Environmental, Nutritional and Endocrine Regulation of Metabolic Processes in Fish

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av

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To Jenny

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Andreas Kullgren, 2011

ABSTRACT

Due to seasonal variations in temperature and food availability, fish in temperate regions should be able to make metabolic adjustments to ensure that enough energy is available for the maintenance of basal processes. The major aim of this thesis was to elucidate how the physiology and lipid metabolism of salmonid fish is affected by temperature and food availability, and to clarify aspects of the endocrine control of lipid metabolism.

In this thesis, the effects of increased temperature or reduced food availability were studied in salmonids by employing a non-prejudiced metabolomics approach to assess the physiological responses. Detailed information on the abundance of specific amino acids, lipid classes, fatty acids and other metabolites in tissue extracts and plasma was obtained by nuclear magnetic resonance (NMR) based metabolomics. NMR-based metabolomics were successfully employed and proved to be applicable to studying metabolic fluxes in fish, providing data on novel and integrated responses. The results show similar changes in lipid metabolism during food deprivation and elevated temperature. The observed responses included increased plasma very-low-density lipoprotein (VLDL) and unsaturated fatty acids (FAs) concurrent with decreased high-density lipoprotein (HDL) and choline. The changes during starvation also involved changes in amino acids and glycogen that indicate that amino acids are used for gluconeogenesis in the liver to preserve glycogen stores.

Growth hormone (GH) has both lipolytic and lipogenic effects. To further elucidate the mechanisms of GH action on salmonid lipid metabolism, the effects of GH *in vivo* on the transcription of several key lipid metabolism enzymes in various tissues were investigated. GH inhibited the hepatic expression of lipoprotein lipase (LPL) thereby decreasing hepatic lipid uptake. Hormone-sensitive lipase (HSL) mRNA expression was not increased by GH in any of the studied tissues, suggesting that the well-known GH-induced lipolysis is regulated on posttranslational levels in rainbow trout. The regulation lipid metabolism in salmonids was further investigated by studying direct effects of FAs and ghrelin on freshly isolated cells from mesenteric adipose tissue and liver. FAs elicited acute negative effects on lipid storage by decreasing lipid uptake via LPL activity in adipose cells as well as by stimulating lipolysis of stored triglycerides (TG) in liver cells.

Together the results presented in this thesis shows that elevated, suboptimal temperature and nutritional may have propound effects on important processes as growth, food intake and the metabolome of salmonid fish, and may lead to a negative energy balance. Metabolic changes may be mediated by hormonal and nutrient factors acting at gene expression or enzyme activity level. The results may contribute to better understand lipid deposition patterns in farmed fish and potential effects of climate change on salmonids in the wild and in aquaculture.

KEYWORDS: lipids, metabolism, temperature, fasting, growth hormone, ghrelin, NMR, metabolomics, *Salmo salar*, *Oncorhynchus mykiss*

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LIST OF PAPERS

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Paper II Kullgren A, Jutfelt F, Fontanillas R, Sundell K, Samuelsson LM, Wiklander K, Kling P, Koppe W, Larsson DGJ, Björnsson BTh, Jönsson E. The impact of temperature on the metabolome and endocrine metabolic signaling in Atlantic salmon (*Salmo salar*). *Accepted for publication in Journal of Comparative Physiology B*

Paper III Kullgren A, Björnsson BTh, Jönsson E. Growth hormone regulates lipid metabolism-related genes in rainbow trout *in vivo*. *Manuscript*

Paper IV Kullgren A, Jönsson E. Lipid uptake and mobilization in isolated rainbow trout hepatocytes and adipocytes are affected by fatty acids and temperature. *Manuscript*

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The most exciting phrase to hear in science, the one that heralds new discoveries, is not Eureka! (I found it!), but rather, “hmm…. that’s funny….”

Isaac Asimov

Environmental, Nutritional and Endocrine Regulation of Metabolic Processes in Fish

Andreas Kullgren

INTRODUCTION

Environmental physiology of animals

Seasons

In temperate regions, the significant and complex seasonal environmental changes provide a challenge for animals which requires physiological adaptations to cope with the changing environment. Apart from temperature, day length and food availability change drastically during the course of the year. Day length (light) is considered the main trigger for several season-related physiological changes, often in combination with temperature (Björnsson et al. 2011).

Temperature

Ectothermic vertebrates such as fish are strongly influenced by environmental temperatures, with impact on physiological processes, metabolism, growth and survival. Within a set tolerance limit, every species have temperature optima for metabolism, food conversion and growth, often changing with life stage and size. Growth, food intake and many metabolic processes increase with temperature up to the optimum, which is process- and species-specific, and a further increase in temperature has a negative impact on these processes. In order to tolerate very low seasonal (winter) temperatures for prolonged periods, many temperate species enter a state of low metabolic rate and growth. In contrast, during warm periods, elevation of temperatures above optimal may quickly reach the upper thermal tolerance limit with deleterious effects as a consequence.

Animal growth is a complex physiological process which may be regulated and affected by many factors (Box 1). The optimal temperature for growth of most salmonids is close to 15°C in the parr stage (Jonsson and Jonsson 2009). In seawater, post-smolt Atlantic salmon (*Salmo salar*) have an optimal temperature for growth at 13°C, but are able to maintain growth up to 19-20°C (Handeland et al. 2003). The capability of converting feed into growth (feed conversion efficiency, FCE) is maximal at about 3°C lower than the temperatures for optimal growth (Handeland et al. 2008). There appears to be little

evolutionary adaptations of temperature tolerance ranges for growth or FCE in salmonid species and the variations are phenotypically plastic (Forseth et al. 2009; Jonsson et al. 2001). However, adaptations to colder environments exist in FCE of Arctic charr (*Salvelinus alpinus*) (Larsson and Berglund 2005) and possibly for Atlantic salmon (Jonsson et al. 2001).

Box 1. Growth

To grow and to reach a large body size is of utmost importance as it increases fitness in many animal species. Fish exhibit indeterminate growth, *i.e.* they do not stop growing at a certain age or size. Growth occurs either through increased cell size (hypertrophy) or numbers (hyperplasia). An increase in body size can occur as length (skeletal) or mass (muscle and fat) growth. The relationship between length and weight is expressed by the condition factor (CF) and is a reflection of the body shape, or leanness, of the fish. Many factors affect growth, *e.g.* appetite, food intake, nutrient uptake and food conversion. Normally, fish do not grow at maximal rates. However, the full growth potential may be reached during catch-up, or compensatory, growth periods (Nicieza and Metcalfe 1997). There are costs associated with growing at elevated rates, which probably explains the restricted growth rates observed in wild fish (Johnsson and Bohlin 2006). There are different hypothesis regarding the mechanisms determining growth rates. The amount of lipid reserves or certain ratios between weight and length or liver size have been suggested as signals responsible for triggering or ending periods of elevated growth rates.

