was supplied to maintain tank water close to 100% saturation (8.8–8.9 mg/l at 13 °C and 7.8–7.9 mg/l at 19 °C). The fish were reared under an 18/6 h light regime (daylight period 05:00–23:00). Final mean body weight (BW) for all fish groups was 196.0 ± 1.5 g (SE) with no difference between groups.

A commercial diet (Optiline) produced by Skretting (Stavanger, Norway; 51% protein, 28% fat and 5% moisture, 4 mm pellets) was provided to fish in three daily meals at 08.15–10.00, 11.30–12.30 and 14.00–15.00 h by way of automatic feeders and to excess of satiation. Only the first meal was given on the 35th day and the ration was increased by 50% to increase the likelihood of all fish consuming feed prior to sampling. Daily feed waste was collected from all tanks. Estimations of daily feed intake were then calculated by subtracting the dried weight of feed waste from the weight of feed offered.

2.2. Sampling (postsmolt 13 °C and 19 °C)

Fish groups were individually weighed at days 1, 15 and 35. A postprandial sampling was conducted on the 35th day, starting 4 h after the morning meal was initiated. This was repeated every 4th hour, up to 24 h, giving a total of 6 sampling points. At each sampling point three fish were sampled from each tank, totalling twelve fish from each temperature. Sampled fish were anaesthetized by placing them in a solution of tricaine methanesulfonate (70 mg/l) in sea water until fish were sedated. Weight and length were then registered before samples of blood and white muscle tissue were taken. Samples were only taken from fish that were determined to have consumed feed in the previous meal after examination of gut contents.

Blood samples were taken from the caudal vein from 3 fish per tank with syringes and transferred to 1.8 ml microcentrifuge tubes each containing 5 μl heparin (5 IU/μl), and then centrifuged at 1250*g* for 10 min before plasma was aspirated. White muscle tissue was excised from medial cutlets of 3 fish per tank for free amino acid analysis (approximately 6 cm^3) and 2 fish per tank for RNA extraction and analysis (approximately 1 cm^3) and immediately flash-frozen in liquid nitrogen. All samples were stored at −80 °C prior to further analyses.

2.3. Experimental design (market sized salmon 12 °C)

A second study was conducted at Lerang Research Station (Skretting ARC) in Forsand, Norway with long-term adapted Atlantic salmon (Salmobreed strain). The fish group was reared in six experimental tanks (approximately 7000 l) at the same temperature (12 °C) and feeding regime for 134 days. The fish had a mean initial BW of 1.71 ± 0.16 kg and a mean BW on the day of sampling of 3.68 ± 0.19 kg (SE). The salinity of the sea water was 33–34 g/l and average oxygen saturation was at 110% (9.7 mg/l).

Optiline (Skretting, Stavanger, Norway; 37.0% fat, 36.1% protein and 5.7% moisture, 9 mm pellets) was offered to fish in all tanks in two meals each day using automatic feeders. Feeding was done to excess of satiation, with approximately 70% of the daily ration in the first meal (07.30–09.30 h) and 30% in the second meal (12.00–14.00 h). Feed waste collection and daily feed intake estimations were conducted following the same principles as in the other study.

2.4. Sampling (market sized salmon 12 °C)

The tanks were divided into two groups of three tanks; one for preprandial and another for postprandial sampling. This ensured that the fish sampled post-prandially were not disturbed by the preprandial sampling and therefore had normal feeding behaviour.

Preprandial sampling of 3 fish per tank was conducted at −4 hours (h), −2 h, −1 h and 0 h before the start of the first daily meal, which was given from 0–2 h, while postprandial sampling of 3 fish per tank was conducted 2 h, 3 h, 4 h and 6 h after the meal was initiated. Fish were partially anaesthetised by applying isoeugenol (AQUI-S) (8 ml/1000 l water) into the tanks before transferring three fish individually to a second anaesthetic container (70 mg tricaine methanesulfonate/l water). Registration of BW and length were done when fish were sedated and samples were only taken from fish that were determined to have consumed feed in the previous meal after examination of gut contents.

Blood samples were taken from the caudal vein from 3 fish per tank with heparinized 4 ml vacutainers (lithium heparin 60 USP) and then centrifuged at 3600*g* for 7 min at 4 °C before plasma was aspirated. White muscle tissue for RNA extraction and free amino acids were cut out in the same way as in the other study. All samples were stored at −80 °C prior to further analysis.

2.5. Analytical methods

Protein in feed was estimated from Kjeldahl Nitrogen (N*6.25, NMKL method no. 6, 4th Ed. 2003), fat by Nuclear Magnetic Resonance (NMR) on a QuickFat analyser (Anvendt Teknologi AS) and dry matter by gravimetric determination after drying (102–105 °C for 16–18 h, NMKL method No.23, 3rd ed. 1991).

The level of amino acids in feed was analysed photometrically after separation by ionic exchange chromatography and reaction with ninhydrin reagent and sodium citrate eluent (EU method, COMMISSION DIRECTIVE 98/64/EC). Free amino acids in plasma and white muscle tissue were also analysed photometrically after separation by ionic exchange chromatography and reaction with a ninhydrin reagent and lithium eluents (Biochrom method AAAFAQ8). The analysis was done on pooled samples from 3 fish taken from the same tank at each sampling time.

Plasma concentrations of IGF-1 and GHRL were measured by using fluoroimmunoassay and radioimmunoassay (RIA), respectively using the methods of Hevrøy (Hevrøy et al., 2013, 2012). For IGF-1 in plasma a dissociation-enhanced lanthanide fluorescence immunoassay (DELFIA) method using Europium (Eu)-labelled IGF-1 tracer (15.9 ng/ml; 1244–302; Perkin Elmer), rabbit anti-barramundi IGF-1 polyclonal antiserum (diluted 1:2072; GroPep, Australia) and recombinant salmon IGF-1 (GroPep, Australia) were used (Hevrøy et al., 2013). GHRL levels in salmon plasma were measured with a heterologous assay using a 125I-radioimmunoassay kit for human ghrelin specific for biologically active (octanoylated) ghrelin (Linco Research Inc., St. Charles, Missouri) validated for use in Atlantic salmon (Hevrøy et al., 2011). IGF-1 and GHRL analyses were conducted on 3 samples taken from the same tank at each sampling time in the postprandial study, while 2 samples per tank at all sampling points, except 6 h after the meal, were analysed in the pre- and postprandial study.

RNA extraction and real-time RT-qPCR (reverse transcription quantitative polymerase chain reaction) were used to measure transcription of the proteins for *ghr* and *igf1* in white muscle tissue by analysing 2 samples taken from the same tank at each time sampling.

Total RNA was extracted using Trizol reagent according to the manufacturer's recommendation (Invitrogen, USA). In order to eliminate genomic DNA, the total RNA sample was subjected to DNase treatment (DNeasy, Ambion), using the manufacturer's protocol. Quantity and quality of RNA were assessed with NanoDrop® ND-1000 UV–vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), where a 260/280 nm absorbance ratio of 1.8–2.0 indicates a pure RNA sample. To evaluate the RNA integrity the 6000 Nano LabChip® kit (Agilent Technologies, Palo Alto, CA, USA) was used, with average RNA integrity numbers (RIN) of 9.5 (SD ± 0.1) muscle tissue.

PCR primers for the target genes *ghr1* and *igf1* were designed with Primer Express software and Biosoft software, as previously described (Hevrøy et al., 2012). β-actin (*actb*), eukaryotic translation elongation factor 1αβ (*ef1ab*) (Hevrøy et al., 2013) and ribosomal protein L13 (*rpl13*) (Olsvik et al., 2013) were used as reference genes.

Reverse transcription (RT) was performed using a modified Multiscribe reverse transcription protocol (GeneAmp PCR 9700, Applied Biosystems, AB), as previously described (Hevrøy et al., 2005). White muscle samples from postsmolt were analysed in triplicate with a total RNA input of 500 ng ± 5% on 96-well plates for the RT and real-time PCR, while white muscles from big salmon were analysed in duplicate. Real-time PCR amplification and analysis were performed using a LightCycler 480 Real-time PCR system (Roche Applied Science, Basel, Switzerland). The real-time PCR SYBR Green Master Mix (LightCycler 480 SYBR Green master mix kit, Roche) contained FastStart DNA polymerase and gene-specific primers at a final concentration of 500 nM. Two microliters of diluted cDNA from each well were transferred to a new plate and 8 μl real-time master mixes were added. PCR was achieved with a 5 min activation and denaturizing step at 95 °C followed by 45 cycles of a 10 s denaturizing step at 95 °C, a 20 s annealing step at 60 °C and 30 s synthesis step at 72 °C. The efficiency of real-time PCR was monitored using two-fold dilution curves of total RNA using 5 points (range, 1000 to 31 ng/μl). Target gene mean normalised expression (MNE) was determined using a normalisation factor calculated with the geNorm software (Vandesompele et al., 2002).

2.6. Calculations

The following calculations were applied:

$$\text{Specific growth rate, SGR, \%} : (\ln \text{final BW} - \ln \text{Initial BW})/\text{days} \times 100$$

BW is body weights (g) and days are number of feeding days from initial to final weighing.

$$\text{Feed conversation ratio, FCR} : \text{Feed eaten, g/weight gain, g}$$

$$\text{Condition factor} : \left(\text{BW, g/Fork length, cm}^3\right) \times 100.$$

2.7. Statistics

All data are presented as mean values ± standard error of the mean (SEM) unless otherwise described. Tanks are used as experimental units. Statistical analyses were conducted using Unistat 6.0 (Unistat Ltd.) and significant differences were accepted at P values < 0.05. Data from the same time point, but from different temperature treatments were subjected to an unpaired t-test while all other data were subjected to analysis of variance (ANOVA). The post hoc test Tukey-HSD was applied if any significant differences were detected by ANOVA.

Results from the study at 12 °C were statistically treated as two sets of data; preprandial and postprandial, as these consisted of different fish tanks. A dotted line is applied in figures to interpolate between pre- and postprandial data sets.

3. Results

3.1. Postsmolt (13 °C and 19 °C)

BW at the intermediate sampling point (day 15) was 140.8 ± 1.6 g at 13 °C and 148.3 ± 2.8 g at 19 °C, while final BW after 35 days was 195.0 ± 1.6 g and 197.0 ± 2.8 g. SGR was not significantly different between temperature treatments (1.81 ± 0.12% at 13 °C and 1.92 ± 0.14% at 19 °C, $P > 0.05$) in the first period (15 days), but was significantly lower in the second period (20 days) for the 19 °C treatment compared to 13 °C (1.78 ± 0.07% and 2.04 ± 0.06%; $P < 0.05$). Mortality was close to zero with only a single fish dying in the 19 °C group.

There were no significant differences in mean feed intake or FCR which ranged from 57 to 73 g per day and 0.66 to 0.79 in the two temperature groups ($P > 0.05$). For fish reared at 19 °C, GHRL peaked at 8 h post-prandially ($P < 0.05$; Fig. 1), while plasma GHRL concentrations from the 13 °C treatment group were stable throughout the day following feeding ($P > 0.05$). Despite these differences there were no statistically significant differences in plasma ghrelin concentration between the two temperature treatments at any particular time point.

Mean total FAA concentration was consistently lower at 19 °C than at 13 °C for both plasma and white muscle tissue throughout the 24 h sampling period, although this difference only reached statistical significance at 12 h and 24 h for plasma and 8 h, 12 h, 16 h and 20 h for white muscle (Fig. 2a and b; $P < 0.05$).

Plasma total FAA concentration (Fig. 2a) peaked 4 to 8 h after the meal at both temperatures, followed by a significant decline to the 12 h sampling point ($P < 0.05$). Plasma FAA values continued to decline over the next 12 h, with the 24 h sampling point giving significantly lower values than 12 h at both temperatures ($P < 0.05$). The corresponding values in white muscle tissue (Fig. 2b) were relatively stable over time with no significant differences between sampling points at either temperature ($P > 0.05$). Plasma level of essential free amino acids (EFAA), which constitute Arginine (Arg), Histidine (His), Isoleucine (Ile), Leucine (Leu), Lysine (Lys), Threonine (Thr), Tryptophan (Trp), Valine (Val), Methionine (Met) and Phenylalanine (Phe), have similar trends to total plasma FAA levels, with significantly higher levels at 13 °C (16 h, 20 h and 24 h; $P < 0.05$) than at 19 °C and higher levels at 4 h and 8 h sampling points, followed by decline from 12 h up to 24 h (Fig. 3a). This was also reflected in all individual plasma EFAA (Table 1a and b) except for Trp. Trp concentrations were lower than any other FAA analysed in plasma and showed no significant differences between temperature treatments, but a significantly higher level at the last sampling point (24 h) compared to the first (4 h) for the 19 °C treatment ($P < 0.05$).

In contrast to the stable total white muscle FAA, the corresponding values for EFAA at 13 °C were significantly elevated at 8 h ($P < 0.05$; Fig. 3b). Mean EFAA levels declined after 16 h at 13 °C with a significantly reduced value for the 24 h sampling point ($P < 0.05$). At 19 °C only the 12 h and 16 h values were significantly higher than the 4 h sampling

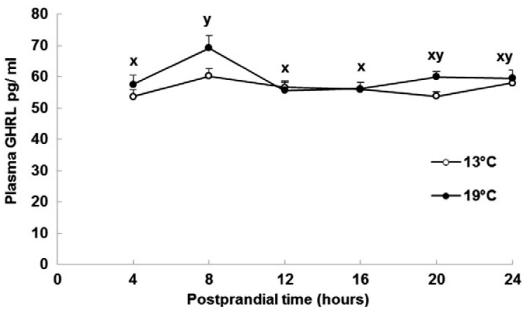

Fig. 1. Concentration (pg/ml) of ghrelin (GHRL) in plasma 4–24 hours (h) post-prandially from Atlantic salmon reared at 13 °C and 19 °C (mean + SEM). Values with different letters at the same temperature (xy at 19 °C) were significantly different ($P < 0.05$). n = 12 except for 16 h and 24 h at 13 °C (n = 11) and 24 h at 19 °C (n = 6).

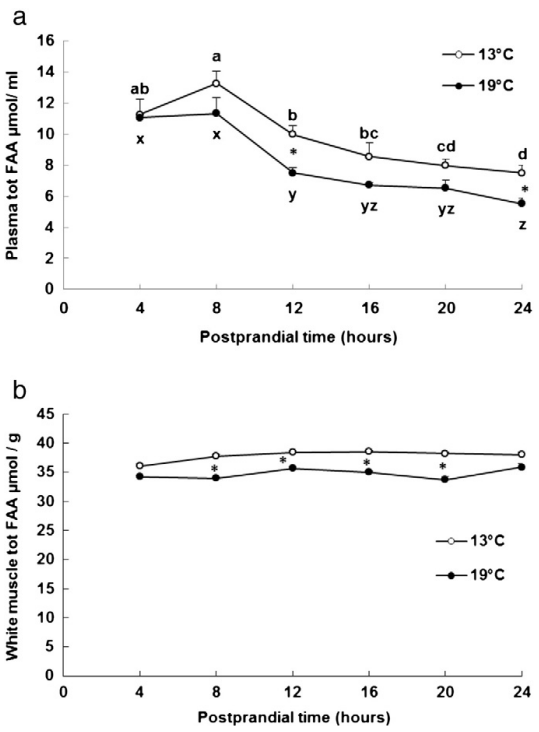

Fig. 2. Total free amino concentration (FAA) in (a) plasma (µmol/ml) and (b) white muscle (µmol/g) 4–24 hours (h) post-prandially in Atlantic salmon reared at 13 °C and 19 °C (mean + SEM). Values with different letters at the same temperature (abcd at 13 °C and xyz at 19 °C) were significantly different ($P < 0.05$) while differences between the temperature groups are indicated with the symbol * ($P < 0.05$). n = 4 except for plasma 8 h at both temperatures (n = 2) (pooled samples of 3 fish per analysis).

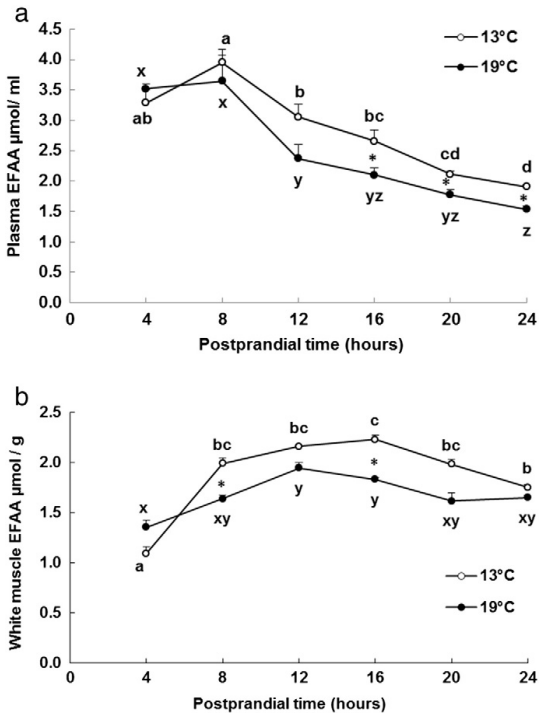

Fig. 3. Total essential free amino acid concentration (EFAA) in (a) plasma (µmol/ml) and (b) white muscle (µmol/g) 4–24 hours (h) post-prandially in Atlantic salmon reared at 13 °C and 19 °C (mean + SEM). Tryptophane was not detected in white muscle. Values with different letters at the same temperature (abcd at 13 °C and xyz at 19 °C) were significantly different ($P < 0.05$) while differences between the temperature groups are indicated with the symbol * ($P < 0.05$). n = 4 except for plasma 8 h at both temperatures (n = 2) (pooled samples of 3 fish per analysis).

time ($P < 0.05$). All other values are intermediate. These postprandial patterns were similar for most individual white muscle EFAA (Table 2a and b), but not in the Lys, His and Thr levels. There were no significant variation or difference in white muscle Lys concentration at either temperatures ($P > 0.05$), except higher Lys for the last sampling point at 13 °C ($P < 0.05$). Free His and Thr in white muscle did not vary significantly between time points at 19 °C ($P > 0.05$), but His was higher at 8 h and 16 h than at 4 h post-prandially for fish at 13 °C ($P < 0.05$). Thr was significantly higher from 12 h post-prandially at 13 °C ($P < 0.05$) and did not show any decline within the sampling range.

There were no significant differences between plasma IGF-1 concentrations in fish reared at 13 °C and 19 °C ($P > 0.05$; Fig. 4) at any particular time point. While there were also no statistically significant differences in plasma IGF-1 concentrations between sampling points at 13 °C, the corresponding values at 19 °C started to decline after 8 h and were significantly lower at 20 h and 24 h sampling points ($P < 0.05$). At 13 °C there were no statistically significant differences in plasma IGF-I concentrations between sampling points ($P > 0.05$). Expressions of *ghr1* mRNA levels (mean normalised expression, MNE) were the same in white muscle samples from both temperatures ($P > 0.05$) with significantly higher level at 4 h than all other sampling points ($P < 0.05$; Fig. 5a). Expressions of *igf1* mRNA were significantly lower at 19 °C than at 13 °C ($P < 0.05$; Fig. 5b) except at 8 h ($P > 0.05$). Within each temperature treatment, there were no significant differences in white muscle *igf1* mRNA expression levels between sampling points ($P > 0.05$).

3.2. Market sized salmon (12 °C)

Daily feed intake was 0.67 ± 0.02% of BW during the 134 days prior to the pre- and postprandial sampling with only a single fish dying. GHRL concentrations in plasma peaked before each adapted mealtime from −2 h to 0 h and at 3 h ($P < 0.05$; Fig. 6).

Total FAA concentration in plasma had no statistically significant peaks ($P > 0.05$) between sampling points pre- or post-prandially (Fig. 7) and values were similar to the basal (24 h) sampling period in the short-term study. White muscle tissue total FAA was also relatively stable with no significant differences post-prandially ($P > 0.05$, Fig. 8), which is consistent with the short-term study. White muscle FAA peaked 1 h prior to feeding, although the magnitude of this change was small and only significantly higher than 2 h prior to feeding.

There was a significant decline ($P < 0.05$) in plasma EFAA between the −4 h sampling point and the 0 h sampling point, when these fish groups usually would have received their first meal of the day (Fig. 7). When divided into individual FAA, this trend is consistent for Lys and Trp ($P < 0.05$) (Table 3) however no significant differences were found between time points for the other individual FAA ($P > 0.05$). Plasma EFAA concentrations found pre-prandially were similar to the short-term study.

Postprandial EFAA levels in big salmon at 12 °C were generally stable in the plasma, with the exceptions of Met, Phe and Leu, which all peaked 2–3 h post-prandially ($P < 0.05$; Table 3). The concentration of EFAA in white muscle tissue was stable with no significant elevations amongst the preprandial or the postprandial sampling points (Fig. 8; $P > 0.05$). The levels of individual EFAA (Table 4) in white muscle were also constant ($P > 0.05$), except Leu which had a decline at 4 h post-prandially ($P < 0.05$).

Plasma IGF-1 concentrations (Fig. 9) were stable with no significant differences between sampling points pre- or postprandially ($P > 0.05$). Expressions of *ghr1* or *igf1* mRNA levels in white muscle (Fig. 10a and b) also did not have statistically significant changes over the time span investigated ($P > 0.05$). Average pre- and postprandial mRNA levels were 0.201 and 0.302 MNE for *ghr1* and 0.330 and 0.183 MNE for *igf1*.

4. Discussion

The current research presents two studies that provide baseline data regarding the endocrinology of appetite and growth in relation to meal times, temperature and nutrient absorption dynamics for postsmolt and market sized Atlantic salmon.

Decreased growth was recorded for salmon postsmolt held at 19 °C compared to those held at 13 °C between the intermediate weighing and the end of the experiment, but not between the start of the experiment and the intermediate weighing point. This indicates that the period of exposure may influence the ability of salmon to cope with high water temperatures.

Contrary to previously described (Kullgren et al., 2013), fish in the present study did not have a decreased feed intake or poorer FCR at 19 °C compared to 13 °C. There were also no differences in GHRL levels between temperatures, indicating that appetite was equivalent for both temperatures. Two previous experiments (Hevrøy et al., 2012; Kullgren et al., 2013) have presented contrasting results regarding the relationship between plasma GHRL and temperature for salmon; one describing no difference between 8 °C, 12 °C and 18 °C (Kullgren et al., 2013) and the other describing depressed levels at 19 °C compared to 14 °C (Hevrøy et al., 2012). Common to both of these previous reports is that they had a single sampling point after the meal, and no description of GHRL changes over time. The current experiment attempted to clarify this discrepancy by repeated postprandial sampling, and thereby demonstrated a clear peak of plasma GHRL concentration 8 h after sampling for the 19 °C treatment, corresponding to the next expected meal. Since this pattern is described by a single sampling point, a better understanding of appropriate timing of GHRL peak levels may be obtained by more

Table 1
Plasma concentration (μmol/ml) of the individual essential free amino acids; Threonine (Thr), Valine (Val), Methionine (Met), Isoleucine (Ile), Leucine (Leu), Phenylalanine (Phe), Lysine (Lys), Histidine (His), Tryptophan (Trp) and Arginine (Arg) 4–24 hours (h) post-prandially in Atlantic salmon reared at 13 °C (a) and 19 °C (b) (mean + SEM). Values with different letters at the same temperature were significantly different ($P < 0.05$). n = 4 except for 8 h (n = 2) (pooled samples of 3 fish per analysis).

a

EFAA plasma 13 °C	Postprandial time (hours)					
	4	8	12	16	20	24
Thr	0.31 ± 0.08	0.33 ± 0.03	0.21 ± 0.03	0.16 ± 0.02	0.12 ± 0.01	0.13 ± 0.01
Val	0.65 ± 0.03^{b}	0.78 ± 0.03^{a}	0.62 ± 0.02^{b}	0.58 ± 0.03^{b}	0.49 ± 0.01^{c}	0.44 ± 0.01^{c}
Met	0.16 ± 0.01^{a}	0.18 ± 0.01^{a}	0.13 ± 0.01^{a,b}	0.10 ± 0.01^{b,c}	0.08 ± 0.01^{c}	0.07 ± 0.00^{c}
Ile	0.33 ± 0.02^{b}	0.45 ± 0.03^{a}	0.34 ± 0.02^{b}	0.31 ± 0.02^{b}	0.23 ± 0.00^{c}	0.20 ± 0.01^{c}
Leu	0.85 ± 0.03^{b}	1.10 ± 0.04^{a}	0.92 ± 0.04^{a,b}	0.87 ± 0.06^{b}	0.65 ± 0.03^{c}	0.52 ± 0.03^{c}
Phe	0.32 ± 0.02^{a,b}	0.41 ± 0.02^{a}	0.32 ± 0.03^{a,b}	0.26 ± 0.02^{b,c}	0.20 ± 0.02^{c,d}	0.17 ± 0.01^{d}
Lys	0.35 ± 0.05^{a}	0.32 ± 0.03^{a}	0.22 ± 0.04^{a,b}	0.15 ± 0.01^{b}	0.12 ± 0.02^{b}	0.14 ± 0.01^{b}
His	0.14 ± 0.04	0.14 ± 0.01	0.09 ± 0.01	0.07 ± 0.01	0.05 ± 0.00	0.06 ± 0.00
Trp	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
Arg	0.17 ± 0.01	0.22 ± 0.02	0.19 ± 0.02	0.13 ± 0.01	0.16 ± 0.03	0.16 ± 0.02

b

EFAA plasma 19 °C	Postprandial time (hours)					
	4	8	12	16	20	24
Thr	0.31 ± 0.01^{a}	0.33 ± 0.03^{a}	0.16 ± 0.03^{b}	0.14 ± 0.01^{b}	0.12 ± 0.00^{b}	0.12 ± 0.01^{b}
Val	0.69 ± 0.01^{a}	0.74 ± 0.07^{a}	0.53 ± 0.05^{b}	0.47 ± 0.02^{b,c}	0.42 ± 0.01^{b,c}	0.39 ± 0.02^{c}
Met	0.16 ± 0.01^{a}	0.17 ± 0.03^{a}	0.11 ± 0.02^{b}	0.09 ± 0.01^{b}	0.07 ± 0.01^{b}	0.06 ± 0.00^{b}
Ile	0.37 ± 0.01^{a}	0.41 ± 0.05^{a}	0.27 ± 0.02^{b}	0.23 ± 0.02^{b,c}	0.18 ± 0.01^{c,d}	0.16 ± 0.01^{d}
Leu	0.89 ± 0.02^{a,b}	0.99 ± 0.10^{a}	0.74 ± 0.05^{b,c}	0.62 ± 0.06^{c,d}	0.50 ± 0.04^{d,e}	0.37 ± 0.02^{e}
Phe	0.35 ± 0.01^{a}	0.35 ± 0.04^{a}	0.24 ± 0.02^{b}	0.22 ± 0.02^{b,c}	0.18 ± 0.01^{c,d}	0.14 ± 0.00^{d}
Lys	0.39 ± 0.01^{a}	0.31 ± 0.06^{a}	0.14 ± 0.04^{b}	0.13 ± 0.01^{b}	0.13 ± 0.01^{b}	0.11 ± 0.01^{b}
His	0.13 ± 0.00^{a}	0.13 ± 0.01^{a}	0.06 ± 0.01^{b}	0.07 ± 0.01^{b}	0.05 ± 0.00^{b}	0.04 ± 0.00^{b}
Trp	0.01 ± 0.00^{a}	0.02 ± 0.00^{a,b}	0.02 ± 0.00^{a,b}	0.02 ± 0.00^{a,b}	0.02 ± 0.00^{a,b}	0.02 ± 0.00^{a,b}
Arg	0.22 ± 0.01^{a}	0.21 ± 0.03^{a}	0.12 ± 0.01^{b}	0.11 ± 0.01^{b}	0.12 ± 0.01^{b}	0.12 ± 0.01^{b}

frequent sampling points. Given its role in meal initiation in other vertebrates and acclimation to feeding regimes (Cummings et al., 2001; Sugino et al., 2002) it was considered pertinent to further examine GHRL concentrations in a second experiment both before and after meal times and in fish that were acclimated to a fixed feeding regime for a long period. This approach revealed that plasma GHRL concentration peaked 1 to 2 h before each expected feeding time, clearly demonstrating anticipation of the meal and indicating that the fish were well habituated to this feeding regime.