Climate change

The predicted changes in environmental conditions, especially the global temperature increase, are believed to affect geographical distribution, population traits (*e.g.* migration, spawning, hatching, smoltification, growth patterns and mortality), susceptibility and dispersal of disease and parasites, as well as food and habitat use of animals. If possible, fish may regulate their body temperature behaviorally by moving away from sub-optimal temperature conditions. As a consequence of global warming, changing spatial distribution of some marine fish populations has already been observed (Lenoir et al. 2011). As the predicted temperature increase is expected to be greater over land (*i.e.* in lakes, rivers and streams) than in the oceans (IPCC 2007), freshwater stages/species are likely to be affected most. During the warmest summer months, juvenile rainbow trout (*Oncorhynchus mykiss*) decreased their food intake, growth rate and feed conversion when subjected to a simulated global warming (2°C increase above the natural water temperature (Morgan et al. 2001)).

The altered conditions caused by climate change will likely have strong impact on aquaculture (Pankhurst and King 2010) as well as on population sizes of wild fish stocks, with serious economic and ecological consequences.

Salmonid fish

Two salmonid species, Atlantic salmon and rainbow trout, were studied in this thesis. Many salmonid species, *e.g.* Atlantic salmon and brown trout (*Salmo trutta*), commonly have anadromous life cycles, *i.e.* they spawn and hatch in fresh water, but spend most of their adult life in the sea (Box 2). However, resident individuals and populations of these species also exist. Salmonid fish encounter many major life-stage transitions and extreme conditions during their life cycle, such as smoltification, over-wintering, migration and spawning. Fish with complex life cycles exhibit major changes in physiology and behavior during the different life stages as well as during the transitions between them. These events are often orchestrated by the endocrine system. Due to these changes, it is important to consider the life stage of an animal when interpreting, discussing or comparing results and while designing experiments.

Salmonid species are globally of great economical and ecological importance. Recreational salmon and trout fishing is a major segment of the tourist/leisure industry in many countries. While highly limited in the Atlantic, commercial salmon fisheries are still a major industry in the Pacific. Both for sustaining recreational and commercial fisheries, as well as for restoring diminished/lost populations, billions of hatchery-raised juvenile salmon and trout are released into the wild each year. 2-2.5 million hatchery-raised Atlantic salmon smolts and 6-7 hundred thousand brown trout juveniles are released into Swedish rivers, and 500-1000 tonnes of rainbow trout are released into Swedish lakes each year. Salmonids are highly appreciated for their eating quality and several salmonid species are aquacultured around the world for food production. By far the largest production volume is in Atlantic salmon aquaculture, with 1.36 million tonnes produced in 2009, in North America, UK, Chile and especially in Norway, which produced close to 800 thousand tonnes. In Sweden, rainbow trout is the most important aquacultured fish species for food production, with annual production about 6500 tonnes in 2009, while the total rainbow trout production in the EU was about 200 thousand tonnes. In addition, Arctic charr is aquacultured in Sweden (about 700 tonnes in 2008), Norway and Iceland, as a more extreme cold-water species.

Knowledge on growth, metabolism and environmental physiology has great applied value and may be used to improve husbandry practices, improving fish welfare and perhaps most importantly, resource utilization in aquaculture, as feed costs are generally of critical importance in aquaculture production.

Domestication of rainbow trout started for more than 100 years ago. Since then, selective breeding mostly focusing on growth rate has greatly altered many characteristics of domesticated compared with wild fish. Elevated growth rate and appetite as well as increased amounts of mesenteric fat deposits (Figure 1) characterize aquacultured rainbow trout. The fish investigated in this thesis originate from aquaculture strains. Several breeding selection programs for Atlantic salmon have been ongoing for 20-30 years, both in Norway and UK, primarily focusing on growth rate for the first generations, but subsequently also considering other traits such as late maturation disease resistance and flesh quality.

Box 2. The Atlantic salmon anadromous lifecycle

The life cycle begins with the hatching of yolk-sac **alevins** in a freshwater stream. The alevins remain in the gravel bed until the yolk sack is depleted before they emerge and are now referred to as **fry**. Soon after they develop camouflaging dark marks on the side of the body and are referred to as **parr**. They linger in the stream for one to five years before they migrate to sea. During the seawards migration, a suite of physiological and morphological changes are initiated. The smoltification process, also called the parr-smolt transformation, prepares the fish for the marine environment. **Smolts** have a leaner body shape and a silvery appearance and osmoregulation is changed to meet the hyperosmotic (saline) conditions of the marine environment. The salmon normally spend one to two years in the ocean until the fish have obtained sufficient size and/or energy reserves. At this point, the fish enter puberty. After sexual maturation is initiated, a spawning migration to the native stream is commenced. During spawning in late autumn, females compete for favorable spawning grounds, and males compete for access to females. The eggs are fertilized in a depression in the stream floor and are covered with gravel. The eggs incubate during the winter and hatching occurs in spring.

Integrated metabolism

Lipid metabolism

Lipids are preferably stored as triglycerides (TG) in lipid droplets within cells. Salmonid fish are considered among the "fatty" fish species, as they have significant amount of fat stored in muscle. For aquacultured salmonids, fillet fat content is often 10-15%. In salmonid fish, mesenteric adipose tissue is considered most important for long-term storage, whereas liver and muscle are used for more short-term storage (Sheridan 1994). The carcass (head, skin, fins and skeleton) also contain a substantial amount of lipids, which may be mobilized during increased energy needs (Jörgensen et al. 1997; Jobling et al. 1998). The liver has an important role in lipid processing, by assembling very low-density lipoproteins (VLDL) for release into the plasma for transport to other tissues. Upon lipid mobilization, TG is hydrolyzed yielding three fatty acids (FA) and one glycerol. Mobilized FA may then be transported in the plasma bound to albumin or other proteins to other tissues. FA from lipid stores may also be used for β-oxidation within the cells (Figure 2).

Plasma lipoproteins in fish are similar to those in mammals (Skinner and Rogie 1978). Of the lipoproteins, VLDL contains most TG and as the TG content decreases by uptake into cells, VLDL turns into low-density lipoprotein (LDL). LDL and high-density lipoprotein (HDL), which contain most cholesterol, can be taken up by the liver and re-assembled into VLDL.

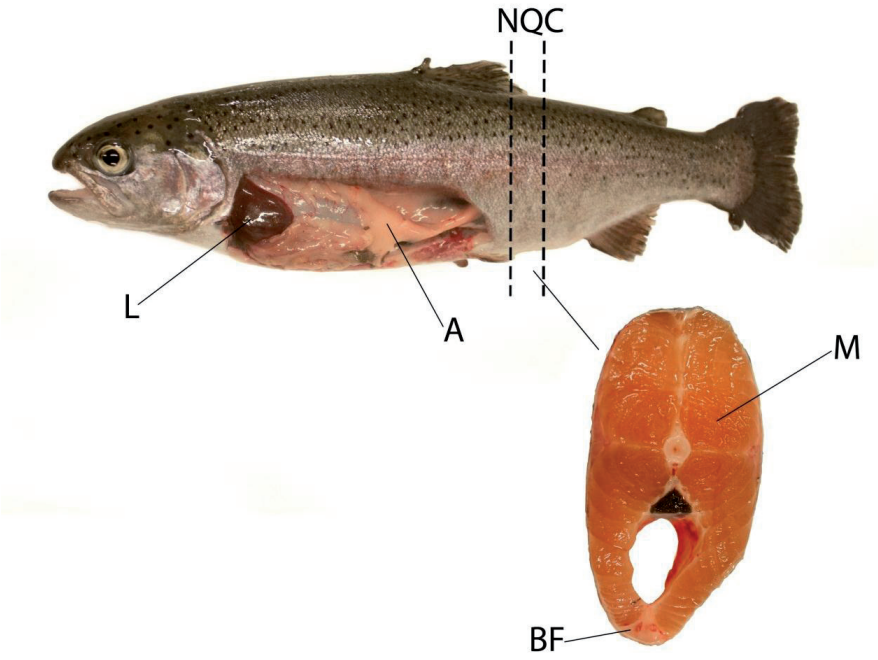

Figure 1. Photograph of rainbow trout showing mesenteric adipose tissue (A), liver (L) as well as the position of muscle (M) and belly flap (BF) samples from the Norwegian quality cut (NQC) used in the thesis. Photo: Gabriella Johansson.

Lipoprotein lipase (LPL) is responsible for the lipolysis of lipoprotein TG preceding FA and glycerol uptake and reassembly of TG inside the cells. The active form of LPL is as dimers present on the outside of cells, in contact with capillary blood. LPL, with similar function as in mammals, is present also in fish, *e.g.* rainbow trout (Skinner and Youssef 1982). The mechanisms of LPL activity regulation is largely unknown, but may encompass many steps, from translation to the binding to the cell surface. It has been suggested that hormones may affect LPL by stabilizing LPL mRNA (Raynolds et al. 1990). Both activity and gene expression of LPL is regulated by insulin in gilthead sea bream adipose tissue (Albalat et al. 2007). In red sea bream, LPL gene expression differentially regulated by unsaturated FA through increased mRNA levels in liver and decreased in mesenteric adipose tissue (Liang et al. 2002). Adipose tissue LPL activity is reduced during fasting and is elevated when feeding resumes (Albalat et al. 2007). Body shape and LPL expression appear to correlate, with lean gilthead sea bream having lower LPL mRNA levels than fish with higher CF (Cruz-Garcia et al. 2009).

In mammals, a large proportion of FAs from LPL lipolysis is not taken up by the nearby cells, but "leaks" to the venous blood and may be transported to other tissues (Frayn et al. 1995), but whether similar mechanisms exist in fish is unknown.

FAs may serve as regulators or signals of metabolic status. FAs exert negative feedback on LPL activity in rat adipocytes (Amri et al. 1996). In rainbow trout, unsaturated FA reduce lipogenesis (Alvarez et al. 2000) and decrease expression of the transcription factor liver X receptor (LXR) thereby also reducing the expression of LPL (Cruz-Garcia et al. 2011).

The liver is the "hub" of lipid processing, responsible for coordinating lipid distribution by organizing lipoprotein assembly and degradation. In many fish species, the liver, apart from supplying the blood with VLDL, is also an important lipid storage site. This is probably especially important for "lean" fish species such as *e.g.* Atlantic cod, which stores little lipid in muscle. In the liver, TG and cholesterol are loaded into VLDL and exported to tissues for storage or oxidation (Babin and Vernier 1989). FAs involved in hepatic VLDL production may originate from different sources, *e.g.* intracellular TG stores, plasma FFA, *de novo* lipogenesis and incoming lipoproteins. In mammals, TGs which are incorporated into VLDL are recruited from cytosolic TG stores rather than direct use of plasma FFA (Gibbons et al. 1992). This implies that FFA need to be taken up by the hepatocytes and incorporated into TG in order to become available for VLDL synthesis. An alternative enzyme system has been proposed in mammals in which the lipolytic enzymes arylacetamide deacetylase (AADA) and/or triacylglycerol hydrolase (TGH) are responsible for TG lipolysis targeted for VLDL assembly (Gibbons et al. 2000; Gilham et al. 2003). However, the existence or relevance of these enzymes has not been investigated in fish.

The intracellular TG stores consist of lipid droplets surrounded by special proteins called perilipins. Lipolysis is activated by phosphorylation of perilipin and HSL by protein kinase A (pKA). Phosphorylation of perilipins exposes the lipid droplet TG for HSL (Kraemer and Shen 2006) and activation of HSL, through cAMP-mediated phosphorylation, increases lipolysis in adipose tissue (Michelsen et al. 1994). Further, glucagon stimulates whereas insulin decreases lipolysis by phosphorylation of HSL in rainbow trout liver (Harmon et al. 1993). It has been suggested that the majority of FA from HSL lipolysis is not directed towards synthesis or export, but rather used for oxidation within the cell for energy production (Pease et al. 1999). In mammals, several other lipases have been identified, *e.g.* the adipose specific enzyme adipose triglyceride lipase (ATGL) which has a similar role as HSL (Zimmermann et al. 2004).

The liver has a high capacity for *de novo* FA synthesis and this is also the tissue where the majority of the lipogenesis occurs (Lin et al. 1977). Fatty acid synthase (FAS) synthesizes FA *de novo* from malonil-CoA which originates from glucose or amino acid metabolism. Liver FAS activity decreases with increasing fat intake (Dias et al. 1999).