Analyses of plasma and muscle from postsmolt revealed a clear postprandial pattern of FAA entering the plasma, with values peaking between 4 h and 8 h post-prandially. Muscle EFAA levels also increased

Table 2
White muscle concentration (μmol/g) of the individual essential free amino acid; Threonine (Thr), Valine (Val), Methionine (Met), Isoleucine (Ile), Leucine (Leu), Phenylalanine (Phe), Lysine (Lys), Histidine (His) and Arginine (Arg) 4–24 hours (h) post-prandially in Atlantic salmon reared at 13 °C (a) and 19 °C (b) (mean + SEM). Tryptophan was not detected. Values with different letters at the same temperature were significantly different ($P < 0.05$). n = 4 (pooled samples of 3 fish per analysis).

a

EFAA white muscle 13 °C	Postprandial time (hours)					
	4	8	12	16	20	24
Thr	0.29 ± 0.01^{a}	0.36 ± 0.04^{a,b}	0.41 ± 0.01^{b}	0.43 ± 0.02^{b}	0.44 ± 0.02^{b}	0.42 ± 0.02^{b}
Val	0.11 ± 0.02^{a}	0.21 ± 0.01^{b}	0.30 ± 0.00^{d}	0.29 ± 0.02^{c,d}	0.25 ± 0.01^{b,c,d}	0.23 ± 0.02^{b,c}
Met	0.04 ± 0.00^{a,b}	0.05 ± 0.00^{a,b}	0.05 ± 0.00^{b}	0.07 ± 0.01^{c}	0.05 ± 0.00^{a,b}	0.04 ± 0.00^{a}
Ile	0.04 ± 0.01^{a}	0.10 ± 0.00^{b,c}	0.15 ± 0.00^{d}	0.12 ± 0.01^{c,d}	0.11 ± 0.00^{c}	0.09 ± 0.00^{b}
Leu	0.28 ± 0.01^{a}	0.44 ± 0.01^{c}	0.52 ± 0.01^{d}	0.47 ± 0.01^{c}	0.38 ± 0.01^{b}	0.31 ± 0.01^{a}
Phe	0.10 ± 0.01^{a}	0.15 ± 0.00^{b}	0.15 ± 0.01^{b}	0.15 ± 0.00^{b}	0.10 ± 0.00^{a}	0.08 ± 0.01^{a}
Lys	0.05 ± 0.01	0.06 ± 0.01	0.04 ± 0.00	0.07 ± 0.00	0.06 ± 0.01	0.06 ± 0.01
His	0.12 ± 0.02^{a}	0.33 ± 0.07^{b}	0.22 ± 0.02^{a,b}	0.30 ± 0.04^{b}	0.27 ± 0.03^{a,b}	0.28 ± 0.03^{a,b}
Arg	0.15 ± 0.02^{a}	0.28 ± 0.01^{b}	0.32 ± 0.02^{b}	0.33 ± 0.02^{b}	0.32 ± 0.03^{b}	0.25 ± 0.01^{b}

b

EFAA white muscle 19 °C	Postprandial time (hours)					
	4	8	12	16	20	24
Thr	0.25 ± 0.25	0.29 ± 0.29	0.30 ± 0.30	0.30 ± 0.30	0.32 ± 0.32	0.34 ± 0.34
Val	0.11 ± 0.11^{a}	0.17 ± 0.17^{a,b}	0.24 ± 0.24^{b,c}	0.25 ± 0.25^{c}	0.20 ± 0.20^{c}	0.23 ± 0.23^{c}
Met	0.05 ± 0.05	0.05 ± 0.05	0.06 ± 0.06	0.06 ± 0.06	0.04 ± 0.04	0.05 ± 0.05
Ile	0.05 ± 0.05^{a}	0.08 ± 0.08^{b}	0.10 ± 0.10^{c}	0.10 ± 0.10^{b,c}	0.08 ± 0.08^{b}	0.09 ± 0.09^{b,c}
Leu	0.27 ± 0.27^{a}	0.36 ± 0.36^{b}	0.40 ± 0.40^{b}	0.35 ± 0.35^{b}	0.26 ± 0.26^{a}	0.26 ± 0.26^{a}
Phe	0.09 ± 0.09^{a}	0.12 ± 0.12^{a}	0.12 ± 0.12^{a}	0.12 ± 0.12^{b}	0.08 ± 0.08^{b}	0.08 ± 0.08^{b}
Lys	0.14 ± 0.14	0.07 ± 0.07	0.06 ± 0.06	0.06 ± 0.06	0.06 ± 0.06	0.13 ± 0.13
His	0.25 ± 0.25	0.28 ± 0.28	0.34 ± 0.34	0.29 ± 0.29	0.28 ± 0.28	0.22 ± 0.22
Arg	0.17 ± 0.17^{a}	0.22 ± 0.22^{a,b}	0.31 ± 0.31^{c}	0.31 ± 0.31^{c}	0.30 ± 0.30^{c}	0.26 ± 0.26^{a,b}

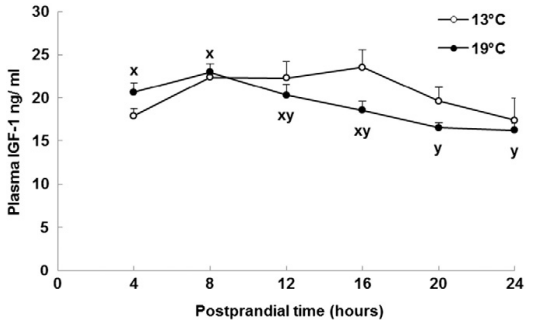

Fig. 4. Concentration (ng/ml) of insulin like growth factor-1 (IGF-1) in plasma 4–24 hours (h) post-prandially from Atlantic salmon reared at 13 °C and 19 °C (mean + SEM). Values with different letters at the same temperature (xy at 19 °C) were significantly different ($P < 0.05$). n = 12 (4 h), n = 9 (8 h), n = 8 (12 h), n = 5 (16 h and 20 h) and n = 3 (24 h) at 13 °C. n = 12 (4 h), n = 7 (8 h), n = 8 (12 h), n = 6 (16 h), n = 8 (20 h) and n = 9 (24 h) at 19 °C.

during this time, but with a later peak, 12–16 h post-prandially. This peak value was more pronounced for fish kept at 13 °C. While total FAA have a very similar trend to EFAA in plasma, total FAA in muscle did not vary with postprandial time. At colder temperatures (8.6 ± 0.6 °C and 10.8 ± 0.1 °C), two studies with postsmolt (95 ± 18 g and 100 g BW), both found the highest plasma EFAA concentrations from

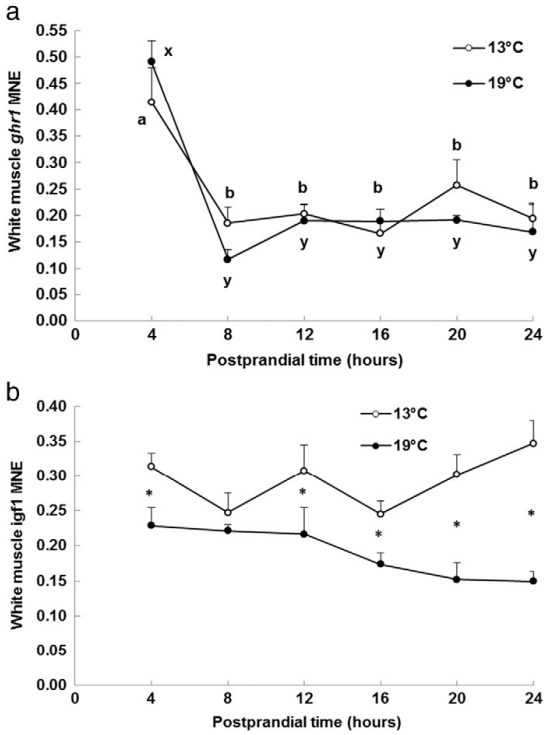

Fig. 5. Growth hormone receptor-1 (*ghr1*) (a) and insulin like growth factor-1 (*igf1*) (b) mRNA levels (mean normalised expression, MNE) in white muscle 4–24 hours (h) post-prandially from Atlantic salmon reared at 13 °C and 19 °C (mean + SEM). Values with different letters at the same temperature (ab at 13 °C and xy at 19 °C for *ghr1*) were significantly different ($P < 0.05$), while significant differences between the temperature groups are indicated with the symbol * ($P < 0.05$). n = 7–8.

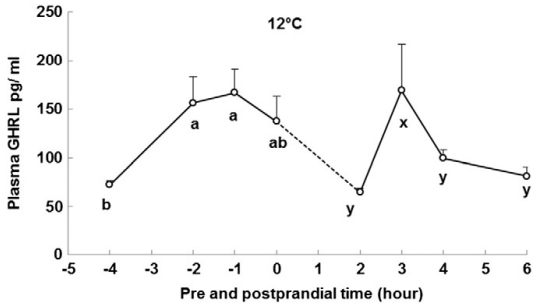

Fig. 6. Concentration (pg/ml) of ghrelin (GHRL) in plasma −4–0 hours (h) pre-prandially and 2–6 h post-prandially from Atlantic salmon reared at 12 °C (mean + SEM). Values with unlike letters were significantly different ($P < 0.05$) within pre- or postprandial sampling groups. Dotted line interpolates between the datasets. n = 7–9.

6 h to 12 h after a 1 h long meal were finished (Espe et al., 1993; Torrison et al., 1995). One of these studies (Torrison et al., 1995) also measured the highest total plasma FAA at 6 h, but statistical significance was not reported. Since the present study at warmer temperatures found peak values even sooner, 4–8 h after the meal was commenced, it is likely that timing of peak plasma FAA levels differ with temperature in a similar way to gut transit time (Fauconneau et al., 1983; Fänge and Grove, 1979; Miegel et al., 2010; Pérez-Casanova et al., 2009; Usher et al., 1991).

The clear pattern that is obvious for total FAA in muscle is a consistent depression at 19 °C compared to 13 °C, showing that FAA muscle pools are lower at high temperatures irrespective of meal inputs. Similar trends are evident for muscle EFAA, plasma FAA and plasma EFAA, indicating a general decrease in amino acid pools in both plasma and muscle associated with high water temperature. The time-course of sampling employed in the present study has revealed that the greatest difference between temperatures occurred not at the time of peak nutrient absorption, but rather at 12–24 h post-prandially when FAA levels were descending. This is pertinent data for further experimentation in this area, as the tendency of previously published work (Carter et al., 2008; Hevrøy et al., 2011) has been to select a single sampling point at peak nutrient absorption (4–8 h), which has prevented this trend from becoming clear.

Previously reported data in this field has suggested that FAA can accumulate in the muscle at high temperature due to down-regulation of *igf1* mRNA levels which depresses protein synthesis (Hevrøy et al., 2011; Hunskår, 2010). The present study has not found data consistent with this result. Muscle FAA levels were consistently lower at 19 °C than

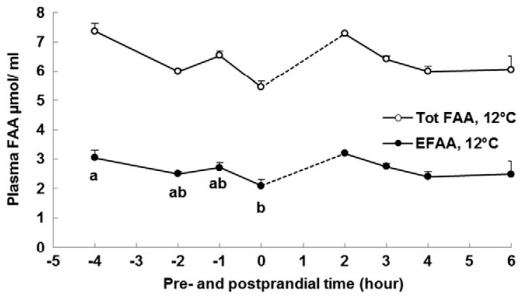

Fig. 7. Plasma concentration (µmol/ml) of total free amino acid (FAA) and total essential free amino acids (EFAA) −4–0 hours (h) pre-prandially and 2–6 h post-prandially in Atlantic salmon reared at 12 °C (mean + SEM). Significantly different values ($P < 0.05$) are indicated with different letters (ab in preprandial group). Dotted line interpolates between the datasets. n = 3 (pooled samples of 3 fish per analysis).

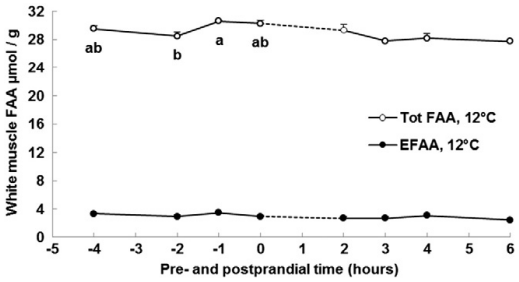

Fig. 8. White muscle concentration (µmol/g) of total free amino acids (FAA) and total essential free amino acid (EFAA) −4–0 hours (h) pre-prandially and 2–6 h post-prandially in Atlantic salmon reared at 12 °C (mean + SEM). Significantly different values ($P < 0.05$) are indicated with different letters (ab in preprandial group for total FAA). Dotted line interpolates between the datasets. n = 3 (pooled samples of 3 fish per analysis).

at 13 °C which is consistent with plasma FAA data and indicates a chronic disruption of homeostasis at 19 °C that has a greater impact on FAA pools than postprandial amino acid influx.

Concentrations of total FAA in white muscle did not vary over time at either temperature, indicating that the majority of FAA was incorporated into muscle or catabolised at the same rate as they moved into the muscle pool. A lack of postprandial variation in muscle FAA concentration is consistent with data from Mente et al. (2003), who claimed that a regular feeding regime before postprandial sampling resulted in a steady supply of FAA to tissues, which continued long after plasma FAA levels had returned to a basal state. Carter et al. (2000) reported increases in muscle FAA levels up to 15 h post-prandially to a single meal, following seven days starvation, which appears to support the hypothesis of Mente et al. (2003). What is unclear now is how long it takes for muscle FAA levels to begin to decrease after a meal. The present data suggest that such a decline requires more than 24 h for both temperatures.

Very few significant differences or peaks in FAA were detected at 13 °C for the long-term adapted salmon in contrast to the short-term study with postsmolt. However, concentration patterns of total FAA and EFAA resemble each other where these two experiments overlap in sampling points. Lack of significant differences in absorption and storage of FAA over time in the long-term study can also be due to different fish sizes and other demands to feed composition; with a lower protein requirement for larger fish. There were also less daily meals offered in the long-term study (two vs. three meals) which may explain the lack of detectable FAA changes in plasma and white muscle from big salmon.

Of the individual EFAA, Leu has a clear influence over the general trends, since it is not only the most abundant EFAA in both plasma and muscle pools, but it is also the EFAA that is most affected by temperature. Leu stands out as a FAA documented to have a particular stimulating role in muscle protein regulation (Garlick, 2005; Kimball and Jefferson, 2004; Suryawan et al., 2011). The effect of temperature was also seen for white muscle Thr with higher levels at 13 °C than at 19 °C. Results from the study at 13 °C and 19 °C are consistent with Kullgren et al. (2013), who reported significant declines in several plasma EFAA at 18 °C compared to 8 °C and 12 °C for postsmolt, but also show that FAA in both plasma and muscle can be affected by high temperature from an early stage post-prandially.

Leu, Met and Phe dropped in plasma and white muscle concentration post-prandially at 12 °C for big salmon, up to the 3 h sampling, followed by stable concentrations or an increase. FAA plays an intermediate signalling role between appetite regulating and growth regulating processes. The only FAA to peak pre-prandially at 12 °C is Trp in plasma, which has received considerable attention for its role in appetite regulation in higher vertebrates, including humans (Hill and Blundell, 1988; Hrboticky et al., 1985; Wurtman et al., 1981). Since GHRL suppresses release of serotonin from the hypothalamus (Brunetti et al., 2002), this Trp peak is likely to be caused by a reduced conversion of Trp to serotonin and therefore indicative of GHRL action. Postprandial FAA signal for nutrient availability and increase anabolic processes mediated by IGF-1. Branched-chain amino acids (BCAA), in particular Leu, have been well documented as anabolic stimulants (Thissen et al., 1994). BCAA are consistently depressed at 19 °C compared to 13 °C in both white muscle and plasma and present possible mechanism for reduced *igf1* mRNA in muscle. Concentration of Arg in plasma and white muscle was in general lower in 19 °C treatment groups than at 13 °C. Arg stimulates the release and activity of many pancreatic hormones, including IGF-1 and insulin, which stimulate growth and metabolism of protein and fat (Mommsen, 2001). Arg is also reported to have a positive impact on growth regulation in trout when given as intraperitoneal injections (Planas et al., 2000). The decreased plasma Arg concentration found under high temperature conditions, may contribute to the depressed *igf1* mRNA expression in white muscle and plasma concentration at 19 °C.

White muscle *ghr1* mRNA activity was not affected by temperature, but rather peaked after the meal in the short term study with postsmolt. The lack of temperature effect implies that *ghr1* is more closely coupled to meal times and appetite rather than temperature. This trend was not as clear in the long-term study with big salmon, where there was an approximately two-fold rise in *ghr1* mRNA activity after the meal, but more variation within treatment values. White muscle *igf1* mRNA was much more sensitive to temperature than *ghr1*, with significantly lower levels at 19 °C compared to 13 °C. Circulating concentrations of IGF-1 were not affected by temperature. However, the plasma IGF-1 levels at 19 °C started to decline at 12 h post-prandially with significant lower levels at 20–24 h post-prandially, while levels at 13 °C remained stable, indicating that the scope for growth is limited at 19 °C. White muscle *igf1* mRNA and circulating IGF-1 in big salmon at 12 °C showed no significant differences over time and confirms the stable results

Table 3
Plasma concentration (µmol/ml) of the individual essential free amino acids; Threonine (Thr), Valine (Val), Methionine (Met), Isoleucine (Ile), Leucine (Leu), Phenylalanine (Phe), Lysine (Lys), Histidine (His), Tryptophan (Trp) and Arginine (Arg) −4–0 hours (h) pre-prandially and 2–6 h post-prandially in Atlantic salmon reared at 12 °C (mean + SEM). Dotted line interpolates between the datasets. Values with different letters at the same temperature, within each pre or post-prandial group, were significantly different ($P < 0.05$). n = 3 (pooled samples of 3 fish per analysis).

EFAA plasma 12 °C	Preprandial time				Postprandial time (hours)			
	−4	−2	−1	0	2	3	4	6
Thr	0.33 ± 0.02	0.21 ± 0.02	0.27 ± 0.04	0.21 ± 0.05	0.42 ± 0.09	0.23 ± 0.01	0.29 ± 0.05	0.34 ± 0.14
Val	0.65 ± 0.06	0.57 ± 0.01	0.61 ± 0.02	0.49 ± 0.05	0.62 ± 0.01	0.57 ± 0.02	0.51 ± 0.02	0.54 ± 0.09
Met	0.15 ± 0.01	0.13 ± 0.00	0.14 ± 0.02	0.13 ± 0.01	0.16 ± 0.01^{a}	0.15 ± 0.01^{a,b}	0.12 ± 0.00^{b}	0.12 ± 0.01^{b}
Ile	0.33 ± 0.04	0.27 ± 0.01	0.28 ± 0.02	0.21 ± 0.02	0.30 ± 0.00	0.28 ± 0.01	0.23 ± 0.01	0.23 ± 0.04
Leu	0.60 ± 0.07	0.54 ± 0.01	0.53 ± 0.02	0.43 ± 0.04	0.54 ± 0.03^{a}	0.55 ± 0.02^{a}	0.42 ± 0.01^{b}	0.41 ± 0.02^{b}
Phe	0.19 ± 0.01	0.16 ± 0.00	0.17 ± 0.02	0.14 ± 0.01	0.18 ± 0.00^{a}	0.17 ± 0.01^{a}	0.13 ± 0.00^{b}	0.13 ± 0.00^{b}
Lys	0.52 ± 0.03^{a}	0.40 ± 0.02 ^{a,b}	0.45 ± 0.03^{a,b}	0.33 ± 0.05^{b}	0.64 ± 0.05	0.55 ± 0.05	0.49 ± 0.10	0.51 ± 0.11
His	0.06 ± 0.01	0.05 ± 0.00	0.07 ± 0.02	0.05 ± 0.00	0.06 ± 0.01	0.06 ± 0.00	0.05 ± 0.00	0.04 ± 0.01
Trp	0.01 ± 0.00^{b,c}	0.02 ± 0.00^{a}	0.02 ± 0.00^{a,b}	0.01 ± 0.00^{c}	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
Arg	0.20 ± 0.02	0.15 ± 0.02	0.16 ± 0.04	0.10 ± 0.01	0.24 ± 0.02	0.17 ± 0.01	0.14 ± 0.02	0.16 ± 0.04

Table 4
White muscle concentration (μmol/g) of the individual essential free amino acids; Threonine (Thr), Valine (Val), Methionine (Met), Isoleucine (Ile), Leucine (Leu), Phenylalanine (Phe), Lysine (Lys), Histidine (His) and Arginine (Arg) −4–0 hours (h) pre-prandially and 2–6 h post-prandially in Atlantic salmon reared at 12 °C (mean + SEM). Tryptophan was not detected. Dotted line interpolates between the datasets. Values with different letters at the same temperature, within each pre or post-prandial group, were significantly different ($P < 0.05$). n = 3 (pooled samples of 3 fish per analysis).

EFAA white muscle 12 °C	Preprandial time				Postprandial time (hours)			
	−4	−2	−1	0	2	3	4	6
Thr	0.58 ± 0.09	0.45 ± 0.03	0.55 ± 0.09	0.53 ± 0.06	0.54 ± 0.03	0.46 ± 0.05	0.51 ± 0.06	0.47 ± 0.03
Val	0.26 ± 0.03	0.22 ± 0.02	0.24 ± 0.02	0.21 ± 0.04	0.27 ± 0.02	0.22 ± 0.01	0.20 ± 0.02	0.27 ± 0.05
Met	0.09 ± 0.00	0.10 ± 0.02	0.11 ± 0.01	0.11 ± 0.02	0.11 ± 0.01	0.09 ± 0.01	0.08 ± 0.02	0.10 ± 0.01
Ile	0.12 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	0.09 ± 0.02	0.12 ± 0.01	0.10 ± 0.00	0.08 ± 0.02	0.12 ± 0.02
Leu	0.27 ± 0.01	0.27 ± 0.01	0.26 ± 0.01	0.23 ± 0.03	0.27 ± 0.02[a]	0.24 ± 0.00[a,b]	0.19 ± 0.01[b]	0.25 ± 0.02[a,b]
Phe	0.08 ± 0.01	0.08 ± 0.01	0.09 ± 0.02	0.07 ± 0.01	0.09 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.07 ± 0.01
Lys	1.49 ± 0.15	1.30 ± 0.26	1.75 ± 0.09	1.28 ± 0.03	0.87 ± 0.18	1.13 ± 0.27	1.42 ± 0.33	0.81 ± 0.18
His	0.31 ± 0.06	0.31 ± 0.05	0.22 ± 0.04	0.32 ± 0.08	0.38 ± 0.04	0.30 ± 0.04	0.38 ± 0.14	0.33 ± 0.10
Trp	0.19 ± 0.04	0.16 ± 0.05	0.19 ± 0.03	0.17 ± 0.01	0.11 ± 0.02	0.13 ± 0.02	0.21 ± 0.07	0.10 ± 0.03
Arg	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

found for postsmolt at 13 °C. Neither the receptor for GH1 nor the growth stimulating hormone IGF-1, had any significant differences throughout the sampling range at 12 °C, which is considered to be in the optimal temperature range for growth of big salmon (Hevrøy et al., 2013). This shows that fish can have a positive growth response all day at optimal temperature conditions.

In summary, plasma and white muscle FAA levels and growth stimulation (IGF-1) were negatively affected by high temperature. Growth differences between 13 °C and 19 °C were only apparent for the last 20 trial days, which indicates that high water temperature may take several weeks to elicit negative effects which are also previously reported (Hevrøy et al., 2013; Kullgren et al., 2013).

5. Conclusions

High temperature resulted in persistently lower muscle FAA levels, while feed intake was not affected. Plasma FAA peaked 4–8 h postprandially, but effect of temperature was not pronounced until plasma levels were declining. As for plasma FAA, high temperature gave a more rapid decline of IGF-1 concentrations and expression of muscle *igf1* mRNA, indicating a diminished growth potential at 19 °C compared to 13 °C. Since feed intake and GHRL levels were not affected by temperature in the study with short-term adapted postsmolt, this also implicates that nutrient availability may have a greater impact on growth regulation at high temperatures than appetite for Atlantic salmon of this size.