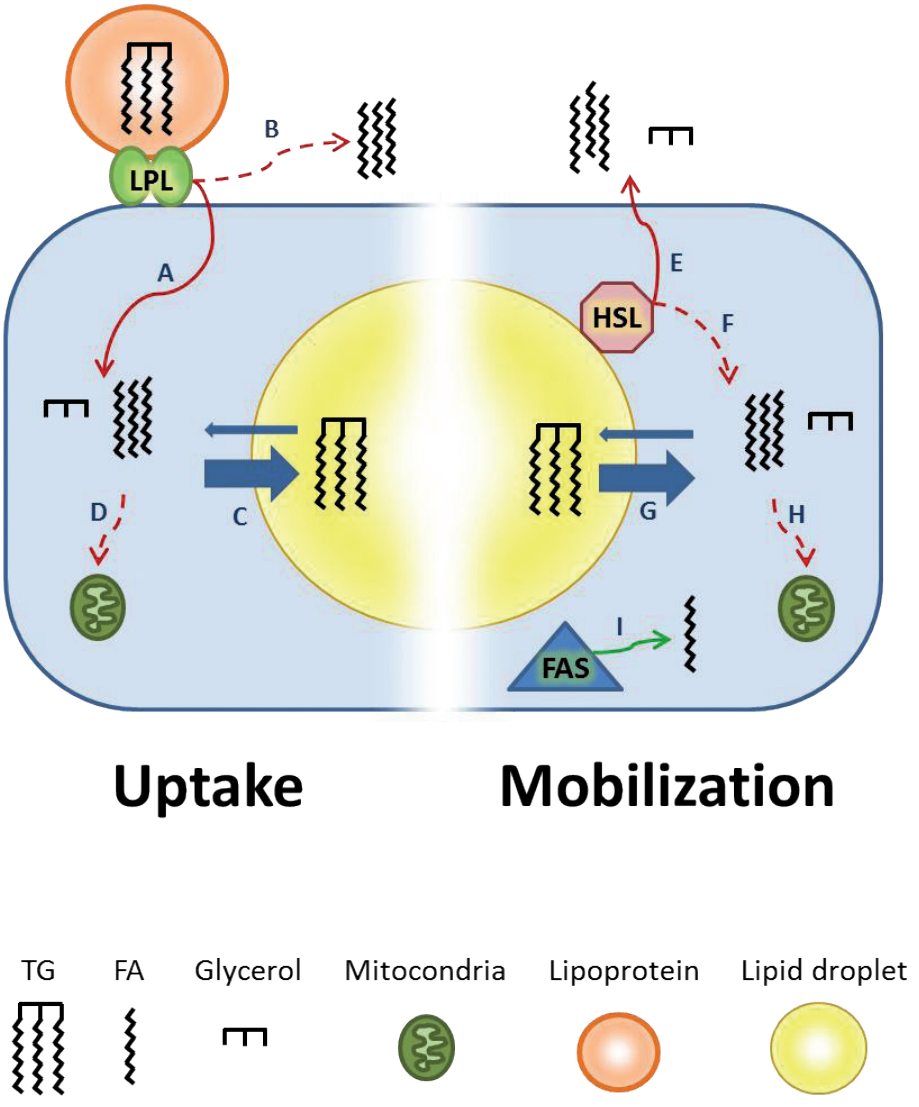

Figure 2. Schematic representation of lipid metabolism in a vertebrate cell. The uptake of lipids from lipoproteins into cells is facilitated by lipoprotein lipase (LPL). Triglycerides (TGs) in the lipoproteins are broken down into fatty acids (FAs) and glycerol. The FAs may then be taken up into the cell (**A**) or transported in the blood (**B**). Inside the cell, the TGs are reassembled from FA and glycerol (**C**) and stored inside lipid droplets. FAs may also be used as substrate for β-oxidation for energy production (**D, H**). Mobilization of stored TGs is mainly performed by hormone-sensitive lipase (HSL) and the resulting FAs are released to the blood (**E**) or used within the cell (**F**). The exchange between TGs in the lipid droplet and FAs in the cytoplasm is a dynamic process (**G**). FAs can be synthesized *de novo* (**I**) in the cell by fatty acid synthase (FAS).

Protein metabolism

In times of negative energy balance, protein is preferably protected at the expense of lipid stores. During prolonged periods of low food intake or fasting, protein catabolism is eventually initiated. Muscle protein is the biggest source for amino acids (AA) which may be used as substrate for synthesis of FA and glucose, thereby providing a link between protein, lipid and carbohydrate metabolism. Released AAs may also be used as an energy source by oxidation.

Carbohydrate metabolism

As the prey items of wild salmonids are mostly invertebrates and fish, the natural diet consists primarily of protein and lipids. Carbohydrate metabolism is thus considered being of less importance (Tocher 2003). Glycogen stores, however, seem to be of significant importance during early response to food deprivation (Sheridan and Mommsen 1991). Glycogen is a polymer of glucose which is better suited for storage than glucose, while glucose is readily incorporated or released from glycogen stores. These are very dynamic processes and glycogen stores are easily accessed during energy needs and rapidly replenished when energy supply is ample. Glucose may also be synthesized from AAs and lactate through the process of gluconeogenesis. Glycolysis is the oxidation of glucose which provides a rapid source of energy.

Endocrine regulation of metabolism

Growth hormone and insulin-like growth factor I

Growth hormone (GH) is a pluripotent hormone which can stimulate both anabolic and catabolic processes. In fish, GH improves growth by elevated appetite and feed conversion (Markert et al. 1977; Johnsson and Björnsson 1994). Some of the growth-promoting effect may arise from increased protein synthesis rates (Foster et al. 1991; Fauconneau et al. 1996). GH stimulates the liver to produce and release insulin-like growth factor I (IGF-I) to the blood. Apart from its effect on endocrine IGF-I release, GH may also stimulate local IGF-I production and paracrine IGF-I signaling in liver, bone and muscle tissue. The growth-promoting effects of GH are thus partly mediated by IGF-I. There is evidence, however, for direct effects of GH on metabolism (Björnsson et al. 2002).

The first evidence of involvement of GH in lipid metabolism was an observed shift in body composition from fat to muscle mass in rats (Lee and Schaffer 1934). GH can be both lipogenic and lipolytic by affecting a pleura of enzymes. The metabolic regulation by GH is very complex and the effect is often tissue- and context dependent (Norrelund 2005).

In salmonids, endogenous levels of GH increase during energy-demanding events, such as during fasting and smoltification, enabling the fish to mobilize fat stores (Sheridan 1986a; Farbridge and Leatherland 1992). GH stimulates hepatic and adipose lipolysis (Albalat et al. 2005a; O'Connor et al. 1993) by elevating the activity of TG lipases (Sheridan 1986a)

causing reduced liver and mesenteric fat stores (Johnsson et al. 2000; Kling et al 2011) and increased circulating FA (Leatherland and Nuti 1981). Uptake of lipids from VLDL in muscles is likely enhanced by GH as shown by increased LPL gene expression in trout myocytes *in vitro* (Cruz-Garcia et al. 2011). Lipid metabolism was not affected by GH treatment in coho salmon smolts (Sheridan et al. 1986) most likely due to already elevated endogenous circulating GH levels.