Circulating GHRL has rapid peaks before anticipated meal times, with two-fold changes occurring within the space of 1 to 2 h. This makes adaptation time to feeding regime, sampling frequency and timing important for detecting meaningful changes in GHRL levels. The effect of high temperature on GHRL remains to be elucidated. Plasma IGF-1 concentrations for salmon at 12 °C were even throughout the sampling period, further indicating that IGF-1 does not respond to feeding time under ideal conditions, but becomes sensitised to short-term nutrient availability under suboptimal conditions.

These data indicate that prolonged periods of warmer conditions, as expected from forecasted global warming, will impact growth and nutrient utilisation. The paper also describes detailed patterns of FAA and regulation of growth and appetite linked hormones for Atlantic salmon in relation to feeding time and temperatures, which can complement and aid future studies on related topics.

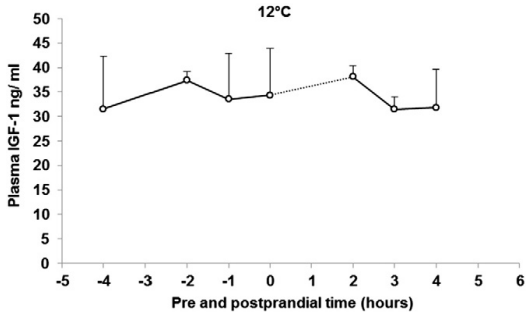

Fig. 9. Concentration (ng/ml) of insulin like growth factor-1 (IGF-1) in plasma −4–0 hours (h) pre-prandially and 2–4 h post-prandially from Atlantic salmon reared at 12 °C (mean + SEM). No significant differences within pre- or postprandial sampling groups ($P > 0.05$). Dotted line interpolates between the datasets. n = 6 except for 0 h (n = 4).

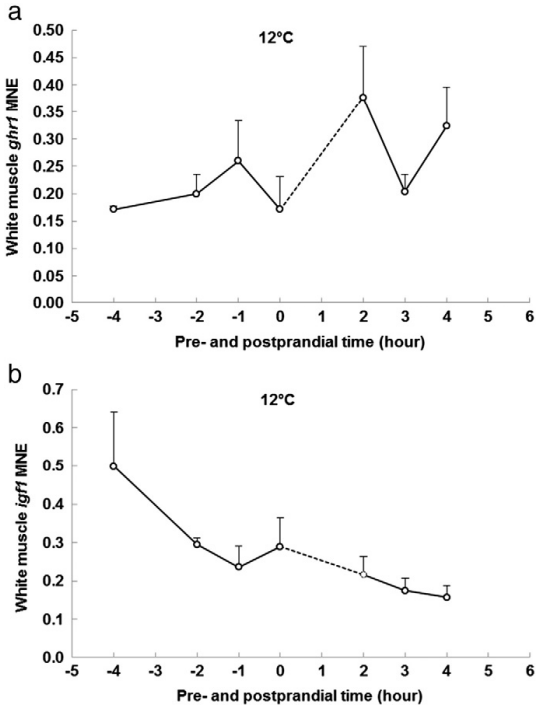

Fig. 10. Growth hormone receptor-1 (*ghr1*) (a) and insulin like growth factor-1 (*igf1*) (b) mRNA levels (mean normalised expression, MNE) in white muscle −4–0 hours (h) pre-prandially and 2–4 h post-prandially from Atlantic salmon reared at 12 °C (mean + SEM). No significant differences within pre- or postprandial sampling groups ($P > 0.05$). Dotted line interpolates between the datasets. n = 6.

Acknowledgements

We thank the staff at Matre Havbruksstasjon (IMR), Lerang Research station (Skretting ARC), NIFES and Skretting ARC laboratory.

The first study at Matre Havbruksstasjon (IMR) was financially supported by the NFR (the Norwegian Research Council, grant number 199683/S40), and the second study at Lerang Research Station by Skretting ARC. The paper is part of the first authors' PhD which is supported by the NFR (grant number 209655/O30), employer Skretting ARC and the collaborating institute NIFES.

References

Attard, M., Thompson, M., Kelly, R., Locatelli, A., 2012. Tamar Estuary Monitoring Report 2012. Natural Resource Management in Northern Tasmania, p. 96 (http://www.nrmnorth.org.au/).

Barrows, F.T., Gaylord, T.G., Stone, D.A.J., Smith, C.E., 2007. Effect of protein source and nutrient density on growth efficiency, histology and plasma amino acid concentration of rainbow trout (*Oncorhynchus mykiss* Walbaum). Aquacult. Res. 38, 1747–1758.

Björnsson, B.T., Johansson, V., Benedet, S., Einarsdottir, I.E., Hildahl, J., Agustsson, T., Jönsson, E., 2002. Growth hormone endocrinology of salmonids: regulatory mechanisms and mode of action. Fish Physiol. Biochem. 27, 227–242.

Brett, J.R., 1979. Environmental Factors and Growth. In: Hoar, W.S., Randall, C.F., Brett, J.R. (Eds.), Fish Physiology. Academic Press, London, pp. 599–667.

Brunetti, L., Recinella, L., Orlando, G., Michelotto, B., Di Nisio, C., Vacca, M., 2002. Effects of ghrelin and amylin on dopamine, norepinephrine and serotonin release in the hypothalamus. Eur. J. Pharmacol. 454, 189–192.

Canosa, L.F., Chang, J.P., Peter, R.E., 2007. Neuroendocrine control of growth hormone in fish. Gen. Comp. Endocrinol. 151, 1–26.

Carter, C.G., He, Z.Y., Houlihan, D.F., McCarthy, I.D., Davidson, I., 1995. Effect of feeding on the tissue free amino acid concentrations in rainbow trout (*Oncorhynchus mykiss* Walbaum). Fish Physiol. Biochem. 14, 153–164.

Carter, C.G., Houlihan, D.F., He, Z.Y., 2000. Changes in tissue free amino acid concentrations in Atlantic salmon, *Salmo salar* L., after consumption of a low ration. Fish Physiol. Biochem. 23, 295–306.

Carter, C.G., Katersky, R.S., Barnes, J.C., Bridle, A.R., Hauler, R.C., 2008. Assessment of fish growth performance under limiting environmental conditions: aquaculture nutrition subprogram. In: Carter, C.G. (Ed.), FRDC Final Report. Tasmanian Aquaculture and Fisheries Institute, Tasmania, Australia, p. 147.

Choi, K., Roh, S.G., Hong, Y.H., Shrestha, Y.B., Hishikawa, D., Chen, C., Kojima, M., Kangawa, K., Sasaki, S., 2003. The role of ghrelin and growth hormone secretagogues receptor on rat adipogenesis. Endocrinology 144, 754–759 (Mar).

Cummings, D.E., Purnell, J.Q., Frayo, R.S., Schmidova, K., Wisse, B.E., Weigle, D.S., 2001. A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans. Diabetes 50, 1714–1719.

Espe, M., Lied, E., Torrissen, K.R., 1993. Changes in plasma and muscle free amino acids in Atlantic salmon (*Salmo salar*) during absorption of diets containing different amounts of hydrolysed cod muscle protein. Comp. Biochem. Physiol. A Physiol. 105, 555–562.

Fänge, R., Grove, D., 1979. 4 Digestion. In: Hoar, W.S., Randall, D.J., Brett, J.R. (Eds.), Fish Physiology. Academic Press, pp. 161–260.

Fauconneau, B., Choubert, G., Blanc, D., Breque, J., Luquet, P., 1983. Influence of environmental temperature on flow rate of foodstuffs through the gastrointestinal tract of rainbow trout. Aquaculture 34, 27–39.

Frøiland, E., Murashita, K., Jørgensen, E.H., Kurokawa, T., 2010. Leptin and ghrelin in anadromous Arctic charr: cloning and change in expressions during a seasonal feeding cycle. Gen. Comp. Endocrinol. 165, 136–143.

Garlick, P.J., 2005. The role of leucine in the regulation of protein metabolism. J. Nutr. 135, 1553S.

Handeland, S.O., Imsland, A.K., Stefansson, S.O., 2008. The effect of temperature and fish size on growth, feed intake, food conversion efficiency and stomach evacuation rate of Atlantic salmon post-smolts. Aquaculture 283, 36–42.

Hevrøy, E.M., Espe, M., Waagbø, R., Sandnes, K., Ruud, M., Hemre, G.I., 2005. Nutrient utilization in Atlantic salmon (*Salmo salar* L.) fed increased levels of fish protein hydrolysate during a period of fast growth. Aquacult. Nutr. 11, 301–313.

Hevrøy, E.M., Azpeleta, C., Shimizu, M., Lanzén, A., Kaiya, H., Espe, M., Olsvik, P.A., 2011. Effects of short-term starvation on ghrelin, GH–IGF system, and IGF-binding proteins in Atlantic salmon. Fish Physiol. Biochem. 37, 217–232.

Hevrøy, E.M., Waagbø, R., Torstensen, B.E., Takle, H., Stubhaug, I., Jørgensen, S.M., Torgersen, T., Tvenning, L., Susort, S., Breck, O., Hansen, T., 2012. Ghrelin is involved in voluntary anorexia in Atlantic salmon raised at elevated sea temperatures. Gen. Comp. Endocrinol. 175, 118–134.

Hevrøy, E.M., Hunskår, C., Gelder, S., Shimizu, M., Waagbø, R., Breck, O., Takle, H., Susort, S., Hansen, T., 2013. GH–IGF system regulation of attenuated muscle growth and lipolysis in Atlantic salmon reared at elevated sea temperatures. J. Comp. Physiol. B. 183, 243–259.

Hill, A.J., Blundell, J.E., 1988. Role of Amino Acids in Appetite Control in Man. In: Huether, G. (Ed.), Amino acid availability and brain function in health and disease. Proceedings of the Advanced Research.

Hrboticky, N., Leiter, L.A., Anderson, G.H., 1985. Effects of L-tryptophan on short-term food-intake in lean men. Nutr. Res. 5, 595–607.

Hunskår, C., 2010. Somatic Growth, Nutrient Utilisation And Endocrine Responses of Atlantic Salmon (*Salmo salar* L.) at high sea Temperatures, University of Bergen. University of Bergen, Bergen, Norway, p. 89.

Inui, A., 2001. Ghrelin: an orexigenic and somatotrophic signal from the stomach. Nat. Rev. Neurosci. 2, 551–560.

Inui, A., Asakawa, A., Bowers, C.Y., Mantovani, G., Laviano, A., Meguid, M.M., Fujimiya, M., 2004. Ghrelin, appetite, and gastric motility: the emerging role of the stomach as an endocrine organ. FASEB J. 18, 439–456.

Jönsson, E., 2013. The role of ghrelin in energy balance regulation in fish. Gen. Comp. Endocrinol. 187, 79–85.

Kaiya, H., Mori, T., Miyazato, M., Kangawa, K., 2009. Ghrelin receptor (GHS-R)-like receptor and its genomic organisation in rainbow trout, *Oncorhynchus mykiss*. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 153, 438–450.

Kaushik, S.J., Luquet, P., 1979. Influence of dietary amino acid patterns on the free amino acid contents of blood and muscle of rainbow trout (*Salmo gairdneri* R.). Comp. Biochem. Physiol. B Comp. Biochem. 175–180.

Kimball, S.R., Jefferson, L.S., 2004. Regulation of global and specific mRNA translation by oral administration of branched-chain amino acids. Biochem. Biophys. Res. Commun. 313, 423–427.

Koskela, J., Pirhonen, J., Jobling, M., 1997. Feed intake, growth rate and body composition of juvenile Baltic salmon exposed to different constant temperatures. Aquacult. Int. 5, 351–360.

Kullgren, A., Jutfelt, F., Fontanillas, R., Sundell, K., Samuelsson, L., Wiklander, K., Kling, P., Koppe, W., Larsson, D.G., Bjornsson, B.T., Jonsson, E., 2013. The impact of temperature on the metabolome and endocrine metabolic signals in Atlantic salmon (*Salmo salar*). Comp. Biochem. Physiol. A Mol. Integr. Physiol. 164, 44–53.

Kwasek, K., Zhang, Y., Dabrowski, K., 2010. Utilization of dipeptide/protein based diets in larval and juvenile Koi carp — post-prandial free amino acid levels. J. Anim. Physiol. Anim. Nutr. 94, 35–43.

Larsen, B.K., Dalsgaard, J., Pedersen, P.B., 2012. Effects of plant proteins on postprandial, free plasma amino acid concentrations in rainbow trout (*Oncorhynchus mykiss*). Aquaculture 326–329, 90–98.

Lyndon, A.R., Davidson, I., Houlihan, D.F., 1993. Changes in tissue and plasma free amino acid concentrations after feeding in Atlantic cod. Fish Physiol. Biochem. 10, 365–375.

Matsuda, K., Miura, T., Kaiya, H., Maruyama, K., Shimakura, S.-I., Uchiyama, M., Kangawa, K., Shioda, S., 2006a. Regulation of food intake by acyl and des-acyl ghrelins in the goldfish. Peptides 27, 2321–2325.

Matsuda, K., Miura, T., Kaiya, H., Maruyama, K., Uchiyama, M., Kangawa, K., Shioda, S., 2006b. Stimulatory effect of n-octanoylated ghrelin on locomotor activity in the goldfish, *Carassius auratus*. Peptides 27, 1335–1340.

Mente, E., Deguara, S., Santos, M.B., Houlihan, D., 2003. White muscle free amino acid concentrations following feeding a maize gluten dietary protein in Atlantic salmon (*Salmo salar* L.). Aquaculture 225, 133–147.

Miegel, R.P., Pain, S.J., van Wettere, W.H.E.J., Howarth, G.S., Stone, D.A.J., 2010. Effect of water temperature on gut transit time, digestive enzyme activity and nutrient digestibility in yellowtail kingfish (*Seriola lalandi*). Aquaculture 308, 145–151.

Miura, T., Maruyama, K., Shimakura, S.-I., Kaiya, H., Uchiyama, M., Kangawa, K., Shioda, S., Matsuda, K., 2006. Neuropeptide Y mediates ghrelin-induced feeding in the goldfish, *Carassius auratus*. Neurosci. Lett. 407, 279–283.

Miura, T., Maruyama, K., Shimakura, S.-I., Kaiya, H., Uchiyama, M., Kangawa, K., Shioda, S., Matsuda, K., 2007. Regulation of food intake in the goldfish by interaction between ghrelin and orexin. Peptides 28, 1207–1213.

Mommsen, T.P., 2001. Paradigms of growth in fish. Comp. Biochem. Physiol. B 129, 207–219.

Ogata, H., 1986. Correlations of essential amino acid patterns between the dietary protein and the blood, hepatopancreas, or skeletal muscle in carp [*Cyprinus carpio*]. Bull. Jpn. Soc. Sci. Fish. 52, 307–312.

Oksbjerg, N., Gondret, F., Vestergaard, M., 2004. Basic principles of muscle development and growth in meat-producing mammals as affected by the insulin-like growth factor (IGF) system. Domest. Anim. Endocrinol. 27, 219–240.

Olsvik, P.A., Vikeså, V., Lie, K.K., Hevrøy, E.M., 2013. Transcriptional responses to temperature and low oxygen stress in Atlantic salmon studied with next-generation sequencing technology. BMC Genomics 14.

Pérez-Casanova, J.C., Lall, S.P., Gamperl, A.K., 2009. Effect of feed composition and temperature on food consumption, growth and gastric evacuation of juvenile Atlantic cod (*Gadus morhua* L.) and haddock (*Melanogrammus aeglefinus* L.). Aquaculture 294, 228–235.

Peterson, R.H., Martin-Robichaud, D.J., 1989. First feeding of Atlantic salmon (*Salmo salar* L.) fry as influenced by temperature regime. Aquaculture 78, 35–53.

Planas, J.V., Méndez, E., Baños, N., Capilla, E., Navarro, I., Gutiérrez, J., 2000. Insulin and IGF-I receptors in trout adipose tissue are physiologically regulated by circulating hormone levels. J. Exp. Biol. 203, 1153–1159.

Reinecke, M., 2006. Insulin-Like Growth Factor I and II in Fish. In: Reinecke, M., Zaccone, G., Kapoor, B. (Eds.), Fish Endocrinology. Science Publishers, Enfield, USA, pp. 87–130.

Riley, L.G., Fox, B.K., Kaiya, H., Hirano, T., Grau, E.G., 2005. Long-term treatment of ghrelin stimulates feeding, fat deposition, and alters the GH/IGF-I axis in the tilapia, *Oreochromis mossambicus*. Gen. Comp. Endocrinol. 142, 234–240.

Schlisio, W., Nicolai, B., 1978. Kinetic investigations on the behaviour of free amino acids in the plasma and of two aminotransferases in the liver of rainbow trout (*Salmo gairdneri* Richardson) after feeding on a synthetic composition containing pure amino acids. Comp. Biochem. Physiol. B Comp. Biochem. 59, 373–379.

Solomon, S., Qin, D., Manning, M., Chen, Z., Marquis, M., Averyt, K.B., Tignor, M., Miller, H.L., 2007. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. 2007 IPCC (Intergovernmental Panel on Climate Changes), pp. 1–996.

Sugino, T., Yamaura, J., Yamagishi, M., Ogura, A., Hayashi, R., Kurose, Y., Kojima, M., Kangawa, K., Hasegawa, Y., Terashima, Y., 2002. A transient surge of ghrelin secretion before feeding is modified by different feeding regimens in sheep. Biochem. Biophys. Res. Commun. 298, 785–788.

Sunde, J., Kiessling, A., Higgs, D., Opstvedt, J., Venturini, G., Rungruangsak-Torrissen, K., 2003. Evaluation of feed protein quality by measuring plasma free amino acids in Atlantic salmon (*Salmo salar* L.) after dorsal aorta cannulation. Aquacult. Nutr. 9, 351–360.

Suryawan, A., Orellana, R.A., Fiorotto, M.L., Davis, T.A., 2011. Triennial Growth Symposium: leucine acts as a nutrient signal to stimulate protein synthesis in neonatal pigs. J. Anim. Sci. 89, 2004–2016.

Thissen, J.P., Pucilowska, J.B., Underwood, L.E., 1994. Differential regulation of insulin-like growth factor I (IGF-I) and IGF binding protein-1 messenger ribonucleic acids by amino acid availability and growth hormone in rat hepatocyte primary culture. Endocrinology 134, 1570–1576.

Torrison, K.R., Lied, E., Espe, M., 1995. Differences in Utilization of Dietary Proteins with Varying Degrees of Partial Pre-hydrolysis in Atlantic Salmon (*Salmo salar* L.) with Genetically Different Trypsin Isozymes. Biopolymers and Isozymes: Structure, Functions and Applications, pp. 432–442.

Unniappan, S., Peter, R.E., 2005. Structure, distribution and physiological functions of ghrelin in fish. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 140, 396–408.

Usher, M.L., Talbot, C., Eddy, F.B., 1991. Effects of transfer to seawater on growth and feeding in Atlantic salmon smolts (*Salmo salar* L.). Aquaculture 94, 309–326.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F., 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 3, 18.

Wood, A.W., Duan, C., Bern, H.A., 2005. Insulin-like growth factor signaling in fish. Int. Rev. Cytol. 243, 215–285.

Wurtman, R.J., Hefti, F., Melamed, E., 1981. Precursor control of neurotransmitter synthesis. Pharmacol. Rev. 32, 315–335.

Yamada, S., Simpson, K.L., Tanaka, Y., Katayama, T., 1981. Plasma amino acid changes in rainbow trout *Salmo gairdneri* force-fed casein and a corresponding amino acid mixture. Bull. Jpn. Soc. Sci. Fish. 47, 1035–1040.

Yamamoto, T., Sugita, T., Furuita, H., 2005. Essential amino acid supplementation to fish meal-based diets with low protein to energy ratios improves the protein utilization in juvenile rainbow trout *Oncorhynchus mykiss*. Aquaculture 246, 379–391.

II

Received: 10 March 2016 | Accepted: 19 July 2016

DOI: 10.1111/anu.12488

ORIGINAL ARTICLE

WILEY Aquaculture Nutrition

High dietary energy level stimulates growth hormone receptor and feed utilization in large Atlantic salmon (*Salmo salar* L.) under hypoxic conditions

V. Vikesså[1,2] | L. Nankervis[1] | E.M. Hevrøy[2]

[1]Skretting ARC (Aquaculture Research Centre), Stavanger, Norway

[2]National Institute of Nutrition & Seafood Research (NIFES), Bergen, Norway

Correspondence
Vibeke Vikesså, Skretting ARC, Stavanger, Norway.
Email: vibeke.vikesaa@skretting.com

Abstract

This study examines how appetite and growth regulation of Atlantic salmon are affected by low dissolved oxygen (LO) and dietary digestible energy levels (DE: high [HE] vs. low [LE]). Long-term exposure to LO resulted in a reduced feed intake, growth, digestible protein and fat retention efficiencies and increased feed conversation ratio and plasma ghrelin concentrations ($p < .05$) compared to high dissolved oxygen (HO). Pair-feeding of rations based on the feed intake of the LO groups, but fed at HO, resulted in a 50% growth improvement in HE diet groups. This suggests that the poor growth under LO was not entirely caused by the reduced feed intake. Salmon adapted to LO by increased haemoglobin concentrations, while osmoregulation was affected by increased plasma chloride concentrations ($p < .05$). Plasma ghrelin concentration was unaffected by DE ($p > .05$). Growth regulation was affected by the HE diet, with increased liver and muscle growth hormone receptor *ghr1* mRNA ($p < .05$), regardless of oxygen level. The growth depression due to low oxygen appears to be related to higher metabolic costs, while higher DE upregulates the GH-IGF system at the *ghr1* level and found to be beneficial for growth, feed intake, oxyregulation and osmoregulation under hypoxia.

KEYWORDS

dietary digestible energy, free amino acids, GH-IGF system, ghrelin, growth and appetite regulation, low oxygen

1 | INTRODUCTION

Environmental hypoxia is recognized by a shortage of oxygen below the requirement of physiological functions of an organism (Farrell & Richards, 2009). Salmonids are sensitive to hypoxia, and a minimum value of 85%–120% dissolved oxygen (DO) saturation is suggested for optimal growth of Atlantic salmon (Thorarensen & Farrell, 2011). Saturation level and duration of exposure decide when DO becomes critical (Nilsson & Randall, 2010). Under intensive farming conditions salmon face periods of low and fluctuating DO not only during warm summers but also in connection to shallow water, low tidal current, algae bloom, short day length, a high fish biomass density and during feeding. Understanding appetite and growth regulation under low DO conditions is relevant to salmon farming at present, but also when facing future challenges of the forecasted climate change towards higher temperatures (Solomon et al., 2007).

Water oxygen and temperature are major environmental factors affecting metabolic rate and growth potential of fish. While temperature directly controls metabolic rate, the oxygen availability in water limits energy metabolism of fish below a certain critical level (Brett, 1979) and therefore plays a permissive role. Oxygen is crucial for metabolic energy production and a shift from aerobic towards anaerobic dependency becomes inefficient in terms of ATP yield (Nilsson, 2010). Fish species possess a range of adaptation mechanisms to maintain oxygen supply in hypoxic conditions including physiological, anatomical and behavioural changes (Chapman & McKenzie, 2009). Providing sufficient oxygen supplies for the digestion is important as it is thought to control the voluntary feed intake (Dam & Pauly, 1995).

Oxyregulation by an immediate elevation of the gill ventilation is a common strategy to extract more oxygen from the water (Nilsson, 2007; Nilsson & Randall, 2010; Perry, Jonz, & Gilmour, 2009) and is also reported for salmonids (Barnes, King, & Carter, 2011; Stevens, Sutterlin, & Cook, 1998). Also, fish can oxyregulate by making physiological changes related to the oxygen carrying capacity of their blood (Nilsson & Randall, 2010; Perry & Gilmour, 2010), or by reducing their metabolic rate and thereby the need for oxygen and energy (oxyconforming) (Nilsson & Randall, 2010; Perry et al., 2009). A reduced feed intake of fish is perceived as a consequence of the latter strategy and is thought to be a major cause for depressed growth of fish at low DO conditions, but other mechanisms affecting growth are unclear. Feed intake can be influenced by diet composition such as dietary digestible energy (DE) level (Bendiksen, Jobling, & Arnesen, 2002) which is readily elevated by higher lipid inclusion in the diet. Oxygen consumption decreased for both Nile tilapia (*Oreochromis niloticus*) and rainbow trout (*Oncorhynchus mykiss*) when fed with high DE (as fat) diets at optimal temperatures under normoxia (Saravanan et al., 2012, 2013). Fish also require less oxygen to utilize DE as fat in the diet, resulting in a higher DE intake compared to DE supplied mainly as protein or starch in the diets (Saravanan et al., 2012, 2013). This indicates that growth is less oxygen demanding for fish feeding on a high dietary DE as fat, which can be an advantage for fish under low DO conditions. Fat is the most energy dense macronutrient, and the energy yield is more than double that of proteins or carbohydrates (Schmidt-Nielsen, 1997). Larger fish would benefit most from high fat diets as both oxygen and DE requirement increase with body size (Glencross, 2008; National Research Council Committee on Nutrient Requirements of and Shrimp 2011). It is therefore interesting to find out how high fat diets could potentially impact appetite and growth regulation under reduced DO conditions.