GH exerts its effect by binding to the extracellular domain of a dimerizing GH receptor (GHR). The GHR is associated to the tyrosine protein kinase Janus kinase (JaK2). Upon binding of GH to its receptor, a phosphorylation cascade is initiated, which activates different signaling pathways. GH also activates several transcription factors, e.g. STATs (signal transducers and activators of transcription). Not only enzymatic activities and transcription are regulated by GH, but also mRNA stability (Yin et al. 1998).

The responsiveness to GH can be modulated by differential tissue-specific expression of GHRs. In rainbow trout and Atlantic salmon, two types of GHR exist, GHR1 and GHR2 (Very et al. 2005; Benedet et al. 2005), which are expressed differently during fasting and re-feeding. Fasting-induced lipolysis of adipose lipids is mediated through increased abundance of GHRs (Norbeck et al. 2007). In fasted fish, however, hepatic sensitivity to GH decreases, resulting in lower IGF-I plasma levels and growth (Norbeck et al. 2007). By adjusting the abundance of the two GHRs, the growth-promoting and lipolytic actions of GH can be regulated independently in a tissue-specific manner. In fish, the two GHRs are also differentially regulated by temperature in combination with food availability (Gabillard et al. 2006).

IGF-I is affected by environmental cues, and increased daylight and/or temperature stimulate IGF-I levels (Reinecke 2010). IGF-I also has a function in protecting muscles from degradation during fasting and stimulating compensatory growth during re-feeding (Montserrat et al. 2007).

Ghrelin

Ghrelin was originally discovered as the first circulating hormone that stimulates growth hormone (GH) secretion. It is now considered an important regulator of metabolism and energy balance. The effects of ghrelin are somewhat contradictory, but they include regulation of food intake and adiposity in vertebrates, and most commonly, a stimulatory action has been shown (Kaiya et al. 2008). Ghrelin was identified in fish less than ten years ago (Kaiya et al. 2003). Ghrelin has been shown to stimulate food intake, body weight gain, and fat storage in liver and muscle in tilapia (Riley et al. 2005), increase lipid accumulation in liver in goldfish (Kang et al. 2011), as well as decrease food intake without affecting tissue lipid content in rainbow trout (Jönsson et al. 2010). Ghrelin levels are influenced by nutritional status in several fish species although the results are inconsistent, *e.g* in rainbow trout plasma ghrelin levels were depressed during fasting (Jönsson et al. 2007) while in hybrid striped bass they were increased (Picha et al. 2009). In Arctic charr, a salmonid species that exhibits large variations in appetite and growth between seasons, ghrelin mRNA expression in stomach also varied between seasons (Frøiland et al. 2010). These data support a role of ghrelin in energy balance regulation in

fish. Some effects of ghrelin might arise from the stimulatory effect on GH release, but in mammals, a direct influence on lipid metabolism of adipocytes is also suggested (Miegueu et al. 2011; Rodriguez et al. 2009). However, it is still unknown how ghrelin responds to temperature and what its potential direct action on adiposity or lipid metabolism in fish is.

Leptin

The peptide hormone leptin was discovered in the so-called *ob/ob* mice, which are leptin mutant. These mice become obese and exhibit a very high food intake (Zhang et al. 1994). Leptin has subsequently been shown to have many different physiological functions, but one of its most central and most well studied actions is its inhibiting action on food intake. Leptin was cloned in fish in 2005 (Kurokawa et al. 2005), and recent studies indicate it to also be anorexigenic in fish (Murashita et al. 2008; Li et al. 2010). However, some major differences in leptin endocrinology appear to exist between mammals and fish. Thus, while most leptin is produced in adipose tissue of mammals, in fish, liver appears to be a major site of leptin production as judged by leptin mRNA expression (Kurokawa et al. 2005; Kurokawa and Murashita 2009; Murashita et al. 2008; Gorissen et al. 2009; Huising et al. 2006; Trombley et al. 2011; Kling et al. 2011). Also, while leptin levels decrease rapidly during fasting in mammals, recent data on fish indicate that leptin levels may increase during periods of restricted feeding or fasting (Kling et al 2009, Johnsen et al 2011, Trombley et al 2011). Seasonal variations in leptin expression suggest that also environmental cues may affect the leptin system (Frøiland et al. 2010). Thus, still a lot of questions remain about the biological function of leptin in fish and if this hormone plays a role in lipid metabolism and energy balance.

AIM OF THESIS

The main objective of this thesis was to elucidate how the physiology and lipid metabolism of salmonid fish is affected by temperature and food availability, key environmental factors which change seasonally or are affected by human activities (*e.g.* climate change), and further, to clarify aspects of the endocrine control of lipid metabolism. I have used an integrated approach with focus on the whole animal response, including the interplay between different tissues.

More specific aims were:

To investigate the applicability of nuclear magnetic resonance (NMR) based metabolomics in integrative fish physiology by investigating the effects of fasting (Paper I) and temperature (Paper II) on the metabolome and physiology. This aim included the optimization of techniques for metabolic profiling in fish.

To elucidate the endocrine regulation of lipid metabolic processes and its potential interaction with temperature in salmonids (Papers II, III & IV), with focus on key metabolic and growth regulators; growth hormone (GH), insulin-like growth factor I, leptin and ghrelin.

To better understand the role of GH in lipid metabolism by clarifying the effect of GH on lipid metabolic gene expression *in vivo*.

To clarify if fatty acids (FAs) and ghrelin may have a direct role in lipid uptake or lipid mobilizing processes in adipose and liver.

METHODS

NMR-based metabolomics

Metabolomics is the study of endogenous low molecular weight (< 1 kDa) compounds, *i.e.* metabolites. All metabolites present at a given moment in an animal, tissue or biofluid are collectively referred to as the metabolome. Several analytical methods, most commonly NMR or different mass spectrometry (MS) techniques (*e.g.* GC-MS, LC-MS) are used in metabolomics studies due to the varying chemical properties of different metabolites.

A change in some environmental factor or other outer/inner stimuli (*e.g.* disease, genetic modification) will elicit a response initiating certain processes. This physiological switch will be reflected in a characteristic change in the metabolome that can be detected using metabolomics. As a close relationship exists between the metabolome and the physiological state of the animal, metabolomics is suitable for studies on whole-animal physiology as well as on specific tissues. Metabolic profiling is an approach in which the response pattern of observable metabolite levels is investigated.

The use of metabolomics in fish research is a new approach and relatively few studies exist, focusing on nutrition, handling stress, toxicology, pathology, development and FA composition in tissues (Samuelsson and Larsson 2008). It is possible to identify metabolic "profiles" or "fingerprints" from tissue extracts, intact tissue samples and biofluids (*e.g.* plasma, urine).