Fish growth is regulated by the GH-IGF system which is influenced by environmental factors and nutritional status (Beckman, 2011; Deane & Woo, 2009; MacKenzie, VanPutte, & Leiner, 1998; Pérez-Sánchez, Martí-Palanca, & Kaushik, 1995; Pérez-Sánchez et al., 2002; Pickering, Pottinger, Sumpter, Carragher, & Le Bail, 1991) determined by feed intake and diet composition and stress. GH1 production is under hypothalamic control and release from the pituitary is stimulated by growth hormone (GH)-releasing hormone (Ueno, Yamaguchi, Kangawa, & Nakazato, 2005) or the appetite stimulating hormone ghrelin (Inui et al., 2004; Kaiya et al., 2003; Ueno et al., 2005). GH1 can stimulate tissue growth either directly or indirectly by binding to GH1 receptors (GHR1) and stimulate synthesis of insulin-like growth factor-1 (IGF1) in the liver and other tissues (Beckman, 2011; Pérez-Sánchez, 2000). The GHR1 declines in fasted fish implying a reduced stimulation of IGF1 synthesis and growth (Gray et al., 1992; Pérez Sánchez, Martí-Palanca, & Le Bail, 1994), but also lack of negative feedback to the GH1 synthesis (Duan, 1998; Pérez Sánchez et al., 1994) leading to a GH1 accumulation (Pérez-Sánchez, 2000). GH1 also has a role in metabolic use of energy from fat, which can be impacted by nutritional status and DE level (Company, Calduch-Giner, Kaushik, & Pérez-Sánchez, 1999; Deane & Woo, 2009; Pérez-Sánchez, 2000). Feeding a high fat diet is found to raise the GH1 level in several fish species (Cameron, Gurure, Reddy, Moccia, & Leatherland, 2002; Company et al., 1999; Pérez-Sánchez, 2000) possibly caused by a shift in use of dietary fat as metabolic energy source rather than proteins (a protein sparing effect) (Company et al., 1999; Pérez-Sánchez, 2000) or by an enhanced lipolytic activity of GH1 (Björnsson, 1997; Hevrøy et al., 2013). Elevated plasma GH1 levels under low DO conditions are reported in rainbow trout, but whether this was caused by the low oxygen or reduced feed intake was not investigated (Pickering et al., 1991). At low DO, feed intake in fish is reduced which is considered to be the primary cause of growth reduction (Brett, 1979; Carter, Katersky, Barnes, Bridle, & Hauler, 2008). However, it is unclear whether the low DO directly affects growth regulatory mechanisms in fish.

This study investigates how long-term exposure to low DO affects regulation of appetite and growth and how this is modulated by dietary fat levels. To elucidate this, Atlantic salmon were kept at normal water DO or low water DO levels and fed to apparent satiation either with a high DE or low DE diet. To determine whether low feed intake is the primary cause of depressed growth under low DO conditions, a technique of pair-feeding was applied.

2 | MATERIALS AND METHODS

The experiment was conducted according to guidelines given by Norwegian Regulation on Animal Experimentation and EC Directive 86/609/EEC, and the protocol was approved by the National Animal Research Authority.

2.1 | Experimental conditions

Atlantic salmon (AquaGen strain, under-yearling smolt) were reared at Lerang Research Station, Norway (59°N; Skretting ARC), using standard practices in 8.0 ± 0.2°C (*SD*) seawater and continuous light 24:0 to a body weight (BW) of 1.32 ± 0.32 kg. Sexually immature salmon were intraperitoneally PIT tagged (Passive Integrated Transponder, Trovan Unique glass tags, 2 × 12 mm) and acclimatized to 18 experimental tanks (7,000 L, 12°C seawater) for two weeks prior to the experiment. These tanks were randomly allocated to six experimental treatments in triplicate, four of which being combinations of two diets and two oxygen levels, while the other two treatments were pair-feeding groups to separate the diet influence from that of low DO conditions (Table 1). The pair-fed groups were given feed rations based on the feed intake of the corresponding low dissolved oxygen (LO) group, but were maintained under high dissolved oxygen (HO) conditions. Two experimental diets were produced as 9-mm extruded pellets at Skretting Feed Technology Plant (Stavanger, Norway) differing in digestible energy (DE), high digestible energy (HE) 21.1 MJ kg^{-1} and low digestible energy (LE) 19.3 MJ kg^{-1} (Table 2). Yttrium oxide was added in both diets as a marker to enable determination of nutrient digestibility. Each of the diets (HE and LE) was fed twice daily in excess of satiation, using automatic feeders. Uneaten feed was collected daily, dried to constant weight, re-weighed and used to correct daily feed intake data.

TABLE 1 Experimental treatment set-up: two diet treatments differing in DE level were combined with two different DO regimes and pair-feeding at 12°C

	High oxygen (HO) 7.7 mg L^{-1}	Low oxygen (LO) 5.0–8.0 mg L^{-1}	High oxygen (HO) Pair-feeding of LO rations
High digestible energy (HE), 21.1 MJ kg^{-1}	HE/HO	HE/LO	HE/HO p
Low digestible energy (LE), 19.3 MJ kg^{-1}	LE/HO	LE/LO	LE/HO p

DE, dietary digestible energy level, MJ kg^{-1}; DO, dissolved oxygen level, mg L^{-1}; HE and LE, high or low digestible energy diet; HO and LO, high or low dissolved water oxygen; p, pair-feeding of LO rations.

The HO group was maintained at 7.7 ± 0.7°C mg L^{-1} (88% saturation, Fig. 1) for the experimental period. The low dissolved oxygen (LO) regime was designed to mimic a possible natural/farming situation with the oxygen concentration maintained at approximately 5.0–5.5 mg L^{-1} (57%–63% saturation) with one daily peak up to approximately 8 mg L^{-1} (92% saturation). Elevation of oxygen in the LO groups started at approximately 07.00 hrs, while the decline started at approximately 16.00 hrs. Oxygen was measured in the outlet and in the upper layer of the water column (at approximately 0.5 m depth) in each tank on a daily basis. Automatic measurements were carried out by Oxyguard sensors placed in the outlet and recorded every 15 min by the software Genesis 32 Automation Suite (produced by Iconics Inc). While the first meal was given when the oxygen was just starting to increase (07.30–08.30 hrs), the 2nd meal (12.30–14.00 hrs) coincided with the oxygen peak (12.00–18.00 hrs). The LO profile was made by adjusting the oxygen supply to header tanks providing water to the experimental tanks based on tank outlet oxygen levels and controlled by a Program Logic Controller system (PLC, Moeller, Bonn, Germany). The salinity of the water was 28–30 g L^{-1}, temperature was held at 12.1 ± 0.1°C (*SD*), and light was continuous (24:0).

2.2 | Sampling

All fish were weighed, and their PIT-tag numbers (AEG ARE H5) recorded the day before they were presented to the experimental diets, for sampled fish after 113 feeding days and for remaining fish after 118 feed days. Postprandial sampling of three fish per tank was conducted 4 hrs after the meal was initiated at the 113-day sampling point. Sampled fish were partially anaesthetized by applying isoeugenol (AQUI-S; 8 ml 1,000 L^{-1} water) into the tanks before netting fish individually and transferring to a second anaesthetic container (70 mg tricaine methanesulfonate L^{-1} water). Blood samples were taken from three fish per tank from the caudal blood vessel with heparinized 4 ml vacutainers (lithium heparin 60 USP). Each fish blood sample was centrifuged at 3,600 g for 7 min at 4°C before plasma was aspirated and stored at −20°C for further analysis of free amino acids (FAA) and concentration of hormones. Two remaining samples of whole blood were stored at 4°C for consecutive analyses of pH, gases and ions on sampling site and haematology parameters within the next 24 hrs at NIFES laboratory.

TABLE 2 Ingredient and nutrient composition of experimental diets

	HE	LE
Ingredient (name of producer) (g kg^{-1})		
Wheat (Svenska Lantmannen)	58	56
Wheat gluten (Syral)	60	59
Faba beans (Soufflet)	59	58
Soya protein concentrate (Imcopa)	300	300
Wheat starch (Cargill)	0	66
Fish meal (Welcon)	150	150
Rapeseed oil (Linas Agro)	209	171
Fish oil (FF Skagen)	135	111
DL-Methionine (Evonik)	2.4	2.4
Lysine-HCL 78% (Paik Kwang)	5.7	5.7
Astaxanthin 10% (DSM)	0.4	0.4
Yttrium premix 10 % (Trouw Nutrition)	1.0	1.0
Vitamin premix[a]	1.1	1.1
Mineral premix[a]	18.5	18.5
Sum	1000	1000
Composition		
Moisture (g kg^{-1})	55	65
Protein (g kg^{-1})	381	382
Fat (g kg^{-1} as is)	369	316
Gross energy (GE, MJ kg^{-1})	25.3	24.2
Digestible energy (DE, MJ kg^{-1})	21.1	19.3
Amino acids (mg g^{-1} protein)		
Arginine	61	61
Histidine	25	25
Isoleucine	42	40
Leucine	73	71
Lysine	72	71
Methionine	24	24
Cystine	13	13
Phenylalanine	45	44
Tyrosine	27	27
Threonine	39	38
Valine	44	45
Alanine	44	46
Aspartic acid	98	97
Glutamic acid	178	174
Glycine	44	44
Proline	55	51
Serine	47	47
EFAA	427	420
Total FAA	933	920

HE and LE; high or low digestible energy diet; EFAA, essential free amino acids; Total FAA, sum of all analysed FAA.
[a]Proprietary to Skretting.

White muscle tissue was sampled out from the medial cutlets between the dorsal fin and the lateral line from three sampled fish per tank and stored at −80°C prior to further analysis of FAA (pooled) and gene expressions, respectively. Liver samples were also taken from each sampled fish and stored at −80°C for gene expression analyses.

Nine whole fish from the first day were homogenized and a pooled sample of 200 ml frozen at −20°C for protein, fat and moisture analysis. Pooled whole fish homogenates were similarly processed from six

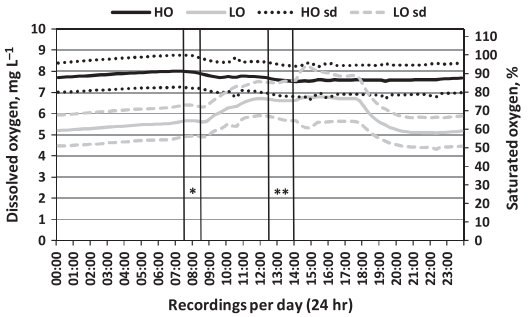

FIGURE 1 Dissolved oxygen levels (mg L^{-1} on the left *y*-axis, % oxygen saturated on the right *y*-axis) in the outlet of experimental tanks with high (HO) and low (LO) dissolved oxygen. Standard deviations (*SD*) are shown in dotted lines for each oxygen treatment. The first and second meal are indicated with * and **, respectively. HO and LO, high or low dissolved water oxygen

fish per tank at the final sampling. Faeces samples, collected from all remaining fish by manual stripping 4 hrs postprandially, and feed samples from the final sampling were stored at −20°C for further analysis of yttrium and gross energy (GE).

2.3 | Mortality

All fish in one tank died after 52 days of feeding due to an accidental mechanical failure in treatment HE/HO p, which left this group with duplicates instead of the intended triplicates. The only other mortality was a single fish in one of the tanks belonging to the LE/LO treatment.

2.4 | Analytical methods

Single samples of whole blood were analysed for the partial pressure of carbon dioxide and oxygen (pCO_2 and pO_2), pH and concentration of Na^+ and Cl^- by a clinical I-STAT analyser using CG8+ cartridges (I-STAT Corporation, Windsor, USA). A second whole blood sample was immediately transferred to disposable heparinized (32 µl Li Hep) capillary pipettes for centrifugation (10,000 ***g*** for 5 min), and haematocrit evaluated using a Micro-Haematocrit graph (Sigma Laborzentrifugen GmbH). A third sample was analysed for red blood cell count (RBC) and concentration of haemoglobin (Hb) with CELL-DYN 400 Hematology Analyzer (Sequoia-Turner Corporation).

Protein in feed and fillets was estimated from Kjeldahl Nitrogen (N x 6.25, NMKL method no. 6, 4th ed. 2003). Dry matter in feed was determined gravimetrically after drying to constant weight, while ash in feed was determined gravimetrically after combustion at 550°C for 16–18 hrs (NMKL method No. 23, 3rd ed. 1991). Yttrium oxide in feed and pooled samples of faeces (per tank) was analysed using inductively coupled plasma mass spectrometry based on the method NS-EN ISO 11885. GE (MJ) in feed and faeces was determined by bomb calorimetry (ISO 9831:1998).

Crude fat analysis of pooled samples of homogenized fish was carried out by a modified version of the Soxhlet method (AOCS Official Method Ba 3-38 re-approved in 2009) applying dichloromethane and an automatic extraction system. Fat in feed was analysed by nuclear magnetic resonance on a QuickFat analyzer (Anvendt Teknologi AS; NMKL method No. 199, 2014).

The level of amino acids in feed was analysed photometrically after separation by ionic exchange chromatography and reaction with ninhydrin reagent and sodium citrate eluent (EU method, Commission Directive 98/64/EC). FAA in pooled samples of plasma and white muscle tissue were also analysed photometrically after separation by ionic exchange chromatography and reaction with a ninhydrin reagent and lithium eluents (Biochrom method AAAFAQ8).

Plasma hormone concentrations of IGF-1 and ghrelin were measured in single samples using fluoroimmunoassay and radioimmunoassay, respectively, using the methods as described in Hevrøy (Hevrøy et al., 2012, 2013). For IGF-1 in plasma, a dissociation-enhanced lanthanide fluorescence immunoassay (DELFIA) method using Europium (Eu)-labelled IGF-1 tracer (15.9 ng ml^{-1}; 1244-302; Perkin Elmer), rabbit antibarramundi IGF-1 polyclonal antiserum (diluted 1:2,072; GroPep, Australia) and recombinant salmon IGF-1 (GroPep, Australia) were used (Hevrøy et al., 2013). Ghrelin levels in salmon plasma were measured with a heterologous assay using a 125I-radioimmunoassay kit for human ghrelin specific for biologically active (octanoylated) ghrelin (Linco Research Inc., St. Charles, Missouri) validated for use in Atlantic salmon (Hevrøy et al., 2011).

RNA extraction and real-time reverse transcription quantitative polymerase chain reaction (RT-qPCR) was used to measure transcription of the mRNA expression for *ghr1* and *igf1* in single samples of liver and white muscle tissue.

Total RNA was extracted using Trizol reagent according to the manufacturer's recommendation (Invitrogen, USA). To eliminate genomic DNA, the total RNA sample was subjected to DNase treatment (DNeasy, Ambion), using the manufacturer's protocol. Quantity and quality of RNA were assessed with NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), where a 260/280 nm absorbance ratio of 1.8 – 2.0 indicates a pure RNA sample. To evaluate the RNA integrity, the 6000 Nano LabChip® kit (Agilent Technologies, Palo Alto, CA, USA) was used, with RNA integrity numbers (RIN) between 9.0 and 10.0 for both tissues.

PCR primers for the target genes *igf1* and *ghr1* were designed with Primer Express software and Biosoft software, as previously described (Hevrøy et al., 2012). Housekeeping genes β-actin (*actb*), eukaryotic translation elongation factor 1αβ (*ef1ab*) and ribosomal protein L13 (*rpl13*) were used as reference genes (Hevrøy et al., 2013).

Reverse transcription (RT) was performed using a modified Multiscribe reverse transcription protocol (GeneAmp PCR 9700; Applied Biosystems, AB), as previously described (Hevrøy et al., 2006). White muscle and liver samples were analysed in duplicate with a total RNA input of 500 ng ± 5% on 96-well plates for the RT and real-time PCR. Real-time PCR amplification and analysis were performed using a LightCycler 480 Real-time PCR system (Roche Applied Science, Basel, Switzerland). The real-time PCR SYBR Green Master Mix (LightCycler 480 SYBR Green master mix kit; Roche) contained

FastStart DNA polymerase and gene-specific primers at a final concentration of 500 nM. Two microlitres of diluted cDNA from each well was transferred to a new plate, and 8 µl real-time master mix was added. PCR was achieved with a 5-min activation and denaturizing step at 95°C followed by 45 cycles of a 10-second denaturizing step at 95°C, a 20-second annealing step at 60°C and 30-second synthesis step at 72°C. The efficiency of real-time PCR was monitored using twofold dilution curves of total RNA using five points (range, 1,000 to 31 ng μl^{-1}). Target gene mean normalized expression (MNE) was determined using a normalization factor calculated with the geNorm software (Vandesompele et al., 2002).

2.5 | Calculations

The following calculations were used:

Specific growth rate (SGR) %: (ln final BW, g − ln initial BW, g)/days × 100.

Feed conversation ratio (FCR) (on dry matter basis [DM]): feed eaten, g (DM)/weight gain, g.

Digestible energy (DE) of diets, MJ kg^{-1}: gross energy in diet (GE), MJ kg^{-1} × Apparent digestibility coefficient of energy in diet.

Protein, fat and energy apparent digestibility coefficient (ADC), %: = 100 − (100 × (yttrium in diet/yttrium in faeces) × (nutrient in faeces/nutrient in diet)).

Yttrium is in mg kg^{-1}. Nutrient is protein (mg kg^{-1}), fat (mg kg^{-1}) or energy (MJ kg^{-1}).

Digestible protein (DP) and fat (DF) retention efficiencies % were calculated according to Saravanan et al. (2012).

Mean red blood cell volume (MCV), fl: (Hct, %/RBC count, 10^{12} L^{-1}) × 1,000.

2.6 | Statistics

All data are presented as mean values ± standard error of the mean (*SEM*) unless otherwise described. Tanks are used as experimental units. Statistical analyses were conducted using Unistat 6.0 (Unistat Ltd.), and significant differences were accepted at p values < .05. All data per DE level were subjected to a one-way analysis of variance (ANOVA) to find the effect of pair-feeding. All other data, excluding the pair-feeding groups, were subjected to a two-way analysis of variance (ANOVA) with interactions of two factors (DO and DE). The post hoc test Tukey HSD was applied if any significant differences were detected by the ANOVA.

3 | RESULTS

3.1 | Feed intake, growth and blood parameters

LO treatments significantly reduced feed intake, SGR, DP and DF retention efficiencies, pH and pCO_2, but increased FCR, Hb in blood and concentration of Cl^- in plasma (p < .05) (Table 3). Only FCR was affected by dietary DE levels with LE diets giving higher values than HE diet groups (p < .05) (Table 3). Successful pair-feeding was reflected in similar daily feed intake (p > .05) between the pair-fed groups (HO p) and the corresponding group at LO conditions (Table 3). Pair-feeding with HE diets at HO conditions gave higher SGR and pH in plasma and lower FCR and Hb concentration in the blood compared to LO conditions (p < .05). When pair-feeding with the LE diet at HO, this positive effect was not detected for SGR, pH or the Hb concentration (p > .05). FCR was also lower when pair-fed with LE diets, but pair-feeding with LE diets also gave higher DF retention efficiency and higher plasma pCO_2 compared to fish groups fed LE diets at LO conditions (p < .05). There were no significant differences in Hct, RBC count and MCV in blood or plasma pO_2 and Na^+ between DO and DE treatment groups (p > .05) (Table 3).

3.2 | Plasma and white muscle FAA

LO conditions resulted in higher white muscle concentrations of isoleucine and leucine than in fish held at HO (p < .05 and p < .01) (Table 4b). No other FAA in plasma and white muscle were significantly affected by DO conditions (p > .05) (Table 4a,b). Dietary DE level did not give any significant differences in FAA in plasma and white muscle (p > .05). Pair-feeding of LE diets at HO conditions gave lower plasma lysine concentration than at LO (p < .05) (Table 4a). The same treatment gave no difference in white muscle isoleucine concentration between the pair-fed and the LO group (p > .05), but the pair-fed group had a higher white muscle concentration than the group fed HE diet in surplus at HO conditions (p < .05) (Table 4b).

3.3 | Plasma ghrelin and IGF-I

There was a significant effect of DO on plasma ghrelin levels. LO treatments resulted in significantly higher plasma ghrelin concentration than HO treatments (p < .05) (Fig. 2). There were no other significant effects of DE, interactions of DE and DO on plasma ghrelin level. Similarly, there was no significant effect of pair-feeding (p > .05) within each energy level. There were no significant effects from DE, DO or pair-feeding treatments on plasma concentrations of IGF-1 (p > .05, mean concentration 36.2 ± 0.7 ng ml^{-1}).

3.4 | Liver and white muscle *ghr1* and *igf-1* mRNA

Fish fed HE diets had significantly higher expression of *ghr1* mRNA in both liver and muscle tissue than feeding with LE diets (p < .05) (Figs 3a & 4). DE levels did not have any significant effect on the mRNA levels of *igf1* in liver (Fig. 3b) or muscle tissue (p > .05, MNE of 0.45 ± 0.03 mRNA). Level of DO did not significantly affect liver or white muscle mRNA expression of *ghr1* and *igf1* when fish were fed in surplus (p > .05) (Figs 3a,b & 4). No significant effects from interaction of DO conditions and DE levels were detected (p > .05).

Pair-feeding with LE diets at HO gave significant higher *ghr1* mRNA levels in the liver than at LO conditions (p < .05) (Fig. 3a), while neither oxygen nor pair-feeding treatment affected liver *igf1* expression for fish fed the LE diet (p > .05) (Fig. 3b). Pair-feeding with HE diets at HO

TABLE 3 Growth performance parameters, haematology and plasma homoeostasis indicators for Atlantic salmon given diets differing in DE level under different DO conditions. n = 3 (except for HE/HO p, n = 2). n = 6 when sorted by DO treatment groups or DE diet groups

	HE/HO	HE/LO	HE/HO p	LE/HO	LE/LO	LE/HO p	DO	DE	DO × DE
BW final, g	$3.85/0.13^{a}$	$3.41/0.05^{b}$	$3.67/0.05^{ab}$	$3.73/0.10^{x}$	$3.33/0.18^{y}$	$3.49/0.08^{xy}$	*		
Feed intake DM, % BW day^{-1}	$1.48/0.04^{a}$	$1.33/0.05^{b}$	$1.34/0.07^{ab}$	1.50/0.06	1.33/0.11	1.32/0.04	*		
SGR, % day^{-1}	$0.91/0.01^{a}$	$0.79/0.02^{c}$	$0.85/0.01^{b}$	0.88/0.02	0.79/0.06	0.82/0.02	*		
FCR DM	$0.89/0.02^{ab}$	$0.94/0.03^{a}$	$0.88/0.02^{b}$	$0.95/0.03^{xy}$	$0.98/0.02^{x}$	$0.92/0.02^{y}$	*	*	
DP retention efficiency, %	46.2/1.6	42.9/2.8	45.4/3.9	45.6/2.1	43.4/0.4	47.3/3.46	*		
DF retention efficiency, %	75.2/2.6	66.5/2.6	73.6/11.1	$75.3/2.8^{y}$	$71.9/1.4^{y}$	$83.7/2.9^{x}$	*		
Hb, g dL^{-1}	$9.9/0.3^{b}$	$10.8/0.2^{a}$	$9.8/0.3^{b}$	10.6/0.2	10.8/0.3	10.0/0.7	*		*
Hct, %	46.3/3.3	48.9/3.2	46.8/1.6	49.4/3.8	48.2/2.5	44.7/2.3			
RBC count, 10^{12} L^{-1}	1.34/0.12	1.39/0.08	1.38/0.12	1.37/0.13	1.38/0.14	1.33/0.19			
MCV, fl	346/6	352/16	353/33	362/13	353/27	340/33			
pH	$7.07/0.04^{ab}$	$7.00/0.03^{b}$	$7.12/0.03^{a}$	7.07/0.00	7.06/0.02	7.04/0.05	*		
pO_2, kPa	1.98/0.64	2.00/0.07	1.97/0.00	1.67/0.67	1.66/0.31	1.69/0.54			
pCO_2, kPa	5.33/0.38	4.48/0.59	4.75/0.02	$5.80/0.19^{x}$	$4.21/0.33^{y}$	$5.71/0.87^{x}$	*		
cNa^+, mM	169/2	175/3	168/3	169/3	171/3	168/3			
cCl^-, mM	$139/2^{b}$	$146/3^{a}$	$139/2^{ab}$	138/3	143/6	139/4	*		

DE, dietary digestible energy level; DO, dissolved oxygen level; HE and LE, high or low digestible energy diet; HO and LO, high or low dissolved water oxygen; p, pair-feeding of LO rations.

^{a,b,c} and ^{x,y} values represent means for HE and LE diet groups, respectively. Different letters per row are significantly different (one-way ANOVA, $p < .05$).

*Values represent means that are significantly different with respect to DO, DE levels or interactions of these (DE × DO) (two-way ANOVA, $p < .05$).

did not give significantly different *igf1* mRNA levels ($p > .05$) in the liver than fish feeding at LO conditions, but was significantly higher than the group fed in surplus at HO conditions ($p < .05$) (Fig. 3b). There was no effect of pair-feeding with HE diets on liver *ghr1* mRNA ($p > .05$) (Fig. 3a). Pair-feeding did not result in any significant differences in muscle mRNA levels of *ghr1* (Fig. 4) and *igf1* ($p > .05$). Mean muscle *igf1* mRNA was not affected by any of the treatments ($p > .05$).

4 | DISCUSSION

This study reports on the role of key appetite and growth regulating mechanisms in Atlantic salmon when exposed to low oxygen concentrations and related physiological responses mediated by dietary nutrients.

The current findings of reduced feed intake, growth, DP and DF retention efficiencies and increased FCR at LO are in line with several previous reports concerning low oxygen conditions for several fish species, including Atlantic salmon (Bernier & Craig, 2005; Brett, 1979; Carter et al., 2008; Foss, Evensen, & Øiestad, 2002; Glencross, 2009; Hansen et al., 2015; Pedersen, 1987; Remen, Oppedal, Torgersen, Imsland, & Olsen, 2012; Remen et al., 2014). Reduced growth under hypoxic conditions has been linked to lower appetite (Buentello, Gatlin, & Neill, 2000; Foss et al., 2002; Pichavant et al., 2001; Thetmeyer, Waller, Black, Inselmann, & Rosenthal, 1999; Tran-Duy, Schrama, van Dam, & Verreth, 2008); however, growth and appetite can be challenging to separate, because one often affects the other. The technique of pair-feeding was therefore employed to circumvent this experimental limitation. By separating data in DE levels, a one-way ANOVA revealed that pair-fed fish at normoxia (HE/HO p) had about 50% greater growth than fish fed at hypoxic conditions (HE/LO). This means that appetite cannot entirely explain the degree of growth reduction exhibited by the fish in LO compared to the HO treatment. A similar trend was seen in the LE diet group, but was not significant. In contrast, similarly performed pair-feeding studies with turbot (*Scophthalmus maximus*) and seabass (*Dicentrarchus labrax*) indicate that growth reduction under low oxygen conditions is caused primarily by a reduced feed intake (Pichavant et al., 2001). The oxygen demand was reduced by the reduced feed intake, and it seems that this way of oxyconforming was sufficient to allow the species to adapt to the LO treatments. The degree of low oxygen challenge is impossible to compare between water temperatures because oxygen solubility varies with temperature. It is therefore yet unclear whether the results from the turbot and seabass study at 17 and 22°C represent a difference between the two species and Atlantic salmon in the present study, which was conducted at 12°C.