NMR-based metabolomics simultaneously distinguishes and quantifies up to 100 different metabolites. Almost all biologically active molecules contain hydrogen and are thus possible to detect by ^{1}H NMR. ^{1}H NMR analysis is based on the properties of hydrogen nuclei (protons) when placed in a magnetic field. Protons can occupy two different spin states with different energies and in NMR spectroscopy a radio frequency pulse is transmitted that will excite the lower energy spin state. The relaxation back to equilibrium gives rise to resonances that are detected and Fourier transformed (from time to frequency domain) into a proton NMR spectrum. The chemical shift, *i.e.* the position of a peak along the x-axis of the NMR spectrum is reported in ppm (parts per million) relative to that of an internal standard, of a peak is determined by the electron shielding properties of the neighboring atoms in the molecule. The peak area (signal intensity) is proportional to the concentration of the molecule (although number of protons contributing to the signal must be taken into consideration). An example of the workflow in NMR-based metabolomics is illustrated in Figure 3.

To discriminate peaks from different metabolites and to assign metabolite identities to the corresponding peaks are some of the challenges in NMR metabolic profiling. The number of detectable molecules is limited by the concentration and overlapping signals from other molecules. This may be partly circumvented by partitioning the samples by extraction or

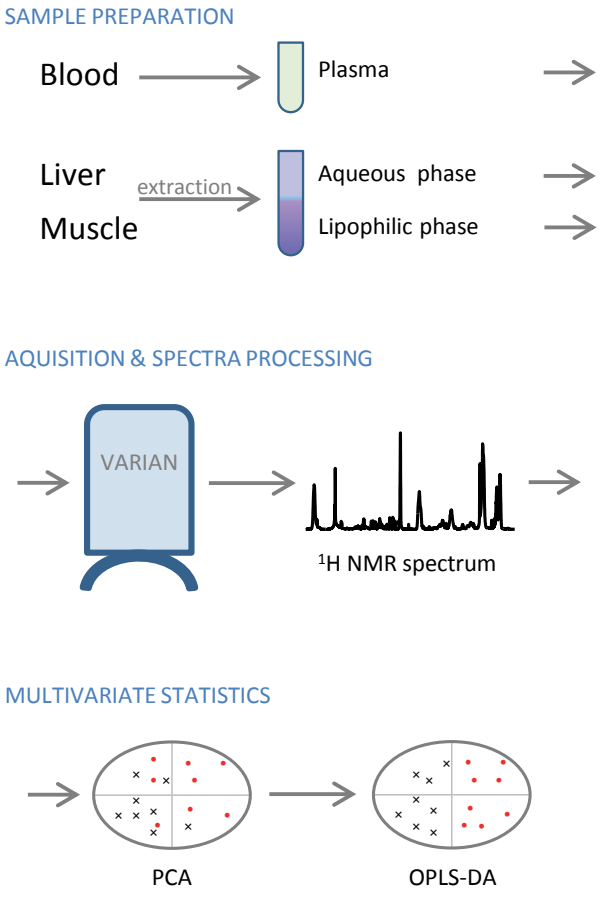

Figure 3. A schematic flowchart illustrating the different steps of nuclear magnetic resonance (NMR) based metabolomics. PCA: Principal component analysis. OPLS-DA: Orthogonal partial least squares discriminant analysis.

by using NMR spectroscopy techniques that attenuate specific signals. One such technique is presaturation, where the otherwise dominating resonance from water (or other solvents) is minimized by signal suppression (presat spectra, Figure 4 A). The Carr-Purcell-Meiboom-Gill (CPMG) sequence (Meiboom and Gill 1958) attenuates resonances from large molecules, *e.g.* proteins and lipids, and thus reveals signals from smaller molecules (CPMG spectra, Figure 4 B). The complexity of samples can be decreased by separating lipophilic and hydrophilic compounds. Examples of ^{1}H NMR spectra of different tissue extracts are presented in Figure 5. Both spectral resolution and sensitivity

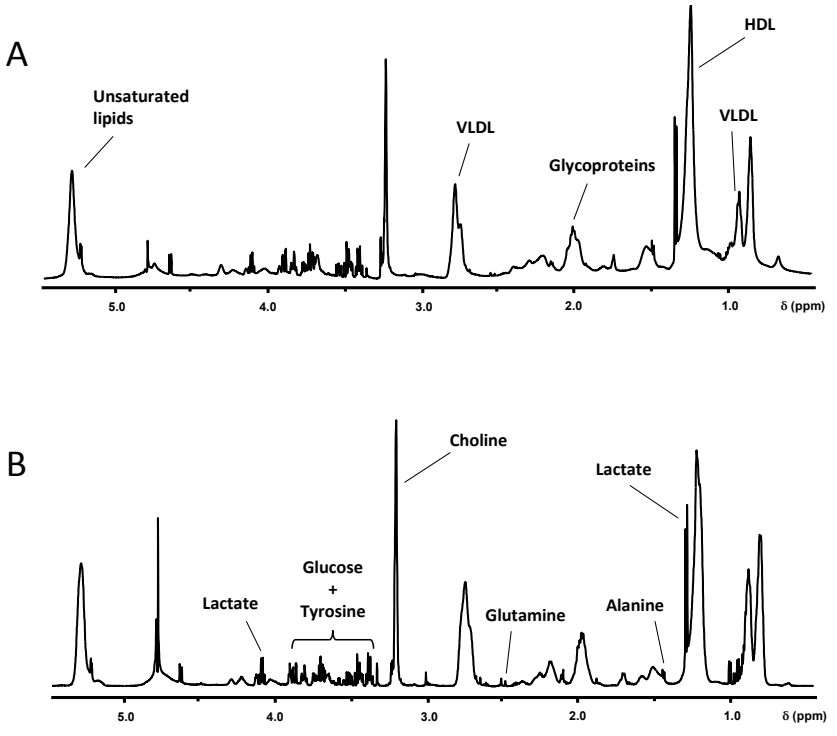

Figure 4. Examples of 600 MHz 1H NMR spectra of rainbow trout plasma; presat (**A**) and CPMG [Carr-Purcell-Meiboom-Gill] (**B**) spectrum. The CPMG sequence attenuates signals from large molecules, *e.g.* proteins and lipids, thus intensifying signals from smaller molecules.

are determined by the magnetic field strength. The sensitivity can be further enhanced by using a cold probe which increases the signal-to-noise ratio allowing detection of metabolites at lower concentrations.