A lack of appetite downregulation under LO conditions in the present study was supported by an increase in circulating ghrelin, which is known to stimulate appetite (Asakawa et al., 2001; Inui, 2001; Nakazato et al., 2001). It is therefore likely that low environmental oxygen has a direct growth inhibitory role separate to appetite suppression. Similar findings are reported for rats (Raff, 2003), where the authors found ghrelin unlikely to be the primary cause of low feed intake and growth at hypoxic conditions. Evidently endocrine cues for appetite were present, as indicated by the elevated ghrelin levels at LO in the present study, but as feed intake was still lower compared to HO

TABLE 4 Mean (a) plasma concentrations (μmol ml^{-1}) and (b) white muscle concentrations (μmol g^{-1}) of single essential FAA, EFFA and total FAA as means per treatment for Atlantic salmon given diets differing in DE level under different DO conditions. *n* = 3 (except for HE/HO p, *n* = 2). *n* = 6 when sorted by DO treatment groups

(a) μmol ml^{-1}	HE/HO	HE/LO	HE/HO p	LE/HO	LE/LO	LE/HO p	
Threonine	0.481/11.994	0.482/5.423	0.417/9.205	0.525/16.580	0.512/4.972	0.428/1.426	
Valine	0.980/24.400	0.855/9.919	0.855/15.970	0.958/16.822	0.953/6.687	0.892/6.112	
Methionine	0.273/4.218	0.264/6.826	0.275/5.168	0.271/3.120	0.272/3.817	0.246/1.327	
Isoleucine	0.487/11.940	0.421/8.074	0.430/9.547	0.479/6.531	0.506/4.094	0.460/1.959	
Leucine	0.807/21.947	0.695/11.000	0.690/13.449	0.788/11.702	0.813/5.968	0.744/3.601	
Phenylalanine	0.272/5.652	0.247/5.700	0.275/4.987	0.277/2.455	0.271/3.181	0.249/2.618	
Lysine	0.833/8.141	0.671/18.020	0.623/20.947	0.788/3.980^{xy}	0.841/7.428^{x}	0.647/5.822^{y}	
Histidine	0.096/1.497	0.096/2.339	0.104/1.623	0.096/1.222	0.100/1.950	0.095/1.314	
Tryptophan	0.023/0.912	0.023/0.242	0.022/0.464	0.024/0.525	0.020/0.500	0.018/0.217	
Arginine	0.295/1.309	0.250/10.639	0.262/7.365	0.308/1.525	0.302/4.133	0.261/7.834	
EFAA	4.548/89.309	4.004/75.393	3.953/87.796	4.514/60.772	4.590/37.629	4.040/2.603	
Total FAA	10.585/217.881	9.433/102.978	9.097/160.672	10.704/182.630	9.657/93.841	9.310/50.741	
(b) μmol g^{-1}	**HE/HO**	**HE/LO**	**HE/HO p**	**LE/HO**	**LE/LO**	**LE/HO p**	**DO**
Threonine	0.886/0.112	0.904/0.103	0.732/0.028	0.786/0.102	0.788/0.106	0.752/0.204	
Valine	0.325/0.014	0.343/0.041	0.314/0.023	0.280/0.023	0.314/0.045	0.342/0.008	
Methionine	0.141/0.017	0.134/0.014	0.121/0.001	0.138/0.026	0.136/0.021	0.142/0.037	
Isoleucine	0.163/0.004	0.168/0.006	0.147/0.014	0.119/0.016^{y}	0.140/0.022^{xy}	0.166/0.008^{x}	*
Leucine	0.289/0.010	0.303/0.029	0.284/0.017	0.224/0.023	0.257/0.045	0.295/0.006	*
Phenylalanine	0.089/0.012	0.086/0.005	0.089/0.008	0.090/0.013	0.090/0.012	0.090/0.003	
Lysine	2.016/0.245	1.537/0.158	1.548/0.506	1.600/0.219	1.495/0.523	1.721/0.085	
Histidine	0.279/0.088	0.223/0.089	0.180/0.054	0.311/0.261	0.320/0.061	0.339/0.167	
Tryptophan	[a]						
Arginine	0.226/0.026	0.200/0.008	0.209/0.053	0.167/0.014	0.201/0.080	0.202/0.028	
EFAA	4.414/0.248	3.898/0.359	3.623/0.524	3.715/0.498	3.741/0.643	4.047/0.448	
Total FAA	31.695/0.467	30.640/0.596	31.099/0.087	30.059/0.577	30.736/1.437	31.408/0.577	

FAA, free amino acids; EFAA, the sum of essential FAA; total FAA, the sum of all analysed FAA; DE, dietary digestible energy level; DO, dissolved oxygen level; HE and LE, high or low digestible energy diet; HO and LO; high or low dissolved water oxygen; p, pair-feeding of LO rations.

$^{x, y}$Values represent means for LE diet groups. Different letters per row are significantly different (one-way ANOVA, $p < .05$).

*Values represent means that are significantly different with respect to DO (two-way ANOVA, $p < .05$).

[a]Not analysed.

groups, other functions of the metabolism must have been limited by the low DO conditions.

There are higher energetic costs related to low water oxygen (Brett, 1979), which in the present study was supported by reduced retention efficiencies of DP and DF, showing that fish were using more of the energy from digested protein and fat rather than depositing it compared to fish at HO. A metabolic downregulation affects functions of the whole metabolism, and there were several signs of a disrupted homoeostasis in the LO fish group as evidenced by an elevated level of Hb and Cl^- and lower pH and pCO_2. These responses were similar between pair-fed groups at HO to that found for satiated fish at HO, indicating that the differences recorded are due to water oxygen level rather than differences in feed intake. A reduced blood pH is a common reaction to an increased anaerobic metabolism in fish exposed to hypoxia (Nilsson & Randall, 2010) and reduces the bloods affinity to oxygen which releases more oxygen into the tissues (Jensen, Fago, & Weber, 1998). However, it cannot readily explain why pCO_2 is also lower in blood from LO-treated groups. As CO_2 excretion increases with fish consuming more oxygen (Kieffer, Alsop, & Wood, 1998), it may also be likely for the inverse situation to be true, with reduced blood CO_2 levels in LO-treated fish due to lower oxygen availability. Changes in haematology and ion concentrations are known as secondary responses to environmental stress (Barton, 2002), and the raised concentration of Cl^- and Na^+ in LO fish may indicate an imbalance of ion regulation. This might also have an impact on the oxygen binding capacity because a higher influx of Na^+ into red blood cells from rainbow trout is reported to inhibit the ability of Hb to bind oxygen and thereby improving the release of oxygen for the metabolism (Ferguson

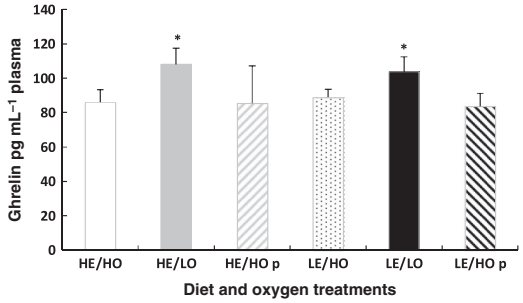

FIGURE 2 Concentration of ghrelin (pg ml^{-1}) in plasma from Atlantic salmon given diets differing in DE level under different DO conditions. The letter p represents pair-feeding of LO rations. Means with the superscript symbol * are significantly different with respect to DO level when fed in surplus (two-way ANOVA, $p < .05$). $n = 6$ when sorted on DO treatment groups. DE, dietary digestible energy level; DO, dissolved oxygen level; LO, low dissolved oxygen

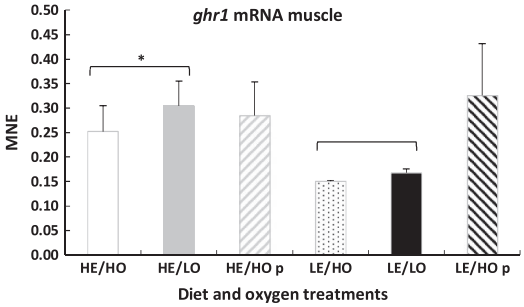

FIGURE 4 Mean normalized expression (MNE) of *ghr1* mRNA levels in white muscle from Atlantic salmon given diets differing in DE level under different DO conditions. The letter p represents pair-feeding of LO rations. Means with the superscript symbols * are significantly different with respect to DE level (two-way ANOVA, $p < .05$). $n = 6$ when sorted on DE diet groups. DE, dietary digestible energy level; DO, dissolved oxygen level; LO, low dissolved water oxygen

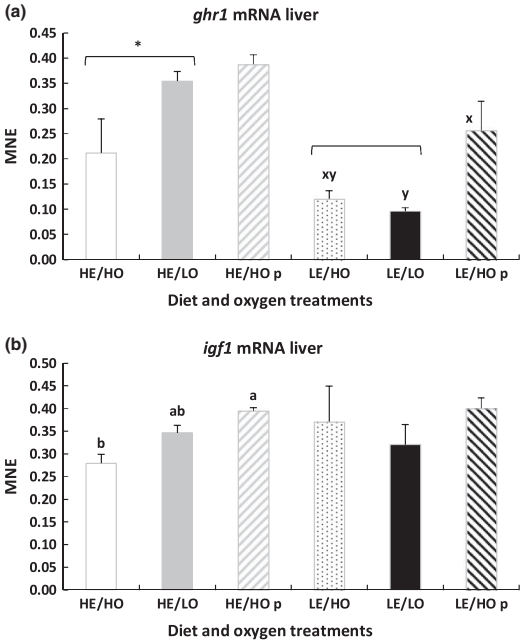

FIGURE 3 Mean normalized expression (MNE) of (a) *ghr1* and (b) *igf1* mRNA levels in livers from Atlantic salmon given diets differing in DE level under different DO conditions. The letter p represents pair-feeding of LO rations. Means per parameter with different superscript letters ((a) x–y for LE diet groups and (b) a–b for HE diet groups) are significantly different (one-way ANOVA, $p < .05$). (a) $n = 3$ (except for HE/HO p, $n = 2$). (b) $n = 3$ (except for HE/HO p, $n = 2$). Means with the superscript symbol * are significantly different with respect to DE level (two-way ANOVA, $p < .05$). $n = 6$ when sorted on DE diet groups. DE, dietary digestible energy level; DO, dissolved oxygen level; HE and LE, high or low digestible energy diet; HO and LO, high or low dissolved water oxygen

& Boutilier, 1989; Ferguson, Tufts, & Boutilier, 1989). Whether this is true for Atlantic salmon would require further studies to confirm. Fish also regulate and maintain oxygen uptake under conditions of decreasing DO by an immediate acceleration of gill ventilation (Nilsson, 2007; Nilsson & Randall, 2010; Perry et al., 2009). This may result in a higher flux of ions through the gills, as indicated by the higher blood Cl^- and Na^+ ion levels. One of the most commonly documented measures of oxyregulation in vertebrates is an increased Hb concentration (Nilsson & Randall, 2010). This was found in LO-treated salmon compared to HO-treated groups and demonstrates a physiological adaptation to a better oxygen carrying capacity and tolerance to low water oxygen. There was also an interaction effect on Hb where fish fed HE diets held under HO conditions had significantly lower Hb concentration than the LO group fed the same diet. This positive effect of HO was not seen in the LE diet groups. Other oxyregulating mechanisms like increased MCV, RBC count or Hct (Perry & Gilmour, 2010) were not significantly affected by low water oxygen conditions in the present study.

In the present study, the DE level had no direct impact on plasma ghrelin and contradicts finding in rainbow trout fed a high fat diet (Jönsson et al., 2007). However, several of the investigated parameters in the present study revealed different responses to low water oxygen when salmon were fed diets with different energy content. Feeding LE diets resulted in a further increase in FCR compared to HE diet groups, which is also reported for rainbow trout (Saravanan et al., 2013).

Liver and muscle *ghr1* mRNA were increased by high DE levels, regardless of the oxygen level. As neither plasma IGF1 levels nor muscle *igf1* mRNA were similarly elevated, we assume that this elevation in receptor activity represents a more direct action of GH. The upregulation of *ghr1* mRNA in HE diet groups allows a possible stimulation of GH1 to promote growth, whereas the unaffected IGF1 levels in plasma and muscle mRNA indicate a limited stimulation of IGF1 release and its impact on growth. Feeding fish with high fat diets is reported to elevate the GH1 levels (Cameron et al., 2002; Company et al., 1999; Holloway,

Sheridan, Van Der Kraak, & Leatherland, 1999; Pérez-Sánchez, 2000) and is explained by a greater use of dietary fat for energy (Björnsson, 1997; Company et al., 1999; Pérez-Sánchez, 2000). There were significantly lower retention efficiencies of both DP and DF at LO conditions in the present study, which indicate a higher use of nutrients as energy rather than deposition at hypoxia compared to normoxia. A direct effect of DE level on the retention efficiencies was not detected. However, when fish were pair-fed at HO conditions with LE diets, the DF retention efficiency was increased, which demonstrates that the salmon energy metabolism can be influenced by feed composition.

The high activity of liver and muscle *ghr1* mRNA in LO-treated fish feeding on HE diets might be a combined response to the DE level and increased plasma ghrelin at LO conditions, as this effect was absent in LE diet groups. A link between growth and response in plasma IGF-1 and liver *igf1* mRNA is reported from other studies (Gaylord, Rawles, & Davis, 2005; Hevrøy et al., 2007), and IGF-1 is considered to be a suitable growth indicator in fish (Beckman, 2011). A clear reflection of the growth result was not seen in plasma IGF-1 or *igf1* mRNA levels as these were not significantly affected by the DO or DE treatments. However, when considering pair-feeding and dietary DE groups, both *ghr1* and i*gf1* mRNA levels responded positively to HO. The pair-fed LE diet group resulted in an increased liver *ghr1* mRNA level when exposed to HO, and this trend was also seen in muscle *ghr1* mRNA in LE diet groups, although not statistically significant. Several factors are listed to influence the growth and IGF-1 relation, there amongst stress, diseases, photoperiod, temperature, salinity, toxicants (Beckman, 2011) and nutritional condition (Duan, 1998). Findings from the present study confirm the latter factor and demonstrate that the response of IGF-1 to feed ration and DO level can be influenced by dietary DE level as seen by the different responses in liver *igf1* mRNA between HE and LE diet groups.

The most predictable occurrence of LO in commercial salmon farming is under conditions of high water temperature. As oxygen has lower solubility in water at higher temperature (Brett, 1979), raising temperatures naturally lowers DO concentration (mg L^{-1}), even if it is maintained at 100% saturation (Nilsson, 2010). By maintaining temperature near optimum for growth (13°C) of large Atlantic salmon (Hevrøy et al., 2013), but varying DO, we have separated these two factors that naturally and inversely co-vary. Low oxygen and high temperature can be considered as two different mechanisms for decreasing growth under summer growth conditions where fish are farmed near the upper end of temperature tolerance levels. In contrast to the present findings, high temperature studies with salmon have demonstrated appetite downregulation through higher leptin levels, while ghrelin levels have been contradictory with reduced plasma ghrelin in large salmon (Hevrøy et al., 2012), but no significant changes in postsmolt (Kullgren et al., 2013). Both studies reported increased leptin levels at elevated temperatures possibly causing anorectic conditions, hence the reduced feed intake and growth at high temperatures (Hevrøy et al., 2012; Kullgren et al., 2013). The difference in plasma ghrelin response to high temperature might be related to fish size and time of sampling (Kullgren et al., 2013), as plasma ghrelin peaks at different time periods postprandially for salmon held at 19 and 13°C (Vikeså, Nankervis, Remø, Waagbø, & Hevrøy, 2015). Even though high temperature studies (Hevrøy et al., 2012; Kullgren et al., 2013) and the present low oxygen study all resulted in reduced growth and feed intake in large salmon, it seems salmon are using different mechanisms to regulate growth under these different suboptimal conditions. The present results indicate that growth is regulated more directly by low oxygen environments by reducing metabolic potential, but indirectly through appetite control at high temperatures.

The majority of white muscle FAA concentrations were unaffected by the low oxygen conditions compared to normoxia, and no differences were detected for plasma FAA concentrations. The reduced feed intake of LO-treated fish groups indicates a lower influx of FAA and less FAA available for protein synthesis. However, these results suggest that salmon were capable of maintaining a sufficient FAA supply to the metabolism, possibly by catabolizing body proteins or fat, which is supported by the reduced DP retention efficiencies at LO conditions. The postprandial FAA absorption could also not be that pronounced at the single sampling point at 4 hrs postprandially as plasma FAA concentrations are previously reported to peak at 4–8 hrs postprandially for salmon reared at both optimal and high temperatures (Vikeså et al., 2015). This does not entirely explain the lack of response in the pair-fed groups exposed to HO conditions though. It is possible that hypoxia also have a belated effect on the absorbed FAA in salmon due to a slower digestive rate than at normoxia similar to that shown for high temperature (Vikeså et al., 2015). The few significant changes in FAA concentrations were detected in the LE diet groups and may signify that energy metabolism was altered and FAA were metabolized as energy at a higher rate than in HE diet groups.

5 | CONCLUSIONS

Appetite regulation (ghrelin) in large salmon was not downregulated under long-term exposure to low water oxygen. A pair-feeding technique showed that growth depression under low oxygen conditions cannot entirely be caused by lower feed intake, but appears to be a combined effect of limited growth regulation and higher basal metabolic cost. Results also indicate that feeding high fat diets can support the energy metabolism in salmon held under low oxygen conditions.

ACKNOWLEDGMENTS

Staff at Lerang Research station (Skretting ARC), NIFES and Skretting ARC laboratory. Review and comments on the manuscript by Dr Saravanan Subramanian were very helpful and highly appreciated. The experiment was financially supported by Skretting ARC. The study is part of the first authors' PhD which is supported by the NFR (grant number 209655/O30), employer Skretting ARC and the collaborating institute NIFES.

AUTHOR CONTRIBUTION

Each co-author (L.N. and E.M.H.) has assisted the first author in designing the research, conducting research, sampling and analysing data and preparing the manuscript.

REFERENCES

Asakawa, A., Inui, A., Kaga, O., Yuzuriha, H., Nagata, T., Ueno, N., … Kasuga, M. (2001). Ghrelin is an appetite-stimulatory signal from stomach with structural resemblance to motilin. *Gastroenterology*, *120*, 337–345.

Barnes, R., King, H., & Carter, C. G. (2011). Hypoxia tolerance and oxygen regulation in Atlantic salmon, *Salmo salar* from a Tasmanian population. *Aquaculture*, *318*, 397–401.

Barton, B. A. (2002). Stress in fishes: A diversity of responses with particular reference to changes in circulating corticosteroids 1. *Integrative and Comparative Biology*, *42*, 517–525.

Beckman, B. R. (2011). Perspectives on concordant and discordant relations between insulin-like growth factor 1 (IGF1) and growth in fishes. *General and Comparative Endocrinology*, *170*, 233–252.

Bendiksen, E. Å., Jobling, M., & Arnesen, A. M. (2002). Feed intake of Atlantic salmon parr *Salmo salar* L. in relation to temperature and feed composition. *Aquaculture Research*, *33*, 525–532.

Bernier, N. J., & Craig, P. M. (2005). CRF-related peptides contribute to stress response and regulation of appetite in hypoxic rainbow trout. *American Journal of Physiology: Regulatory, Integrative and Comparative Physiology*, *289*, R982–R990.

Björnsson, B. T. (1997). The biology of salmon growth hormone: From daylight to dominance. *Fish Physiology and Biochemistry*, *17*, 9–24.

Brett, J. R. (1979). Environmental factors and growth. In W. S. Hoar, C. F. Randall & J. R. Brett (Eds.), *Fish physiology*. (pp.599–675). London, UK: Academic Press Inc.

Buentello, J. A., Gatlin, D. M., & Neill, W. H. (2000). Effects of water temperature and dissolved oxygen on daily feed consumption, feed utilization and growth of channel catfish (*Ictalurus punctatus*). *Aquaculture*, *182*, 339–352.

Cameron, C., Gurure, R., Reddy, K., Moccia, R., & Leatherland, J. (2002). Correlation between dietary lipid:protein ratios and plasma growth and thyroid hormone levels in juvenile Arctic charr, *Salvelinus alpinus* (Linnaeus). *Aquaculture Research*, *33*, 383–394.

Carter, C. G., Katersky, R. S., Barnes, J. C., Bridle, A. R., & Hauler, R. C. (2008). Assessment of fish growth performance under limiting environmental conditions: Aquaculture nutrition subprogram. *FRDC Final Report* (pp. 1–147). Launceston, Tas., Australia: Tasmanian Aquaculture and Fisheries Institute.

Chapman, L. J., & McKenzie, D. J. (2009). Behavioral responses and ecological consequences. In G. R. Jeffrey, P. F. Anthony & J. B. Colin (Eds.), *Hypoxia* (1st ed.). Oxford, UK: Academic press.

Company, R., Calduch-Giner, J. A., Kaushik, S., & Pérez-Sánchez, J. (1999). Growth performance and adiposity in gilthead sea bream (*Sparus aurata*): Risks and benefits of high energy diets. *Aquaculture*, *171*, 279–292.

Dam, A. A., & Pauly, D. (1995). Simulation of the effects of oxygen on food consumption and growth of Nile tilapia, *Oreochromis niloticus* (L.). *Aquaculture Research*, *26*, 427–440.

Deane, E. E., & Woo, N. Y. S. (2009). Modulation of fish growth hormone levels by salinity, temperature, pollutants and aquaculture related stress: A review. *Review in Fish Biology and Fisheries*, *19*, 97–120.

Duan, C. (1998). Nutritional and developmental regulation of insulin-like growth factors in fish. *Journal of Nutrition*, *128*, 306S–314S.

Farrell, A. P., & Richards, J. G. (2009). Defining hypoxia. In J. G. Richards, A. P. Farrell & C. J. Brauner (Eds.), *Hypoxia* (1st ed.). Amsterdam, the Netherlands: Elsevier.

Ferguson, R. A., & Boutilier, R. G. (1989). Metabolic-membrane coupling in red blood cells of trout: The effects of anoxia and adrenergic stimulation. *Journal of Experimental Biology*, *143*, 149.

Ferguson, R. A., Tufts, B. L., & Boutilier, R. G. (1989). Energy metabolism in trout red cells: Consequences of adrenergic stimulation in vivo and in vitro. *Journal of Experimental Biology*, *143*, 133.

Foss, A., Evensen, T. H., & Øiestad, V. (2002). Effects of hypoxia and hyperoxia on growth and food conversion efficiency in the spotted wolffish *Anarhichas minor* (Olafsen). *Aquaculture Research*, *33*, 437–444.

Gaylord, T. G., Rawles, S. D., & Davis, K. B. (2005). Dietary tryptophan requirement of hybrid striped bass (*Morone chrysops* × *M. saxatilis*). *Aquaculture Nutrition*, *11*, 367–374.

Glencross, B. D. (2008). A factorial growth and feed utilization model for barramundi, *Lates calcarifer* based on Australian production conditions. *Aquaculture Nutrition*, *14*, 360–373.

Glencross, B. D. (2009). Reduced water oxygen levels affect maximal feed intake, but not protein or energy utilization efficiency of rainbow trout (*Oncorhynchus mykiss*). *Aquacult. Nutr.*, *15*, 1–8.

Gray, E. S., Kelley, K. M., Law, S., Tsai, R., Young, G., & Bern, H. A. (1992). Regulation of hepatic growth hormone receptors in coho salmon (*Oncorhynchus kisutch*). *General and Comparative Endocrinology*, *88*, 243–252.

Hansen, T. J., Olsen, R. E., Stien, L., Oppedal, F., Torgersen, T., Breck, O., … Fjelldal, P. G. (2015). Effect of water oxygen level on performance of diploid and triploid Atlantic salmon post-smolts reared at high temperature. *Aquaculture*, *435*, 354–360.

Hevrøy, E. M., Azpeleta, C., Shimizu, M., Lanzén, A., Kaiya, H., Espe, M., & Olsvik, P. A. (2011). Effects of short-term starvation on ghrelin, GH-IGF system, and IGF-binding proteins in Atlantic salmon. *Fish Physiology and Biochemistry*, *37*, 217–232.

Hevrøy, E. M., El-Mowafi, A., Taylor, R. G., Olsvik, P. A., Norberg, B., & Espe, M. (2007). Lysine intake affects gene expression of anabolic hormones in Atlantic salmon, *Salmo salar*. *General and Comparative Endocrinology*, *152*, 39–46.

Hevrøy, E. M., Jordal, A.E.O., Hordvik, I., Espe, M., Hemre, G.I., & Olsvik, P.A. (2006). Myosin heavy chain mRNA expression correlates higher with muscle protein accretion than growth in Atlantic salmon, *Salmo salar*. *Aquaculture*, *252*, 453–461.

Hevrøy, E. M., Hunskår, C., Gelder, S., Shimizu, M., Waagbø, R., Breck, O., … Hansen, T. (2013). GH–IGF system regulation of attenuated muscle growth and lipolysis in Atlantic salmon reared at elevated sea temperatures. *Journal of Comparative Physiology B*, *183*, 243–259.

Hevrøy, E. M., Waagbø, R., Torstensen, B. E., Takle, H., Stubhaug, I., Jørgensen, S. M., … Hansen, T. (2012). Ghrelin is involved in voluntary anorexia in Atlantic salmon raised at elevated sea temperatures. *General and Comparative Endocrinology*, *175*, 118.

Holloway, A. C., Sheridan, M. A., Van Der Kraak, G., & Leatherland, J. F. (1999). Correlations of plasma growth hormone with somatostatin, gonadal steroid hormones and thyroid hormones in rainbow trout during sexual recrudescence. *Comparative Biochemistry and Physiology – Part B*, *123*, 251–260.

Inui, A. (2001). Ghrelin: An orexigenic and somatotrophic signal from the stomach. *Nature Reviews Neuroscience*, *2*, 551–560.

Inui, A., Asakawa, A., Bowers, C. Y., Mantovani, G., Laviano, A., Meguid, M. M., & Fujimiya, M. (2004). Ghrelin, appetite, and gastric motility: The emerging role of the stomach as an endocrine organ. *The FASEB Journal*, *18*, 439–456.

Jensen, F. B., Fago, A., & Weber, R. E. (1998). 1 - Hemoglobin structure and function. In: Perry, S. F., & Tufts, B. L. (Eds.), *Fish Physiology* (pp. 1–40). San Diego, CA, USA: Academic press.

Jönsson, E., Forsman, A., Einarsdottir, I. E., Kaiya, H., Ruohonen, K., & Björnsson, B. T. (2007). Plasma ghrelin levels in rainbow trout in response to fasting, feeding and food composition, and effects of ghrelin on voluntary food intake. *Comparative Biochemistry and Physiology. Part A, Molecular & Integrative Physiology*, *147*, 1116–1124.

Kaiya, H., Kojima, K., Hosoda, H., Moriyama, S., Takahashi, A., Kawauchi, H., & Kangawa, K. (2003). Peptide purification, complementary deoxyribonucleic acid (DNA) and genomic DNA cloning, and functional characterisation of ghrelin in rainbow trout. *Endocrinology*, *144*, 5215–5226.

Kieffer, J. D., Alsop, D., & Wood, C. M. (1998). A respirometric analysis of fuel use during aerobic swimming at different temperatures in rainbow trout (*Oncorhynchus mykiss*). *Journal of Experimental Biology*, *201*, 3123.

Kullgren, A., Jutfelt, F., Fontanillas, R., Sundell, K., Samuelsson, L., Wiklander, K., … Jonsson, E. (2013). The impact of temperature on the

metabolome and endocrine metabolic signals in Atlantic salmon (*Salmo salar*). *Comparative Biochemistry and Physiology Part A Molecular Integrative Physiology*, *164*, 44–53.

MacKenzie, D. S., VanPutte, C. M., & Leiner, K. A. (1998). Nutrient regulation of endocrine function in fish. *Aquaculture*, *161*, 3–25.

Nakazato, M., Murakami, N., Date, Y., Kojima, M., Matsuo, H., Kangawa, K., & Matsukura, S. (2001). A role for ghrelin in the central regulation of feeding. *Nature*, *409*, 194.

National Research Council Committee on Nutrient Requirements of, F. & Shrimp 2011. *Nutrient requirements of fish and shrimp*. Washington, DC: National Academic Press.

Nilsson, G. E. (2007). Gill remodeling in fish – A new fashion or an ancient secret? *Journal of Experimental Biology*, *210*, 2403.

Nilsson, G. E. (2010). Introduction: Why we need oxygen. In: Nilsson, G. E. (Ed.) *Respiratory physiology of vertebrates* (pp. 3–13). Cambridge, UK: Cambridge University Press.

Nilsson, G. E., & Randall, D. J. (2010). Adaptations to hypoxia in fishes. In: Nilsson, G. E. (Ed.) *Respiratory physiology of vertebrates* (1st ed., pp. 131–173). Cambridge, UK: Cambridge University Press.

Pedersen, L. J. (1987). Energy budgets for juvenile Rainbow trout at various oxygen concentrations. *Aquaculture*, *62*, 289–298.

Pérez Sánchez, J., Martí-Palanca, H., & Le Bail, P. Y. (1994). Homologous growth hormone (GH) binding in gilthead sea bream (*Sparus aurata*). Effect of fasting and refeeding on hepatic GH-binding and plasma somatomedin-like immunoreactivity. *Journal of Fish Biology*, *44*, 287–301.

Pérez-Sánchez, J. (2000). The involvement of growth hormone in growth regulation, energy homeostasis and immune function in the gilthead sea bream (*Sparus aurata*): A short review. *Fish Physiology and Biochemistry*, *22*, 135–144.

Pérez-Sánchez, J., Calduch-Giner, J. A., Mingarro, M., Vega-Rubín de Celis, S., Gómez-Requeni, P., Saera-Vila, A., … Valdivia, M. M. (2002). Overview of fish growth hormone family. New insights in genomic organization and heterogeneity of growth hormone receptors. *Fish Physiology and Biochemistry*, *27*, 243–258.

Pérez-Sánchez, J., Martí-Palanca, H., & Kaushik, S. J. (1995). Ration size and protein intake affect circulating growth hormone concentration, hepatic growth hormone binding and plasma insulin-like growth factor-I immunoreactivity in a marine teleost, the gilthead sea bream (*Sparus aurata*). *Journal of Nutrition*, *125*, 546.

Perry, S. F., & Gilmour, K. M. (2010). Oxygen uptake and transport in water breathers. In: Nilsson, G. E. (Ed.) *Respiratory physiology of vertebrates* (1st ed., pp. 49–94). Cambridge, UK: Cambridge University Press.

Perry, S. F., Jonz, M. G., & Gilmour, K. M. (2009). Oxygen sensing and the hypoxic ventilatory response. In J. G. Richards, A. P. Farrell & C. J. Brauner (Eds.), *Hypoxia* (1st ed., pp. 193–253). London, UK: Academic Press.

Pichavant, K., Person-Le-Ruyet, J., Le Bayon, N., Severe, A., Le Roux, A., & Boeuf, G. (2001). Comparative effects of long-term hypoxia on growth, feeding and oxygen consumption in juvenile turbot and European sea bass. *Journal of Fish Biology*, *59*, 875–883.

Pickering, A. D., Pottinger, T. G., Sumpter, J. P., Carragher, J. F., & Le Bail, P. Y. (1991). Effects of acute and chronic stress on the levels of circulating growth hormone in the rainbow trout, *Oncorhynchus mykiss*. *General and Comparative Endocrinology*, *83*, 86–93.

Raff, H. (2003). Total and active ghrelin in developing rats during hypoxia. *Endocrine*, *21*, 159–161.

Remen, M., Aas, T. S., Vågseth, T., Torgersen, T., Olsen, R. E., Imsland, A., & Oppedal, F. (2014). Production performance of Atlantic salmon (*Salmo salar* L.) postsmolts in cyclic hypoxia, and following compensatory growth. *Aquaculture Research*, *45*, 1355–1366.

Remen, M., Oppedal, F., Torgersen, T., Imsland, A. K., & Olsen, R. E. (2012). Effects of cyclic environmental hypoxia on physiology and feed intake of post-smolt Atlantic salmon: Initial responses and acclimation. *Aquaculture*, *326–329*, 148–155.

Saravanan, S., Geurden, I., Figueiredo-Silva, A. C., Kaushik, S. J., Haidar, M. N., Verreth, J. A. J., & Schrama, J. W. (2012). Control of voluntary feed intake in fish: A role for dietary oxygen demand in Nile tilapia (*Oreochromis niloticus*) fed diets with different macronutrient profiles. *British Journal of Nutrition*, *108*, 1519–1529.

Saravanan, S., Geurden, I., Figueiredo-Silva, A. C., Kaushik, S., Verreth, J., & Schrama, J. W. (2013). Voluntary feed intake in rainbow trout is regulated by diet-induced differences in oxygen use. *Journal of Nutrition*, *143*, 781–787.

Schmidt-Nielsen, K. (1997). Energy metabolism. In Schmidt-Nielsen, K. (Ed.), *Animal physiology, adaptation and environment* (5th ed.). Cambridge, UK: Cambridge University Press.

Solomon, S., Qin, D., Manning, M., Chen, Z., Marquis, M., Averyt, K. B., … Miller, H. L. (2007). Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change, 2007 *IPCC (Intergovernmental Panel on Climate Changes)*.

Stevens, E. D., Sutterlin, A., & Cook, T. (1998). Respiratory metabolism and swimming performance in growth hormone transgenic Atlantic salmon. *Canadian Journal of Fisheries and Aquatic Science*, *55*, 2028–2035.

Thetmeyer, H., Waller, U., Black, K. D., Inselmann, S., & Rosenthal, H. (1999). Growth of European sea bass (*Dicentrarchus labrax* L.) under hypoxic and oscillating oxygen conditions. *Aquaculture*, *174*, 355–367.

Thorarensen, H., & Farrell, A. P. (2011). The biological requirements for post-smolt Atlantic salmon in closed-containment systems. *Aquaculture*, *312*, 1–14.

Tran-Duy, A., Schrama, J. W., van Dam, A. A., & Verreth, J. A. J. (2008). Effects of oxygen concentration and body weight on maximum feed intake, growth and hematological parameters of Nile tilapia, *Oreochromis niloticus*. *Aquaculture*, *275*, 152–162.

Ueno, H., Yamaguchi, H., Kangawa, K., & Nakazato, M. (2005). Ghrelin: A gastric peptide that regulates food intake and energy homeostasis. *Regulatory Peptides*, *126*, 11–19.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., & Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology*, *3*, 18.

Vikeså, V., Nankervis, L., Remø, S. C., Waagbø, R., & Hevrøy, E. M. (2015). Pre and postprandial regulation of ghrelin, amino acids and IGF1 in Atlantic salmon (*Salmo salar* L.) at optimal and elevated seawater temperatures. *Aquaculture*, *438*, 159–169.

III

Aquaculture Research

Aquaculture Research, 2016, 1–16 doi: 10.1111/are.13229

Appetite, metabolism and growth regulation in Atlantic salmon (*Salmo salar* L.) exposed to hypoxia at elevated seawater temperature

Vibeke Vikesså[1,2], Leo Nankervis[1] & Ernst Morten Hevrøy[2]

[1]Skretting ARC (Aquaculture Research Centre), Stavanger, Norway

[2]National Institute of Nutrition and Seafood Research (NIFES), Bergen, Norway

Correspondence: V Vikeså, Skretting ARC (Aquaculture Research Centre), P.O. Box 148, 4016 Stavanger, Norway. E-mail: vibeke.vikesaa@skretting.com

Abstract

High temperature combined with low dissolved oxygen (DO) is one of the most challenging environmental conditions farmed fish experience; thus, understanding their impact on growth regulation is of relevance to cultured and wild populations. This study examines appetite- and growth-regulating mechanisms in Atlantic salmon postsmolt exposed to either high (HO) or low oxygen (LO) at a suboptimally high temperature (17°C). Additionally, the effects of high (HE) and low (LE) dietary energy (DE) were examined. After a month of treatment, analyses of hormones, regulating appetite (ghrelin) and growth (growth hormone receptor *ghr1* and insulin-like growth factor IGF-1), and free amino acids (FAA) were measured pre- and postprandially at −4, −2, 0, 2, 4 and 6 h. No preprandial ghrelin peaks were detected despite a significant reduction in feed intake and growth under hypoxia compared to normoxia. LO treatment also had an overall negative effect on survival compared to HO, while nutrient retention efficiency, FCR and plasma FAA concentrations were unaffected ($P > 0.05$). Feeding HE diet resulted in increased growth (+17%) and improved FCR (−14%) and energy retention efficiency (+26%) independent of DO. Plasma FAA concentrations were unaffected by LO treatment and DE ($P > 0.05$). Growth regulatory gene expressions possibly reflect an overall lower growth at a high temperature overriding the impacts of DO and DE. This study also indicates that optimal adaptation time to environmental conditions and feeding regime is crucial for establishing a regular hormonal appetite signalling that reflects real feeding anticipation in salmon.

Keywords: GH-IGF system, ghrelin, dietary digestible energy, hypoxia, high temperature

Introduction

Water temperature and oxygen are key environmental factors influencing welfare and growth of farmed fish and impose physiological challenges under typical summer conditions. The existing summer thermal stress is expected to exacerbate in several areas where Atlantic salmon is farmed due to predicted trends for increased global seawater temperatures (Solomon, Qin, Manning, Chen, Marquis, Averyt, Tignor & Miller 2007).

Temperature is the major controlling factor of fish growth as it directly affects metabolic rate and oxygen requirement (Brett 1979). Atlantic salmon freshwater stages tolerate a wide range of temperatures (0–28°C) (Elliott & Elliott 2010), but optimal temperature for growth is size and life stage dependent. Large salmon (1.6–2.9 kg) in seawater grow well at 13°C (Hevrøy, Hunskår, Gelder, Shimizu, Waagbø, Breck, Takle, Susort & Hansen 2013), while optimal growth of parr (typically 20–100 g) is within the range of 16–20°C compared to 13–14°C for postsmolt (typically 50–200 g) (Peterson & Martin-Robichaud 1989; Elliott & Hurley 1997; Koskela, Pirhonen & Jobling 1997; Forseth, Hurley, Jensen & Elliott 2001; Handeland, Imsland & Stefansson 2008). A rise in water temperature increases oxygen consumption (Barnes, King & Carter 2011) and metabolic rate in fish,

but also reduces feed intake when temperature rises above the preferred thermal optimum values (Brett 1979). Critical water oxygen level needed to maintain the metabolism increases proportionally with increasing water temperature (Brett 1979; Barnes *et al.* 2011).

Oxygen is considered as a limiting factor for fish metabolism, as it is crucial for synthesis of ATP and therefore regulates energy availability for metabolism. As water temperature increases, limitation on water oxygen solubility and availability directly restricts aerobic energy production (Richards 2009). When exposed to hypoxic conditions, fish follow two coping strategies depending on degree and duration: oxyconforming and oxyregulation. The former entails reduction in metabolic rate so that oxygen is used more sparingly (Ultsch, Jackson & Moalli 1981; Perry, Jonz & Gilmour 2009; Nilsson & Randall 2010), along with reduction in feed intake and growth. The latter mechanism is defined by behavioural, physiological and anatomical adaptation without compromising metabolic rate (Farrell & Richards 2009). Elevation of gill ventilation and increased blood haemoglobin concentration are examples of common mechanisms of oxyregulation to improve oxygen uptake and tolerance to hypoxia (Stevens, Sutterlin & Cook 1998; Nilsson 2007; Perry *et al.* 2009; Nilsson & Randall 2010). Atlantic salmon postsmolt show greater ability to oxyregulate under hypoxia at 14°C, while use of oxyconforming mechanisms dominates at higher temperatures (18–22°C) (Barnes *et al.* 2011).

Dietary fat contributes approximately twice as much energy compared to the same mass of either protein or carbohydrate (Schmidt-Nielsen 1997). Using fat as dietary energy source also requires less oxygen to utilize for growth than protein or starch (Saravanan, Geurden, Figueiredo-Silva, Kaushik, Haidar, Verreth & Schrama 2012; Saravanan, Geurden, Figueiredo-Silva, Kaushik, Verreth & Schrama 2013). This implies that high-energy diets can benefit fish under hypoxic conditions. Dietary factors such as digestible energy (DE) level influence feed intake (Bendiksen, Jobling & Arnesen 2002), and feeding Atlantic salmon with high-DE diets (21.1 versus 19.3 MJ kg^{-1}) benefitted metabolism and growth performance under long-term hypoxia at optimal water temperature of 12°C (Vikeså, Nankervis & Hevroy 2016). Raising temperature above optimum under hypoxia puts an even further demanding challenge on to the salmon, and it is therefore interesting to find out how high-fat diets could potentially impact appetite and growth regulation under such conditions.

Growth regulation in fish is stimulated through the GH-IGF system and is altered by nutritional and environmental factors (Pickering, Pottinger, Sumpter, Carragher & Le Bail 1991; Pérez-Sánchez, Martí-Palanca & Kaushik 1995; MacKenzie, VanPutte & Leiner 1998; Pérez-Sánchez, Calduch-Giner, Mingarro, Vega-Rubín de Celis, Gómez-Requeni, Saera-Vila, Astola & Valdivia 2002; Deane & Woo 2009; Beckman 2011). GH1 stimulates growth directly by binding to its receptor (GHR) (Reinecke, Björnsson, Dickhoff, McCormick, Navarro, Power & Gutiérrez 2005) and indirectly by promoting synthesis and secretion of insulin-like growth factor-1 (IGF-1) in several tissues including liver and muscle (Pérez-Sánchez 2000; Reinecke 2010; Beckman 2011). IGF-1 and growth often respond similarly to external factors and so IGF-1 is considered an useful growth indicator in fish (Pérez-Sánchez & Le Bail 1999; Beckman, Shimizu, Gadberry, Parkins & Cooper 2004; Dyer, Barlow, Bransden, Carter, Glencross, Richardson, Thomas, Williams & Carragher 2004; Wilkinson, Porter, Woolcott, Longland & Carragher 2006; De-Santis & Jerry 2007; Picha, Turano, Beckman & Borski 2008; Beckman 2011). IGF-1 also has a negative feedback function to regulate GH secretion under catabolic conditions (Duan 1998; Reinecke 2010). Growth studies would therefore gain from examining both hormones taking part in the GH-IGF system as their direct and interrelated actions are essential to describe how growth mechanisms are regulated. There are several studies on the response of IGF-1 to elevated temperatures (Beckman, Larsen, Moriyama, Lee-Pawlak & Dickhoff 1998; Gabillard, Weil, Rescan, Navarro, Gutiérrez & Le Bail 2003; Gabillard, Weil, Rescan, Navarro & Gutierrez 2005; Imsland, Björnsson, Gunnarsson, Foss & Stefansson 2007; Hevrøy *et al.* 2013; Vikeså, Nankervis, Remø, Waagbø & Hevrøy 2015), and our earlier study reports on regulation of IGF-1 in Atlantic salmon under hypoxia at an optimal temperature for growth (Vikeså *et al.* 2016). However, to our knowledge, there are no reported studies on the effect of hypoxia combined with high temperatures on regulation of IGF-1.

Ghrelin appetite signalling encourages fish to eat and thus provides nutrients essential for

growth, but it also stimulates release of GH1 (Kaiya, Kojima, Hosoda, Moriyama, Takahashi, Kawauchi & Kangawa 2003a; Kaiya, Kojima, Hosoda, Riley, Hirano, Gordon Grau & Kangawa 2003b; Inui, Asakawa, Bowers, Mantovani, Laviano, Meguid & Fujimiya 2004) and holds a well-known role in energy balance in both fish and higher vertebrates (Choi, Roh, Hong, Shrestha, Hishikawa, Chen, Kojima, Kangawa & Sasaki 2003; Cummings & Shannon 2003; Inui *et al.* 2004; Riley, Fox, Kaiya, Hirano & Grau 2005; Ueno, Yamaguchi, Kangawa & Nakazato 2005; Unniappan & Peter 2005; Cummings 2006; Frøiland, Murashita, Jørgensen & Kurokawa 2010; Hevrøy, Waagbø, Torstensen, Takle, Stubhaug, Jørgensen, Torgersen, Tvenning, Susort, Breck & Hansen 2012; Jönsson 2013). This underlines the importance of studying both ghrelin and the GH-IGF system to elucidate appetite and growth regulation in fish under warm hypoxic conditions.

This study aims at investigating feed intake and growth regulation in Atlantic salmon postsmolt exposed to normoxic and hypoxic conditions at a suboptimally high water temperature (17°C). Additionally, salmon were fed diets of different DE levels to find out how diet affects appetite and growth under warm hypoxic conditions.

Materials and methods

This study presents an experiment conducted at Matre Havbruksstasjon (Institute of Marine Research) in Matredal, Norway, with seawater-adapted Atlantic salmon postsmolt (0+) from an AquaGen strain.

Experimental conditions

Salmon with an initial BW of 264 ± 1.1 g (SE) were reared in 16 experimental tanks (1 m^3), each with 40 fish, and seawater of a salinity of 28–30 g L^{-1} and a photoperiod regime of 18L:6D h (day light period: 0500–2300 hours). Two experimental diets were produced as 4-mm extruded pellets at Skretting Feed Technology Plant (Stavanger, Norway) differing in digestible energy (DE): high digestible energy (HE) 20 MJ kg^{-1} and low digestible energy (LE) 18 MJ kg^{-1} (Table 1).

Quadruplicate tanks were randomly allocated to four experimental treatments which were combinations of the two diets (HE and LE) and two oxygen saturation levels (high oxygen: HO and low

Table 1 Ingredient and nutrient composition (analysed) of experimental diets

	HE	LE
Ingredient (name of producer) (g kg^{-1})		
Wheat (Hage, Raiffeisen Hauptgenossenschaft Nord)	52	167
Wheat gluten (Henan)	200	194
Soy protein concentrate (Marine Protein Holdings)	211	185
Faba beans whole (Frontier Agriculture)	80	80
Fish meal (Welcon)	200	200
Rapeseed oil (Rosenkrantz)	113	71
Fish oil (Welcon)	113	71
DL-Methionine (Evonik)	1.1	1.1
Lysine-HCL 78% (Trouw Nutrition)	7.2	7.8
Astaxanthin 10% (DSM)	0.5	0.5
Yttrium premix 10% (Trouw Nutrition)	1.0	1.0
Vitamin mix*	1.0	1.0
Mineral mix*	20.8	21.1
Composition		
	58.0	70.0
Moisture (g kg^{-1})		
	475.0	455.0
Protein (g kg^{-1})		
	276.0	198.0
Fat (g kg^{-1})		
	23.7	21.8
Gross energy (GE, MJ kg^{-1})		
	20.0	18.0
Digestible energy (DE, MJ kg^{-1})		
Amino acids (mg g^{-1} protein)		
Arginine	55	55
Histidine	23	22
Isoleucine	38	38
Leucine	71	70
Lysine	58	60
Methionine	20	20
Cystine	14	13
Phenylalanine	42	39
Tyrosine	29	31
Threonine	36	34
Valine	43	43
Alanine	42	42
Aspartic acid	79	77
Glutamic acid	219	222
Glycine	44	45
Proline	72	72
Serine	47	47
EFAA	387	383
Total FAA	932	931

HE and LE, high or low digestible energy diet.
EFAA, essential free amino acids; Total FAA, sum of all analysed FAA.
*Proprietary to skretting.

oxygen; LO). Fish were adapted to 11.5°C for 8 days before temperature was raised and reached 16.7°C after another 10 days (Fig. 1). Oxygen was then at 100% saturation in all tanks, but was reduced towards 72% in the LO treatment groups over three consecutive days. Oxygenated water was supplied to maintain saturation close to 100% in the HO treatment tanks. Temperature was kept at approximately 17°C in all tanks for the remaining experimental period of 31 days.

Feeding was conducted by use of automatic belt feeders, and the daily ration consisted of 1% of the BW and 15% overfeeding. Fish were given two meals a day with approximately 60% of the ration in the first meal (0830–1030 hours) and approximately 40% of the ration in the second meal (1200–1400 hours). Uneaten feed was collected up to 15 min after feeding from Monday to Friday weekly, dried to constant weight, reweighed and used to correct daily feed intake data for all tanks. Feed intake for Saturdays and Sundays was estimated using the feed waste values from the day before or after respectively. A two-phase feeding regime was introduced for the eleven final days to prepare for a pre-and postprandial fish sampling from all tanks. The two daily meals were split in two, where the 1st meal was fed at 0800–1000 hours and 0900–1100 hours, with half of the tanks per treatment and diet group at each time interval, while the 2nd meal was fed at 1130–1300 hours and 1230–1400 hours the same way.

Sampling

Pooled samples of whole fish were taken at the start (n = 6) and end of the experiment (three fish per tank). Preprandial sampling and postprandial sampling of fish were performed after 31 days of diet and oxygen treatment, over a period of 2 days. This enabled a 24-h break in between the samplings, in which fish were offered feed as usual. The preprandial sampling was conducted at −4, −2 and 0 h before the start of the first daily meal, which was given at 0–2 h, while postprandial sampling was conducted 2, 4 and 6 h after the first meal was initiated. The second meal was given at 3.5–5.0 h after the start of the first meal. At each sampling point, two fish were sampled from each tank, totalling 16 fish from each oxygen treatment per diet. Sampled fish were anaesthetized by placing them in a solution of 70 mg tricaine methanesulfonate L^{-1} seawater. Registration of BW was done when fish were sedated and further samples were only taken from fish that were determined to have consumed feed in the previous meal after examination of gut contents.

Blood samples were taken from the caudal blood vessels from three fish per tank with syringes and transferred to 1.8-mL microcentrifuge tubes each containing 5 μL heparin (5 IU μL^{-1}) and then centrifuged at 1250 *g* for 10 min before plasma was aspirated. Liver samples (approximately 1 cm^3) and white muscle tissue (approximately 1 cm^3, excised from medial cutlets) were taken from three fish per tank for RNA extraction and analysis. Samples were immediately flash-frozen in liquid nitrogen.

Analytical methods

Protein in feed and fillets was estimated from Kjeldahl nitrogen (N × 6.25, NMKL method no. 6, 4th ed. 2003). Dry matter in feed was determined

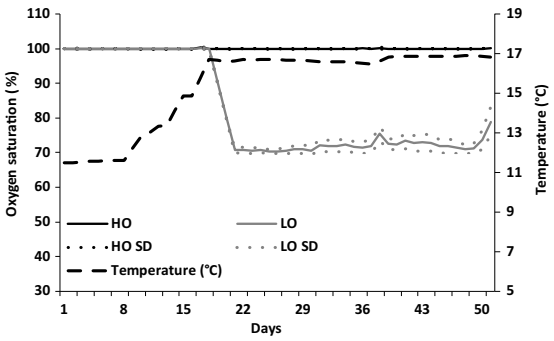

Figure 1 Mean water oxygen saturation levels (%) on the left *y*-axis and water temperature (°C) on the right *y*-axis measured daily in the outlet of experimental tanks with high (HO) and low (LO) dissolved oxygen.

gravimetrically after drying to constant weight, while ash in feed was determined gravimetrically after combustion at 550°C for 16–18 h (NMKL method no. 23, 3rd ed.1991). Crude fat analysis of pooled samples of homogenized fish was performed by a modified version of the Soxhlet method (AOCS Official Method Ba 3-38 re-approved in 2009) applying dichloromethane and an automatic extraction system. Fat in feed was estimated by nuclear magnetic resonance (NMR) on a QuickFat analyser (Anvendt Teknologi AS) (NMKL method no. 199, 2014). Gross energy (GE, MJ kg^{-1}) in feed was determined by bomb calorimetry (ISO 9831:1998).

The level of amino acids in feed was analysed photometrically after separation by ionic exchange chromatography and reaction with ninhydrin reagent and sodium citrate eluent (EU method, COMMISSION DIRECTIVE 98/64/EC). Free amino acids in plasma were also analysed photometrically after separation by ionic exchange chromatography and reaction with a ninhydrin reagent and lithium eluents (Biochrom method AAAFAQ8). The analysis was performed on pooled samples from two fish taken from the same tank at the 0-, 4- and 6-h sampling.