An important part of metabolomics is the statistical analysis as large amounts of data are generated. In the case of NMR-based metabolomics each spectrum is usually divided into hundreds of small parts referred to as bins or buckets. The signal(s) in each bucket is integrated and then treated as a variable in the statistical analysis. Due to the large number of dependent variables, sometimes exceeding 1000, a multivariate approach is common. Initially a so-called unsupervised method can be employed to gain an overview of the data. Principal component analysis (PCA) is a commonly used unsupervised methodWold et al. 1984). PCA is a multivariate projection approach in which the data are condensed into fewer dimensions, providing improved visualization of the data. The relationship and variation among individuals is visualized in a scores plot (Figure 5 A). Similarly, the variables are visualized in a loadings plot. PCA is used to look for outliers and separation

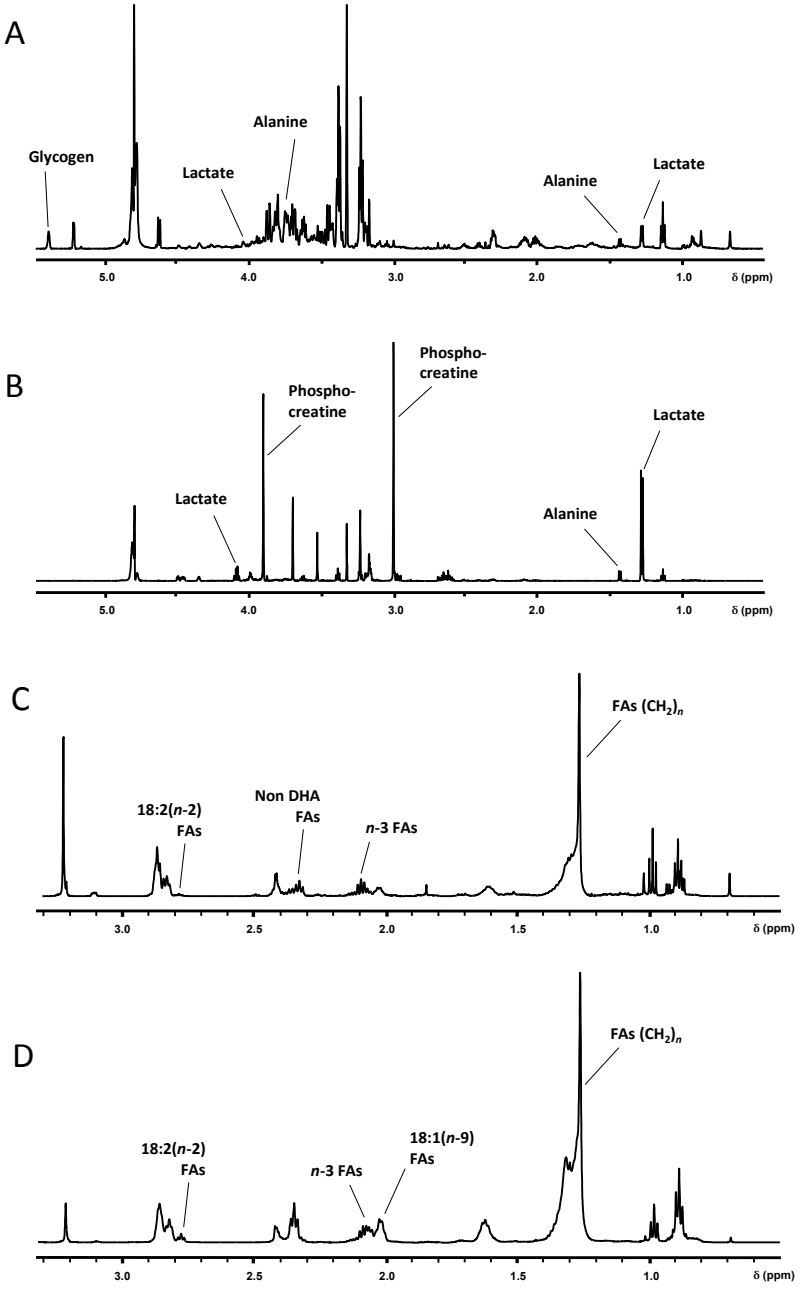

Figure 5. Examples of 600 MHz 1H NMR spectra of rainbow trout tissue extracts; liver (**A**) and muscle (**B**) aqueous extract as well as liver (**C**) and muscle (**D**) lipid extracts. FAs: fatty acids.

trends among different treatment classes and to decide whether to proceed with supervised methods. When information on class membership is included in the model it is a supervised analysis. Partial least squares-discriminant analysis (PLS-DA) is such a supervised method in which the difference between two classes is maximized in the first component and all other variation follows in succeeding components (Figure 5 B). A further development of PLS-DA is the orthogonal PLS-DA, OPLS-DA (Trygg and Wold 2002), where all confounding variation (*i.e.* variation not related to class membership) is removed from the first component (Figure 5 C). From such supervised models the spectral buckets that differ in intensity between treatment groups can be determined. Then identities of the metabolites corresponding to those buckets then need to be identified

Assigning the correct metabolite identities to the different peaks is normally accomplished by running and analyzing 2D NMR spectra, comparison with published data and tables as well as spectral databases, and by spiking samples with authentic metabolites. In 2D NMR additional structural information can be obtained. Overlapping peaks in the 1D spectrum become less of a problem by spreading them out in a second dimension. Connectivity between different nuclei is observed: *e.g.* in ^{1}H-^{1}H COSY spectra, information about which protons are situated next to each other in the molecule is obtained, and ^{13}C-^{1}H HSQC spectra show which protons are attached to which carbons. Together, these 2D NMR methods are used to obtain the identity of the metabolome.

Lipid quantification

By quantifying lipid content in different tissues it is possible to indirectly assess changes in lipid dynamics as changes in amounts of stored lipids. Isopropanol-hexane-water (IHW) extraction yields more TG than the traditional chloroform-methanol extraction (Schwartz and Wolins 2007). Further, this method is more accurate and allows faster extraction procedure and high throughput analysis/quantification of tissue TG (Schwartz and Wolins 2007). The lipid extracts can easily be resuspended in a reaction solution and the TG content quantified by colorimetric analysis of glycerol before and after lipase treatment. Gravimetrical quantification of extracted lipids has been extensively used. However, this approach is not as sensitive as colorimetric quantification and it requires more tissue and a relatively large sample size. It is also possible to use NMR for quantification of tissue lipid stores by lipid profiling focusing on resonances from specific lipid classes. Further, when spectroscopy is not automated, NMR is more time consuming than colorimetric analysis in a 96-well format. However, NMR is more sensitive and can provide detailed information, e.g. different classes of lipids or specific FAs. This