Plasma hormone concentrations of IGF-1 and ghrelin were measured in single samples using fluoroimmunoassay and radioimmunoassay (RIA), respectively, using the methods as described by Hevrøy *et al.* (2012, 2013). For IGF-1 in plasma, a dissociation-enhanced lanthanide fluorescence immunoassay (DELFIA) method using europium (Eu)-labelled IGF-1 tracer (15.9 ng mL^{-1}; 1244-302; Perkin Elmer, Waltham, MA, USA), rabbit anti-barramundi IGF-1 polyclonal antiserum (diluted 1:2072; GroPep, Adelaide, SA, Australia) and recombinant salmon IGF-1 (GroPep) was used (Hevrøy *et al.* 2013). Ghrelin levels in salmon plasma were measured with a heterologous assay using a 125I-radioimmunoassay kit for human ghrelin specific for biologically active (octanoylated) ghrelin (Linco Research, St. Charles, Missouri, USA) validated for use in Atlantic salmon (Hevrøy, Azpeleta, Shimizu, Lanzén, Kaiya, Espe & Olsvik 2011). IGF-1 and ghrelin analyses were conducted on two samples from the same tank at the 0-, 4- and 6-h sampling, while ghrelin was additionally analysed in samples from the −4, −2, 2 h sampling.

RNA extraction and real-time RT-qPCR (reverse transcription–quantitative polymerase chain reaction) were used to measure transcription of *ghr1* and *igf1* in white muscle tissue by analysing two samples from the same tank at the 0-, 4- and 6-h sampling.

Total RNA was extracted using TRIzol reagent according to the manufacturer's recommendation (Invitrogen, USA). To eliminate genomic DNA, the total RNA sample was subjected to **DNase** treatment (DNeasy, Ambion), using the manufacturer's protocol. Quantity and quality of RNA were assessed with NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), where a 260/280 nm absorbance ratio of 1.8–2.0 indicates a pure RNA sample. To evaluate the RNA integrity, the 6000 Nano LabChip® kit (Agilent Technologies, Palo Alto, CA, USA) was used, with average RNA integrity numbers (RIN) of 9.3 (SD ± 0.3) in muscle tissue.

PCR primers for the target genes *igf1* and *ghr1* were designed with Primer Express software and Biosoft software, as previously described (Hevrøy *et al.* 2012). Housekeeping genes β-actin (*actb*), eukaryotic translation elongation factor 1αβ (*ef1ab*) and ribosomal protein L13 (*rpl13*) were used as reference genes (Hevrøy *et al.* 2013).

Reverse transcription (RT) was performed using a modified Multiscribe reverse transcription protocol (GeneAmp PCR 9700, Applied Biosystems, Foster City, CA, USA), as previously described (Hevrøy, Espe, Waagbø, Sandnes, Ruud & Hemre 2005). White muscle samples were analysed in duplicate with a total RNA of 500 ng (±5%) on 96-well plates for the RT and real-time PCR. Real-time PCR amplification and analysis were performed using a LightCycler 480 Real-time PCR system (Roche Applied Science, Basel, Switzerland). The real-time PCR SYBR Green Master Mix (LightCycler 480 SYBR Green master mix kit, Roche) contained FastStart DNA polymerase and gene-specific primers at a final concentration of 500 nM. Two microlitres of diluted cDNA from each well was transferred to a new plate, and 8 μL real-time master mix was added. PCR was achieved with a 5-min activation and denaturizing step at 95°C followed by 45 cycles of a 10-s denaturizing step at 95°C, a 20-s annealing step at 60°C and 30-s synthesis step at 72°C. The efficiency of real-time PCR was monitored using two-fold dilution curves of total RNA using five points (range: 1000 to 31 ng μL^{-1}). Target gene mean

normalized expression (MNE) was determined using a normalization factor calculated with the geNorm software (Vandesompele, De Preter, Pattyn, Poppe, Van Roy, De Paepe & Speleman 2002).

Calculations

The following calculations were applied:

$$\text{Specific growth rate, SGR, \%} : (\ln \text{final BW} - \ln \text{initial BW})/\text{days} \times 100$$

$$\text{Feed conversation ratio, FCR (on dry matter basis, DM)} : \text{Feed eaten, g(DM)}/\text{weight gain, g}$$

$$\text{Protein and energy retention efficiency of diet, \%} : (\text{nutrient gain}/\text{consumed nutrient}) \times 100;$$

where nutrient is protein or energy.

Statistics

All data are presented as mean ± standard error of the mean (SEM) unless otherwise described. Tanks are used as experimental units. Statistical analyses were conducted using UNISTAT 6.0 (Unistat, London, UK), and significant differences were accepted at P values <0.05. All data were subjected to a two-way analysis of variance (ANOVA) with interactions of two factors (DO and DE). Results from different time points were also subjected to repeated analyses to look for time effects. The post hoc test Tukey HSD was applied if any significant differences were detected by ANOVA.

Results

Growth performance and feed utilization

Low oxygen (DO) and low dietary energy (DE) treatment both decreased growth (final BW and SGR) significantly ($P < 0.05$) (Table 2). The LO treatment also resulted in a significantly lower feed intake and higher mortality ($P < 0.05$). FCR and energy retention efficiency were unaffected by the DO level ($P > 0.05$), but were both negatively affected by feeding the LE diet, with a significantly increased FCR and a reduced energy retention efficiency ($P < 0.05$) (Table 2).

Protein retention efficiency was not significantly affected by either treatment ($P > 0.05$), and there were no significant interactions ($P > 0.05$) between DO and DE on any of the investigated parameters.

Pre- and postprandial analyses: −4, −2, 0, 4 and 6 h

Plasma ghrelin concentration in salmon was unaffected by the DO and DE treatments at all analysed pre- and postprandial sampling points ($P > 0.05$) (Fig. 2). There were no preprandial peaks detected for plasma ghrelin concentrations ($P > 0.05$), but concentrations measured at 6 h postprandially were significantly higher than 2 and 0 h preprandially ($P < 0.05$).

Circulating IGF-1 concentrations were significantly higher in LO groups 4 h postprandially than in fish held at HO conditions ($P < 0.05$) (Fig. 3). There were no other significant effects detected from any of the DO and DE treatments or from time of sampling ($P > 0.05$).

Gene expression of *ghr1* and *igf1* mRNA in white muscle tissue was affected by time, with a significant decrease ($P < 0.05$) at 4 and 6 h postprandially compared to just before meal time at 0 h (Fig. 4a,b).

No significant effects of DO and DE treatments were detected for muscle *ghr1* and *igf1* mRNA levels ($P > 0.05$).

Plasma free amino acid (FAA) concentration was significantly increased 4 and 6 h postprandially compared to 0 h, that is just before feeding the first daily meal ($P < 0.05$) (Table 3a,b). Tryptophan (Trp) was the only exception to this ($P > 0.05$). Neither DO or DE treatment had a significant impact on plasma FAA levels ($P > 0.05$) except for histidine (His) which was significantly increased in LE diet groups ($P < 0.05$).

Discussion

This study reports on appetite- and growth-regulating mechanisms in Atlantic salmon exposed to low oxygen concentrations at a suboptimally high-temperature condition and given diets differing in digestible energy level. We have previously demonstrated that growth is impaired for Atlantic salmon at low oxygen conditions at 12°C and that this was not caused by low appetite alone (Vikeså *et al.* 2016). Hevrøy *et al.* (2012) also found that high temperature reduced appetite-regulating cues in salmon at 19°C under normoxia. The present

Table 2 Growth performance and feed utilization parameters for Atlantic salmon given diets differing in DE level under different DO conditions at 17°C, $n = 4$

	HE/HO	HE/LO	LE/HO	LE/LO	DO	DE	DO × DE
BW final (g)	418.8/7.7^{a}	380.9/8.6^{b}c	394.3/3.5^{ab}	358.3/5.2^{c}	*	*	ns.
Feed intake DM (% BW day^{-1})	0.88/0.03^{a}	0.65/0.05^{b}	0.85/0.01^{a}	0.64/0.02^{b}	*	ns.	ns.
SGR (% day^{-1})	0.93/0.03^{a}	0.70/0.06^{bc}	0.78/0.02^{ab}	0.61/0.04^{c}	*	*	ns.
FCR (DM)	0.75/0.03	0.78/0.05	0.88/0.02	0.89/0.05	ns.	*	ns.
Mortality (%)	9.4/2.6^{b}	16.4/1.7^{ab}	10.0/2.0b	23.7/1.8^{a}	*	ns.	ns.
Protein retention efficiency (%)	53.9/2.4	55.7/4.2	48.6/1.4	50.9/1.9	ns.	ns.	ns.
Energy retention efficiency (%)	49.3/2.5	50.5/6.6	40.6/2.0	38.5/4.9	ns.	*	ns.

The letters a, b and c indicate significant different means for all four treatment groups (one-way ANOVA, $P < 0.05$). The symbol * indicates significant effects from DE or DO (two-way ANOVA, $P < 0.05$). Non-significant differences are indicated with ns ($P > 0.05$). DE, dietary digestible energy level; DO, dissolved oxygen level; DO × DE, interaction between DO and DE; HE and LE, high or low digestible energy diet; HO and LO, high or low dissolved water oxygen.

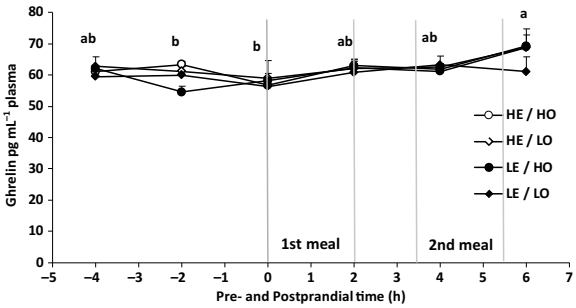

Figure 2 Mean concentration of ghrelin in plasma (pg mL^{-1}) sampled −4, −2 and 0 h pre- and 2 and 6 h postprandially from Atlantic salmon given diets differing in DE level under different DO conditions at 17°C ($n = 4$). The letters a and b indicate significant differences between the mean of each sampling point (two-way ANOVA with repeated analyses, $P < 0.05$). Meal times are indicated within dotted vertical lines.

study brings together both of these concepts to examine appetite and growth regulation at different oxygen levels (approximately 70% versus 100% saturation) combined with high temperature (about 12 versus 17°C).

Growth and feed utilization

Similar to previous studies on the impact of low dissolved oxygen on feed intake and growth in fish (Brett 1979; Pedersen 1987; Thetmeyer, Waller, Black, Inselmann & Rosenthal 1999; Foss, Evensen & Øiestad 2002; Bernier & Craig 2005; Carter, Katersky, Barnes, Bridle & Hauler 2008; Glencross 2009; Remen, Oppedal, Torgersen, Imsland & Olsen 2012; Burt, Hamoutene, Perez-Casanova, Kurt Gamperl & Volkoff 2014; Remen, Aas, Vågseth, Torgersen, Olsen, Imsland & Oppedal 2014; Hansen, Olsen, Stien, Oppedal, Torgersen, Breck, Remen, Vågseth & Fjelldal 2015), salmon in the present study had significantly reduced feed intake and growth in the LO group compared to HO group. Limited availability of dissolved oxygen to salmon leads to compromises in feed intake and growth and is exacerbated by thermal stress. Performance of salmon under hypoxia differs with different water temperatures (Barnes *et al.* 2011), and, by way of a pair-feeding technique, we have previously shown that a reduction in feed intake did not solely cause the growth reduction in salmon held under hypoxia at an optimal growth temperature (12°C) (Vikeså *et al.* 2016). The reduced growth is suggested to be a direct effect of hypoxia on growth mechanisms which may vary with fish species and environmental factors such as temperature, oxygen, CO_2 and photoperiod. Nutrient retention efficiency and FCR were not affected by LO in the present study, which is in

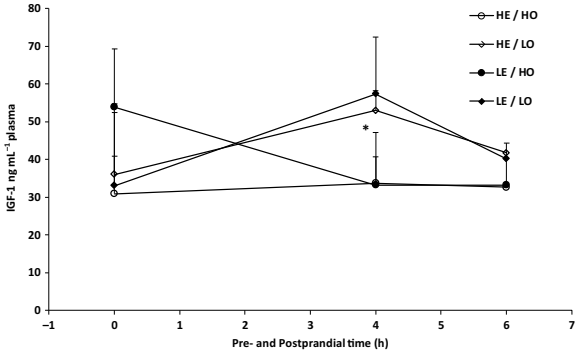

Figure 3 Concentration of IGF1 in plasma (ng mL^{-1}) sampled 0 h pre- and 4 and 6 h postprandially from Atlantic salmon given diets differing in DE level under different DO conditions at 17°C (n = 4). The symbol * indicates a significant effect of DO at the 4-h sampling point (two-way ANOVA, $P < 0.05$).

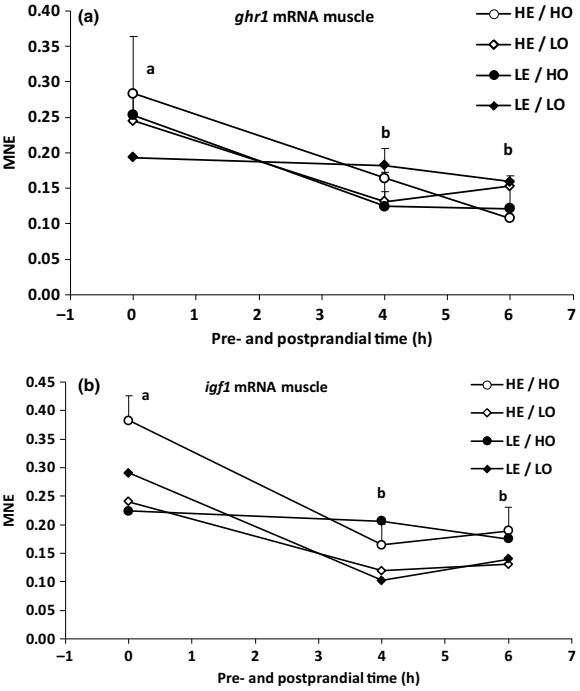

Figure 4 Mean normalized expression (MNE) of (a) *ghr1* and (b) *igf1* mRNA levels in white muscle sampled 0 h pre- and 4 and 6 h postprandially from Atlantic salmon given diets differing in DE level under different DO conditions at 17°C (n = 4). The letters a and b indicate significant differences between the means for each sampling point (two-way ANOVA with repeated analyses, $P < 0.05$).

line with several studies on hypoxia (Brett 1979; Caldwell & Hinshaw 1994; Thetmeyer *et al.* 1999; Foss *et al.* 2002; Carter *et al.* 2008; Glencross 2009; Remen *et al.* 2012, 2014; Hansen *et al.* 2015). In contrast, we have formerly shown that feeding salmon with HE or LE diets under hypoxia (LO) at 12°C resulted in an overall higher FCR and reduced protein and energy retention (Vikeså *et al.* 2016) than under normoxia (HO). Feeding HE diets under hypoxia improved FCR and energy retention efficiency at both 12°C and in the present study at 17°C. High dietary energy also increased growth (final BW and SGR) and concurs with previous results (Vikeså *et al.* 2016).

A separation of the effects from temperature and oxygen can be difficult as the combination of the

Table 3 Mean plasma concentration (μmol mL^{-1}) of individual essential free amino acids (EFAA) sampled 0 h pre- and 4 and 6 h postprandially from Atlantic salmon given diets differing in DE level (a: HE diet groups and b: LE diet groups) under different DO conditions at 17°C (n = 4)

	(a)					
	HE/HO			HE/LO		
	0 h	4 h	6 h	0 h	4 h	6 h
Thr	0.142/0.009*	0.263/0.034	0.248/0.008	0.176/0.018*	0.277/0.025	0.274/0.039
Val	0.439/0.049*	0.557/0.035	0.567/0.023	0.513/0.049*	0.606/0.025	0.620/0.052
Met	0.069/0.005*	0.122/0.014	0.128/0.010	0.074/0.004*	0.138/0.010	0.115/0.020
Ile	0.176/0.025*	0.245/0.018	0.262/0.015	0.203/0.021*	0.273/0.009	0.272/0.018
Leu	0.322/0.025*	0.435/0.035	0.471/0.035	0.375/0.020*	0.499/0.013	0.468/0.036
Phe	0.117/0.004*	0.178/0.016	0.210/0.013	0.133/0.004*	0.202/0.008	0.165/0.024
Lys	0.220/0.024*	0.446/0.039	0.387/0.033	0.198/0.045*	0.419/0.033	0.403/0.040
His	0.051/0.006*	0.073/0.003	0.088/0.010	0.060/0.005*	0.074/0.001	0.080/0.007
Trp	0.013/0.001	0.016/0.001	0.016/0.000	0.017/0.001	0.021/0.002	0.014/0.001
Arg	0.113/0.010*	0.247/0.033	0.252/0.031	0.104/0.011*	0.253/0.022	0.215/0.036
Tot FAA	5.783/0.384*	7.758/0.316	7.259/0.259	6.318/0.113*	7.631/0.144	7.510/0.928
EFAA	1.664/0.123*	2.581/0.176	2.629/0.069	1.852/0.163*	2.763/0.116	2.626/0.205

	(b)						
	LE/HO			LE/LO			
	0 h	4 h	6 h	0 h	4 h	6 h	DE
Thr	0.181/0.034*	0.306/0.016	0.282/0.048	0.180/0.024*	0.246/0.006	0.310/0.019	**
Val	0.548/0.131*	0.559/0.007	0.579/0.054	0.436/0.033*	0.656/0.047	0.654/0.021	
Met	0.067/0.006*	0.165/0.008	0.141/0.018	0.073/0.004*	0.119/0.014	0.136/0.008	
Ile	0.227/0.062*	0.257/0.004	0.276/0.035	0.165/0.014*	0.297/0.018	0.305/0.014	
Leu	0.389/0.094*	0.485/0.010	0.512/0.067	0.314/0.016*	0.505/0.028	0.544/0.019	
Phe	0.147/0.023*	0.213/0.013	0.213/0.025	0.129/0.005*	0.181/0.027	0.211/0.008	
Lys	0.372/0.168*	0.471/0.024	0.434/0.038	0.215/0.048*	0.516/0.077	0.436/0.013	
His	0.070/0.005*	0.092/0.004	0.085/0.002	0.070/0.013*	0.088/0.003	0.084/0.005	**
Trp	0.019/0.005	0.017/0.000	0.018/0.003	0.016/0.001	0.019/0.002	0.020/0.002	
Arg	0.134/0.034*	0.304/0.004	0.302/0.036	0.103/0.011*	0.243/0.025	0.272/0.022	
Tot FAA	6.103/0.894*	8.049/0.076	8.073/0.411	5.783/0.337*	7.668/0.310	7.896/0.185	
EFAA	2.154/0.557*	2.869/0.027	2.841/0.294	1.700/0.137*	2.870/0.146	2.973/0.09	

The symbol * indicates significant differences between sampling time points for each treatment group per FAA (two-way ANOVA with repeated analyses, $P < 0.05$). A significant effect of DE on histidine is indicated by the symbols ** (two-way ANOVA, $P < 0.05$).

two environmental factors has a confounding effect on the metabolism, which may explain some of the response inconsistency in feed conversion and nutrient retention between the present hypoxia study at 17°C and former at 12°C (Vikeså *et al.* 2016).

Mortality

Fish mortality (~15%) was generally high in all treatment groups which probably reflects a more severe reaction to the rise in temperature from 12 to 17°C during the initial phase of the study. Similarly, a high mortality (~10%) has been reported for postsmolts under hypoxic (~90% and ~60% saturated oxygen) and warm conditions (19°C), and fed diets with DE level equivalent to the present HE diet (Carter *et al.* 2008). The LO treatment had an overall negative effect on survival, while a significant effect from DE level was not detected. However, it is interesting to see that the numerical difference in mortality under LO conditions was 44% higher in fish groups fed the LE diets compared to HE diet groups.

Plasma ghrelin

The present study did not find any significant differences in circulating ghrelin concentrations due to diet or oxygen treatments. Mean concentration

was higher at 6 h postprandially compared to −2 and 0 h preprandially, but no preprandial ghrelin peaks were detected, even though sampling covered both a pre- and postprandial time range. Similar results with respect to time have previously been reported for postsmolt at 13 and 19°C under normoxic conditions (Vikeså *et al.* 2015); however, a second experiment in the same publication showed clear peaks before meal times with large salmon at 12°C under normoxia (Vikeså *et al.* 2015). Elevated plasma ghrelin levels (samples 4 h postprandially only) are also reported for postsmolt due to hypoxia at 12°C, in spite of a reduced feed intake compared to those at normoxic conditions (Vikeså *et al.* 2016). DE level did not result in different plasma ghrelin concentrations, which is in line with previous studies with salmon (Vikeså *et al.* 2016, Hevrøy *et al.* 2012). Plasma ghrelin levels did not differ between groups of postsmolt held at 13 and 19°C (Vikeså *et al.* 2015), while Hevrøy *et al.* (2012) reported suppressed plasma ghrelin in large salmon at 19°C compared to 14°C, all under normoxia.

Seasonal changes in light regime, feeding schedules or ambient water temperature can interfere with physiological functions and behavioural features regulated by the circadian rhythms, also in fish (Boujard & Leatherland 1992; Rensing & Ruoff 2002; Kulczykowska & Sánchez Vázquez 2010; Feliciano, Vivas, de Pedro, Delgado, Velarde & Isorna 2011; Hoskins 2011; Nisembaum, Velarde, Tinoco, Azpeleta, De Pedro, Alonso-Gmez, Delgado & Isorna 2012; Vera, Negrini, Zagatti, Frigato, Snchez-Vzquez & Bertolucci 2013), including Atlantic salmon (Betancor, McStay, Minghetti, Migaud, Tocher & Davie 2014). It is therefore possible that differences in time course and feeding regime between studies can impact on the timing and extent of ghrelin peaks and feed anticipation as in (Yannielli, Molyneux, Harrington & Golombek 2007; Nisembaum, de Pedro, Delgado & Isorna 2014; Sanchez-Bretano, Blanco, Unniappan, Kah, Gueguen, Bertucci, Alonso-Gomez, Valenciano, Isorna & Delgado 2015). Experimental parameters differ between the present and referred studies, but two common conditions for studies with positive ghrelin responses are that they were both long term (2–4 months) prior to sampling and included large salmon (Hevrøy *et al.* 2012; Vikeså *et al.* 2015, 2016). Hevrøy *et al.* (2012) even involved a 1.5-month acclimation period prior to onset of experimental treatments. Salmon in the present study were exposed to experimental conditions for 1 month, and a two-phase feeding regime was applied only the two last weeks before blood sampling. It is likely that the adaptation period was insufficient for the fish circadian system to develop and establish a regular feeding anticipation regime, resulting in the unexpected preprandial plasma ghrelin concentrations.

Plasma IGF-1 and white muscle ghr1 and igf1 mRNA

Circulating IGF-1 levels were higher at 4 h postprandially in salmon treated with LO conditions compared to HO conditions at a high temperature, while dietary DE level had no effect on plasma IGF-1. In contrast, DO did not affect plasma IGF-1 for a similar postprandial sampling point when large salmon were held at 12°C, even though other growth regulation parameters were negatively affected by low oxygen in the same study (Vikeså *et al.* 2016). White muscle *igf1* and *ghr1* mRNA expressions were unaffected by the DO and DE treatments, but both showed differences in pre- and postprandial levels with a significant drop at 4 and 6 h postprandially compared to just before feeding (0 h).

We have previously demonstrated that high temperature (19°C) downregulates *igf1* mRNA expression irrespective of postprandial time, resulting in a more rapidly declining plasma concentration after the peak at approximately 16 h postprandially (Vikeså *et al.* 2015), reflecting a lower growth potential at high temperature. This may have had an overriding influence on the present treatments, possibly explaining the different response to HO by plasma IGF-1 at 17 versus 12°C and the reduced postprandial mRNA levels of *igf1* and *ghr1*. However, similar postprandial mRNA levels do not necessarily mean that there was a post-transcriptional limitation on the GH1 and IGF-1 synthesis in all treatment groups. As plasma IGF-1 is influenced by oxygen as shown by lower levels at HO, this might indicate a higher clearance rate and possibly a higher receptor activity, reflected by the better growth of HO-treated fish.

For IGF-1 to mediate its function as a growth stimulant, it not only depends on circulating GH1 to be synthesized, but also on IGF-1-binding proteins (IGFBPs) which regulate the binding of IGF-1 to receptors in target tissues (Reinecke 2010).

IGFBPs are stimulated by nutritional cues (Shimizu, Dickey, Fukada & Dickhoff 2005; Shimizu, Beckman, Hara & Dickhoff 2006), and variations in IGFBP levels in fish are connected to other hormones, metabolic status (e.g. starvation and stress), developmental stage and environmental conditions such as temperature and oxygen availability (Wood, Duan & Bern 2005). Postprandial plasma levels of IGFBPs are also reported to react differently to starvation/fasting (Kelley, Haigwood, Perez & Galima 2001; Shimizu, Cooper, Dickhoff & Beckman 2009; Hevrøy *et al.* 2011), stress (Kelley *et al.* 2001; Davis & Peterson 2006) and temperature (Davis & Peterson 2006; Hevrøy *et al.* 2013), which can alter the levels of IGFBPs in circulation. A complete understanding of IGFBP role in the fish metabolism remains to be described (Shimizu *et al.* 2009), but it is possible that adverse environmental conditions such as elevated temperature combined with hypoxia impose an imbalance in IGFBPs and the receptor modulation of the IGF1 activity, while synthesis of IGF-1 is being stimulated by the feed intake and GH1. This might explain the present accumulation of plasma IGF1 in LO-treated fish groups.

Plasma IGF-1 levels respond to food intake, and refeeding after 3-week fasting of coho salmon resulted in a rapid increase in plasma IGF-1 after only 2 h with a decline 14 h postprandially (Shimizu *et al.* 2009). Even a single day of feed deprivation affected the postprandial plasma levels of GH1, IGF-1 and IGFBPs (Shimizu *et al.* 2009). Whether a low feed intake at hypoxia and handling stress from sampling can have a similar short-term postprandial effect on plasma IGF-1 and IGFBPs as described for fasting (Shimizu *et al.* 2009), hence the increased plasma IGF-1 at 4 h postprandially, needs to be further verified.

Ghrelin is a well-known appetite stimulant, but it is also involved in growth regulation as it was first discovered as a GH secretagogue (Kojima, Hosoda, Date, Nakazato, Matsuo & Kangawa 1999). It is therefore possible that the lack of response in the GH-IGF system to the treatments could be linked to the present ghrelin results. The results could also indicate that the combination of hypoxia at a suboptimally high temperature represents catabolic rather than anabolic conditions for growth and appetite regulation in salmon. Reports on inheritable differences in salmon tolerance to critical temperature and oxygen levels (Barnes *et al.* 2011; Anttila, Dhillon, Boulding, Anthony, Glebe, Elliott, Wolters & Schulte 2013) bring in an aspect to future research that can be valuable for breeding salmon to tackle a warmer climate.

Plasma FAA

As expected, postprandial plasma EFAA concentrations were significantly higher than just before feeding time (0 h) for all treatments ($P < 0.05$), except for Trp ($P > 0.05$). However, this should be interpreted with care as plasma Trp is known to be bound to protein during analysis. Plasma FAA concentrations were unaffected by the low oxygen conditions and dietary energy at both 0 h preprandially and 4 to 6 h postprandially ($P > 0.05$). A lower His level in LE diet groups was the only exception ($P < 0.05$). Our previous study showed that the effect of a high temperature is not evident until after 12 h postprandially, where plasma EFAA levels were absorbed/declined at a faster rate at 19°C than at 13°C (Vikeså *et al.* 2015). LO-treated salmon had a reduced mean feed intake over the experimental period which indicates a lower supply of FAA for the synthesis of protein and muscle growth. Although sampling of fish represents only 1 day of the experiment, the unaffected plasma EFAA concentrations and protein retention efficiency suggest an increased protein turnover at hypoxia compared to normoxia. The lack of a postprandial peak in circulating FAA levels at either 4 and 6 h for any of the treatment groups ($P > 0.05$) is in line with previous findings for 4 and 8 h for postsmolt held at 13 and 19°C (Vikeså *et al.* 2015). However, the plasma FAA level tended to peak at 8 h in the lowest temperature group (Vikeså *et al.* 2015). This is comparable to findings from postsmolt held at even colder temperatures (8.6 ± 0.6°C and 10.8 ± 0.1°C), where plasma EFAA concentrations were highest from 6 to 12 h compared to 0 and 24–72 h postprandially (Espe, Lied & Torrissen 1993; Torrisen, Lied & Espe 1995). The comparison of the present plasma EFAA results at 17°C to previous postprandial studies (Espe *et al.* 1993; Torrisen *et al.* 1995; Vikeså *et al.* 2015) indicates a less pronounced absorption of FAA into plasma and a faster depletion rate at higher temperatures than at colder temperatures. This has implications for muscle growth and may explain some of the reduced growth seen for fish at higher temperatures.

Conclusions

The present study confirms that LO under warm conditions also reduces feed intake and growth with no impact on feed utilization, which is well known for several fish species, including salmon, under hypoxia at temperatures considered optimal for growth. Reduced growth under hypoxia can be explained by an increased breakdown of protein from internal sources to maintain the FAA supply, as plasma EFAA concentrations were similar in the treatment groups, despite the reduced feed intake under hypoxia. Feeding HE diets under hypoxia at a warm temperature indicates that it is possible to improve survival of salmon and should be further investigated.

Variable differences in pre- and postprandial plasma IGF-1 levels and white muscle gene expressions of *igf1* and *ghr1* demonstrated a low response to the present DO and DE treatments, which were possibly overruled by the influence of high temperature representing a generally lower growth potential.

Pre- and postprandial measurements failed to demonstrate preprandial ghrelin peaks in relation to meal times, suggesting that a sufficient adaptation to feeding regime is essential to develop an endogenous feeding rhythm and to correlate ghrelin levels to feed anticipation and appetite regulation. It also demonstrates that disruptions of feeding regimes can potentially interfere with appetite signalling with consequences for fish growth.

Acknowledgments

The authors would like to thank staff at Matre Havbruksstasjon (Institute of Marine Research), NIFES and Skretting ARC laboratory. Review and comments on the manuscript by Dr Saravanan Subramanian were very helpful and highly appreciated. The experiment was financially supported by the Norwegian Research Council (NFR, grant number 199683/S40). The study is part of the first authors' PhD which is supported by the NFR (grant number 209655/O30), employer Skretting ARC and the collaborating institute NIFES.

References

Anttila K., Dhillon R.S., Boulding E.G., Farrell A.P., Glebe B.D., Elliott J.A.K., Wolters W.R. & Schulte P.M. (2013) Variation in temperature tolerance among families of Atlantic salmon (*Salmo salar*) is associated with hypoxia tolerance, ventricle size and myoglobin level. *Journal of Experimental Biology* **216**, 1183–1190.

Barnes R., King H. & Carter C.G. (2011) Hypoxia tolerance and oxygen regulation in Atlantic salmon, Salmo salar from a Tasmanian population. *Aquaculture* **318**, 397–401.

Beckman B.R. (2011) Perspectives on concordant and discordant relations between insulin-like growth factor 1 (IGF1) and growth in fishes. *General and Comparative Endocrinology* **170**, 233–252.

Beckman B.R., Larsen D.A., Moriyama S., Lee-Pawlak B. & Dickhoff W.W. (1998) Insulin-like growth factor-I and environmental modulation of growth during smoltification of spring chinook salmon (*Oncorhynchus tshawystscha*). *General and Comparative Endocrinology* **109**, 325–335.

Beckman B.R., Shimizu M., Gadberry B.A., Parkins P.J. & Cooper K.A. (2004) The effect of temperature change on the relations among plasma IGF-I, 41-kDa IGFBP, and growth rate in postsmolt coho salmon. *Aquaculture* **241**, 601–619.

Bendiksen E.Å., Jobling M. & Arnesen A.M. (2002) Feed intake of Atlantic salmon parr *Salmo salar* L. in relation to temperature and feed composition. *Aquaculture Research* **33**, 525–532.

Bernier N.J. & Craig P.M. (2005) CRF-related peptides contribute to stress response and regulation of appetite in hypoxic rainbow trout. *American Journal of Physiology: Regulatory, Integrative and Comparative Physiology* **289**, R982–R990.

Betancor M., McStay E., Minghetti M., Migaud H., Tocher D. & Davie A. (2014) Daily rhythms in expression of genes of hepatic lipid metabolism in Atlantic Salmon (*Salmo salar* L.). *PLoS ONE* **9**, e106739, 1–9.

Boujard T. & Leatherland J. (1992) Circadian rhythms and feeding time in fishes. *Environmental Biology of Fishes* **35**, 109–131.

Brett J.R. (1979) Environmental factors and growth. In: *Fish Physiology* (ed. by W.S. Hoar, C.F. Randall & J.R. Brett). Academic Press, London, UK.

Burt K., Hamoutene D., Perez-Casanova J., Kurt Gamperl A. & Volkoff H. (2014) The effect of intermittent hypoxia on growth, appetite and some aspects of the immune response of Atlantic salmon (*Salmo salar*). *Aquaculture Research* **45**, 124–137.

Caldwell C.A. & Hinshaw J. (1994) Physiological and haematological responses in rainbow trout subjected to supplemental dissolved oxygen in fish culture. *Aquaculture* **126**, 183–193.

Carter C.G., Katersky R.S., Barnes J.C., Bridle A.R. & Hauler R.C. (2008) *Assessment of Fish Growth Performance under Limiting Environmental Conditions: Aquaculture Nutrition Subprogram. FRDC Final Report*. Tasmanian Aquaculture and Fisheries Institute, Tasmania, Australia.

Choi K., Roh S.G., Hong Y.H., Shrestha Y.B., Hishikawa D., Chen C., Kojima M., Kangawa K. & Sasaki S. (2003) The role of ghrelin and growth hormone secretagogues receptor on rat adipogenesis. *Endocrinology* **144**, 754–759.

Cummings D.E. (2006) Ghrelin and the short- and long-term regulation of appetite and body weight. *Physiology & Behavior* **89**, 71–84.

Cummings D.E. & Shannon M.H. (2003) Roles for ghrelin in the regulation of appetite and body weight. *Archives of Surgery (Chicago, IL: 1960)* **138**, 389–396.

Davis K.B. & Peterson B.C. (2006) The effect of temperature, stress, and cortisol on plasma IGF-I and IGFBPs in sunshine bass. *General and Comparative Endocrinology* **149**, 219–225.

Deane E.E. & Woo N.Y.S. (2009) Modulation of fish growth hormone levels by salinity, temperature, pollutants and aquaculture related stress: a review. *Review in Fish Biology and Fisheries* **19**, 97–120.

De-Santis C. & Jerry D.R. (2007) Candidate growth genes in finfish – where should we be looking? *Aquaculture* **272**, 22–38.

Duan C. (1998) Nutritional and developmental regulation of insulin-like growth factors in fish. *Journal of Nutrition* **128**, 306S–314S.

Dyer A.R., Barlow C.G., Bransden M.P., Carter C.G., Glencross B.D., Richardson N., Thomas P.M., Williams K.C. & Carragher J.F. (2004) Correlation of plasma IGF-I concentrations and growth rate in aquacultured finfish: a tool for assessing the potential of new diets. *Aquaculture* **236**, 583–592.

Elliott J.M. & Elliott J.A. (2010) Temperature requirements of Atlantic salmon *Salmo salar*, brown trout *Salmo trutta* and Arctic charr *Salvelinus alpinus*: predicting the effects of climate change. *Journal of Fish Biology* **77**, 1793–1817.

Elliott J.M. & Hurley M.A. (1997) A functional model for maximum growth of Atlantic salmon parr, *Salmo salar*, from two populations in northwest England. *Functional Ecology* **11**, 592–603.

Espe M., Lied E. & Torrissen K.R. (1993) Changes in plasma and muscle free amino acids in Atlantic salmon (*Salmo salar*) during absorption of diets containing different amounts of hydrolysed cod muscle protein. *Comparative Biochemistry and Physiology Part A: Physiology* **105**, 555–562.

Farrell A.P. & Richards J.G. (2009) Defining hypoxia. In: *Hypoxia*(1st edn) (ed. by J.G. Richards, A.P. Farrell & C.J. Brauner). Elsevier, Amsterdam, the Netherlands.

Feliciano A., Vivas Y., de Pedro N., Delgado M.J., Velarde E. & Isorna E. (2011) Feeding time synchronizes clock gene rhythmic expression in brain and liver of goldfish (*Carassius auratus*). *Journal of Biological Rhythms* **26**, 24–33.

Forseth T., Hurley M.A., Jensen A.J. & Elliott J.M. (2001) Functional models for growth and food consumption of Atlantic salmon parr, *Salmo salar*, from a Norwegian river. *Freshwater Biology* **46**, 173–186.

Foss A., Evensen T.H. & Øiestad V. (2002) Effects of hypoxia and hyperoxia on growth and food conversion efficiency in the spotted wolffish *Anarhichas minor* (Olafsen). *Aquaculture Research* **33**, 437–444.

Frøiland E., Murashita K., Jørgensen E.H. & Kurokawa T. (2010) Leptin and ghrelin in anadromous Arctic charr: cloning and change in expressions during a seasonal feeding cycle. *General and Comparative Endocrinology* **165**, 136–143.

Gabillard J.C., Weil C., Rescan P.Y., Navarro I., Gutiérrez J. & Le Bail P.Y. (2003) Environmental temperature increases plasma GH levels independently of nutritional status in rainbow trout (*Oncorhynchus mykiss*). *General and Comparative Endocrinology* **133**, 17–26.

Gabillard J.C., Weil C., Rescan P.Y., Navarro I. & Gutierrez J. (2005) Does the GH/IGF system mediate the effect of water temperature on fish growth? *A Review Cybium* **29**, 107–117.

Glencross B.D. (2009) Reduced water oxygen levels affect maximal feed intake, but not protein or energy utilization efficiency of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture Nutrition* **15**, 1–8.

Handeland S.O., Imsland A.K. & Stefansson S.O. (2008) The effect of temperature and fish size on growth, feed intake, food conversion efficiency and stomach evacuation rate of Atlantic salmon post-smolts. *Aquaculture* **283**, 36–42.

Hansen T.J., Olsen R.E., Stien L., Oppedal F., Torgersen T., Breck O., Remen M., Vågseth T. & Fjelldal P.G. (2015) Effect of water oxygen level on performance of diploid and triploid Atlantic salmon post-smolts reared at high temperature. *Aquaculture* **435**, 354–360.

Hevrøy E.M., Espe M., Waagbø R., Sandnes K., Ruud M. & Hemre G.I. (2005) Nutrient utilization in Atlantic salmon (*Salmo salar* L.) fed increased levels of fish protein hydrolysate during a period of fast growth. *Aquaculture Nutrition* **11**, 301–313.

Hevrøy E.M., Azpeleta C., Shimizu M., Lanzén A., Kaiya H., Espe M. & Olsvik P.A. (2011) Effects of short-term starvation on ghrelin, GH-IGF system, and IGF-binding proteins in Atlantic salmon. *Fish Physiology and Biochemistry* **37**, 217–232.

Hevrøy E.M., Waagbø R., Torstensen B.E., Takle H., Stubhaug I., Jørgensen S.M., Torgersen T., Tvenning L., Susort S., Breck O. & Hansen T. (2012) Ghrelin is involved in voluntary anorexia in Atlantic salmon raised at elevated sea temperatures. *General and Comparative Endocrinology* **175**, 118.

Hevrøy E.M., Hunskår C., Gelder S., Shimizu M., Waagbø R., Breck O., Takle H., Susort S. & Hansen T. (2013) GH–IGF system regulation of attenuated muscle growth and lipolysis in Atlantic salmon reared at elevated sea temperatures. *Journal of Comparative Physiology B* **183**, 243–259.

Hoskins L. (2011) *Characterization of Appetite-Related Neuropeptides in Goldfish (Carassius auratus) and Atlantic cod (Gadus morhua): Daily Rhythms in Gene Expression and Effects of Long-Term Administration*. MSc, Memorial University of Newfoundland, St John's, Newfoundland and Labrador, Canada, USA.

Imsland A.K., Björnsson B.T., Gunnarsson S., Foss A. & Stefansson S.O. (2007) Temperature and salinity effects on plasma insulin-like growth factor-I concentrations and growth in juvenile turbot (*Scophthalmus maximus*). *Aquaculture* **271**, 546–552.

Inui A., Asakawa A., Bowers C.Y., Mantovani G., Laviano A., Meguid M.M. & Fujimiya M. (2004) Ghrelin, appetite, and gastric motility: the emerging role of the stomach as an endocrine organ. *The FASEB Journal* **18**, 439–456.

Jönsson E. (2013) The role of ghrelin in energy balance regulation in fish. *General and Comparative Endocrinology* **187**, 79–85.

Kaiya H., Kojima K., Hosoda H., Moriyama S., Takahashi A., Kawauchi H. & Kangawa K. (2003a) Peptide purification, complementary deoxyribonucleic acid (DNA) and genomic DNA cloning, and functional characterisation of Ghrelin in rainbow trout. *Endocrinology* **144**, 5215–5226.

Kaiya H., Kojima K., Hosoda H., Riley L.G., Hirano T., Gordon Grau E. & Kangawa K. (2003b) Identification of tilapia ghrelin and its effects on growth hormone and prolactin release in the tilapia, *Oreochromis mossambicus*. *Comparative Biochemistry and Physiology, Part B* **135**, 421–429.

Kelley K.M., Haigwood J.T., Perez M. & Galima M.M. (2001) Serum insulin-like growth factor binding proteins (IGFBPs) as markers for anabolic/catabolic condition in fishes. *Comparative Biochemistry and Physiology – Part B* **129**, 229–236.

Kojima M., Hosoda H., Date Y., Nakazato M., Matsuo H. & Kangawa K. (1999) Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* **402**, 656–660.

Koskela J., Pirhonen J. & Jobling M. (1997) Feed intake, growth rate and body composition of juvenile Baltic salmon exposed to different constant temperatures. *Aquaculture International* **5**, 351–360.

Kulczykowska E. & Sánchez Vázquez F.J. (2010) Neurohormonal regulation of feed intake and response to nutrients in fish: aspects of feeding rhythm and stress. *Aquaculture Research* **41**, 654–667.

MacKenzie D.S., VanPutte C.M. & Leiner K.A. (1998) Nutrient regulation of endocrine function in fish. *Aquaculture* **161**, 3–25.

Nilsson G.E. (2007) Gill remodeling in fish – a new fashion or an ancient secret? *Journal of Experimental Biology* **210**, 2403–2409.

Nilsson G.E. & Randall D.J. (2010) Adaptations to hypoxia in fishes. In: *Respiratory Physoiology of Vertebrates*(1st edn) (ed. by G.E. Nilsson). Cambridge University Press, Cambridge, UK.

Nisembaum L.G., Velarde E., Tinoco A.B., Azpeleta C., De Pedro N., Alonso-Gmez A.L., Delgado M.J. & Isorna E. (2012) Light-dark cycle and feeding time differentially entrains the gut molecular clock of the goldfish (*Carassius auratus*). *Chronobiology International* **29**, 665–673.

Nisembaum L.G., de Pedro N., Delgado M.J. & Isorna E. (2014) Crosstalking between the "gut-brain" hormone ghrelin and the circadian system in the goldfish. Effects on clock gene expression and food anticipatory activity. *General and Comparative Endocrinology* **205**, 287–295.

Pedersen L.J. (1987) Energy budgets for juvenile Rainbow trout at various oxygen concentrations. *Aquaculture* **62**, 289–298.

Pérez-Sánchez J. (2000) The involvement of growth hormone in growth regulation, energy homeostasis and immune function in the gilthead sea bream (*Sparus aurata*): a short review. *Fish Physiology and Biochemistry* **22**, 135–144.

Pérez-Sánchez J. & Le Bail P.-Y. (1999) Growth hormone axis as marker of nutritional status and growth performance in fish. *Aquaculture* **177**, 117–128.

Pérez-Sánchez J., Martí-Palanca H. & Kaushik S.J. (1995) Ration size and protein intake affect circulating growth hormone concentration, hepatic growth hormone binding and plasma insulin-like growth factor-I immunoreactivity in a marine teleost, the gilthead sea bream (*Sparus aurata*). *Journal of Nutrition* **125**, 546.

Pérez-Sánchez J., Calduch-Giner J.A., Mingarro M., Vega-Rubín de Celis S., Gómez-Requeni P., Saera-Vila A., Astola A. & Valdivia M.M. (2002) Overview of fish growth hormone family. New insights in genomic organization and heterogeneity of growth hormone receptors. *Fish Physiology and Biochemistry* **27**, 243–258.

Perry S.F., Jonz M.G. & Gilmour K.M. (2009) Oxygen sensing and the hypoxic ventilatory response. In: *Hypoxia*(1st edn) (ed. by J.G. Richards, A.P. Farrell & C.J. Brauner). Academic Press, London, UK.

Peterson R.H. & Martin-Robichaud D.J. (1989) First feeding of Atlantic salmon (*Salmo salar* L.) fry as influenced by temperature regime. *Aquaculture* **78**, 35–53.

Picha M., Turano M., Beckman B. & Borski R. (2008) Endocrine biomarkers of growth and applications to aquaculture: a minireview of growth hormone, insulin-like growth factor (IGF)-I, and IGF-binding proteins as potential growth indicators in fish. *North American Journal of Aquaculture* **70**, 196–211.

Pickering A.D., Pottinger T.G., Sumpter J.P., Carragher J.F. & Le Bail P.Y. (1991) Effects of acute and chronic stress on the levels of circulating growth hormone in the rainbow trout, *Oncorhynchus mykiss*. *General and Comparative Endocrinology* **83**, 86–93.

Reinecke M. (2010) Influences of the environment on the endocrine and paracrine fish growth hormone–insulin-like growth factor-I system. *Journal of Fish Biology* **76**, 1233–1254.

Reinecke M., Björnsson B.T., Dickhoff W.W., McCormick S.D., Navarro I., Power D.M. & Gutiérrez J. (2005) Growth hormone and insulin-like growth factors in fish: where we are and where to go. *General and Comparative Endocrinology* **142**, 20–24.

Remen M., Oppedal F., Torgersen T., Imsland A.K. & Olsen R.E. (2012) Effects of cyclic environmental hypoxia on physiology and feed intake of post-smolt Atlantic salmon: Initial responses and acclimation. *Aquaculture* **326–329**, 148–155.

Remen M., Aas T.S., Vägseth T., Torgersen T., Olsen R.E., Imsland A. & Oppedal F. (2014) Production performance of Atlantic salmon (*Salmo salar* L.) postsmolts in cyclic hypoxia, and following compensatory growth. *Aquaculture Research* **45**, 1355–1366.

Rensing L. & Ruoff P. (2002) Temperature effect on entrainment, phase shifting, and amplitude of circadian clocks and its molecular bases. *Chronobiology International* **19**, 807–864.

Richards J.G. (2009) Metabolic and molecular responses of fish to hypoxia. In: *Hypoxia*(1st edn) (ed. by G.R. Jeffrey, P.F. Anthony & J.B. Colin). Elsevier, Amsterdam, the Netherlands.

Riley L.G., Fox B.K., Kaiya H., Hirano T. & Grau E.G. (2005) Long-term treatment of ghrelin stimulates feeding, fat deposition, and alters the GH/IGF-I axis in the tilapia, *Oreochromis mossambicus*. *General and Comparative Endocrinology* **142**, 234–240.

Sanchez-Bretano A., Blanco A.M., Unniappan S., Kah O., Gueguen M.M., Bertucci J.I., Alonso-Gomez A.L., Valenciano A.I., Isorna E. & Delgado M.J. (2015) In situ localization and rhythmic expression of ghrelin and ghs-r1 ghrelin receptor in the brain and gastrointestinal tract of goldfish (*Carassius auratus*). *PLoS ONE* **10**, 1–21.

Saravanan S., Geurden I., Figueiredo-Silva A.C., Kaushik S.J., Haidar M.N., Verreth J.A.J. & Schrama J.W. (2012) Control of voluntary feed intake in fish: a role for dietary oxygen demand in Nile tilapia (*Oreochromis niloticus*) fed diets with different macronutrient profiles. *British Journal of Nutrition* **108**, 1519–1529.

Saravanan S., Geurden I., Figueiredo-Silva A.C., Kaushik S., Verreth J. & Schrama J.W. (2013) Voluntary feed intake in rainbow trout is regulated by diet-induced differences in oxygen use. *Journal of Nutrition* **143**, 781–787.

Schmidt-Nielsen K. (1997) Energy metabolism. In: *Animal Physiology, Adaptation and Environment*(5th edn) (ed. by K. Schmidt-Nielsen). Cambridge University Press, Cambridge, UK.

Shimizu M., Dickey J.T., Fukada H. & Dickhoff W.W. (2005) Salmon serum 22 kDa insulin-like growth factor-binding protein (IGFBP) is IGFBP-1. *Journal of Endocrinology* **184**, 267–276.

Shimizu M., Beckman B.R., Hara A. & Dickhoff W.W. (2006) Measurement of circulating salmon IGF binding protein-1: assay development, response to feeding ration and temperature, and relation to growth parameters. *Journal of Endocrinology* **188**, 101–110.

Shimizu M., Cooper K.A., Dickhoff W.W. & Beckman B.R. (2009) Postprandial changes in plasma growth hormone, insulin, insulin-like growth factor (IGF)-I, and IGF-binding proteins in coho salmon fasted for varying periods. *American Journal of Physiology: Regulatory, Integrative and Comparative Physiology* **297**, R352–R361.

Solomon S., Qin D., Manning M., Chen Z., Marquis M., Averyt K.B., Tignor M. & Miller H.L. (2007) Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change, 2007 *IPCC (Intergovernmental Panel on Climate Changes)*.

Stevens E.D., Sutterlin A. & Cook T. (1998) Respiratory metabolism and swimming performance in growth hormone transgenic Atlantic salmon. *Canadian Journal of Fisheries and Aquatic Science* **55**, 2028–2035.

Thetmeyer H., Waller U., Black K.D., Inselmann S. & Rosenthal H. (1999) Growth of European sea bass (*Dicentrarchus labrax* L.) under hypoxic and oscillating oxygen conditions. *Aquaculture* **174**, 355–367.

Torrisen K.R., Lied E. & Espe M. (1995) Differences in utilization of dietary proteins with varying degrees of partial pre-hydrolysis in Atlantic salmon (*Salmo salar* L.) with genetically different trypsin isozymes. *Biopolymers and Isozymes: Structure, Functions and Applications* 432–442.

Ueno H., Yamaguchi H., Kangawa K. & Nakazato M. (2005) Ghrelin: a gastric peptide that regulates food intake and energy homeostasis. *Regulatory Peptides* **126**, 11–19.

Ultsch G., Jackson D. & Moalli R. (1981) Metabolic oxygen conformity among lower vertebrates: the toadfish revisited. *Journal of Comparative Physiology* **142**, 439–443.

Unniappan S. & Peter R.E. (2005) Structure, distribution and physiological functions of ghrelin in fish. *Comparative Biochemistry and Physiology Part A Molecular Integrative Physiology* **140**, 396–408.

Vandesompele J., De Preter K., Pattyn F., Poppe B., Van Roy N., De Paepe A. & Speleman F. (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology* **3**, 18.

Vera L.M., Negrini P., Zagatti C., Frigato E., Snchez-Vzquez F.J. & Bertolucci C. (2013) Light and feeding entrainment of the molecular circadian clock in a marine teleost (*Sparus aurata*). *Chronobiology International* **30**, 649–661.

Vikeså V., Nankervis L., Remø S.C., Waagbø R. & Hevrøy E.M. (2015) Pre and postprandial regulation of

ghrelin, amino acids and IGF1 in Atlantic salmon (*Salmo salar* L.) at optimal and elevated seawater temperatures. *Aquaculture* **438**, 159–169.

Vikeså V., Nankervis L. & Hevroy E.M. (2016) Appetite, metabolism and growth regulation in Atlantic salmon (*Salmo salar* L.) exposed to hypoxia at elevated seawater temperature. *Aquaculture Nutrition* (In press).

Wilkinson R.J., Porter M., Woolcott H., Longland R. & Carragher J.F. (2006) Effects of aquaculture related stressors and nutritional restriction on circulating growth factors (GH, IGF-I and IGF-II) in Atlantic salmon and rainbow trout. *Comparative Biochemistry and Physiology, Part A* **145**, 214–224.

Wood A.W., Duan C. & Bern H.A. (2005) Insulin-like growth factor signaling in fish. *International Review of Cytology* **243**, 215–285.

Yannielli P.C., Molyneux P.C., Harrington M.E. & Golombek D.A. (2007) Ghrelin effects on the circadian system of mice. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience* **27**, 2890–2895.