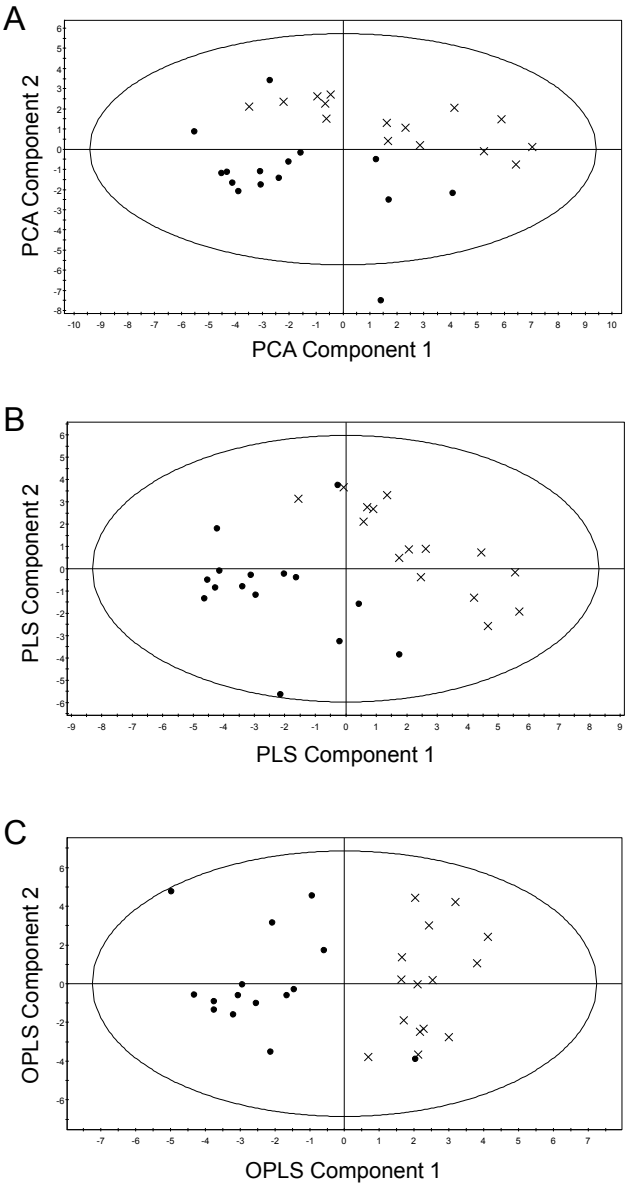

Figure 6. Scores plots from PCA [principal component analysis] (**A**), PLS-DA [partial least squares discriminant analysis] (**B**) and OPLS-DA [orthogonal partial least squares discriminant analysis] (**C**). The different scores plots show the effect of moving from an unsupervised method (PCA) to a supervised (PLS-DA) as well as the improved focusing of treatment-related variation in the 1st component of OPLS-DA.

difference becomes apparent in Paper I, where subtle changes in liver lipids could be detected by NMR, but not with the gravimetric method.

Radioimmunoassays

Radioimmunoassay (RIA) is a common method to measure the concentration of a hormone in plasma or tissue. It is based on the principle that native hormone in the sample competes with added radioactively labeled hormone for the limited binding sites available on the antibody molecule made specifically for the hormone of interest. At the end of the reaction, hormone bound to the antibody is separated from unbound hormone and measured for the radioactivity. The binding of labeled hormone to the antibodies can be estimated and is a function of the total unknown hormone concentration in the plasma**.** Other immunoassays have be developed which avoid the use of radioisotopes as label, but the RIA method is highly sensitive and can detect extremely low concentrations of a substance, which is of particular importance when assessing peptide hormone levels. The RIA method has had an enormous impact on the understanding the basic function of hormonal systems in animals, as well as their involvement in metabolic disorders, nutrient imbalances and suppressed health (Chard 1995). RIAs for GH, IGF-I, leptin and ghrelin validated and established for salmonids (Kling et al. 2009; Jönsson et al. 2007; Bjornsson et al. 1994; Shimizu et al. 2006) were used in Paper II.

Quantitative PCR

In order to quantify transcription of genes, quantitative polymerase chain reaction (qPCR) is commonly used. This method provides relative expression of genes by quantifying messenger RNA (mRNA) levels in an animal, tissue or cell culture. The target mRNA levels are presented as values relative to a reference gene. First, total RNA is extracted and made into double stranded complementary DNA (cDNA). The cDNA of the target gene is then amplified in a PCR reaction using primers targeted towards specific nucleotide sequences in the mRNA of the investigated gene. In Paper III, gene transcription in liver, muscle, belly flap and adipose tissue is assessed using qPCR.

LPL activity

The assay used is based on fluorometric quantification of the product of LPL-mediated lipolysis of an added substrate which is directly proportional to the LPL activity. In Paper IV, the LPL assay kit from Roar Biochemical (New York, NY USA) was used to measure LPL activity in rainbow trout adipocytes and hepatocytes. This assay kit had not been validated for use in fish previously. Only the LPL, which is functional and present at the cell surface can be released by adding heparin.

In Paper IV, the heparin-releasable fraction was analyzed as it represents the physiologically active LPL. Isolated cells or tissue homogenates are placed in a buffer containing heparin and incubated for 40 min before the substrate is added.

Isolated cells

In vitro studies may provide important mechanistic details of physiological responses to different "agents" or treatments in different cell types/tissues. When an *in vivo* approach is used it is often difficult to distinguish direct and indirect systemic induced effects of the treatment. There are numerous approaches to studying effects on a cellular level. A tissue may be divided into small pieces (*e.g.* liver slices or fat pads) which are then incubated in a buffer or medium for a short period of time (hours). A further step is to treat tissue pieces with collagenase, followed by cell separation to obtain a single-cell suspension which may also be used for acute studies. Under the right conditions, such primary cell cultures can be studied for a longer time (days).

In Paper IV, cells from the liver (hepatocytes) and mesenteric fat (adipocytes) were isolated and acute effects were studied by incubation for 6 hours.

MAIN FINDINGS AND DISCUSSION

This section provides an overview of the main findings and discussions of the different papers (Papers I to IV) included in the thesis.

The influence of fasting on metabolic profiles in plasma, muscle and liver (Paper I)

Juvenile rainbow trout were deprived of food for 4 weeks to investigate effects on metabolism and energy reserves. This fasting resulted in decreased body weight and mesenteric fat weight, but there was still a slight increase in length. The fasting responses elucidated by NMR-based metabolomics in Paper I mostly encompassed alterations in lipid metabolism. The observed responses include elevated plasma VLDL levels, concurrent with decreased levels of HDL as well as mobilization of liver and muscle FA stores. However, the 4-week fasted fish still had substantial mesenteric fat reserves, and the proportion of mesenteric fat per body weight had actually increased. These results are in line with other studies showing that salmonids may tolerate several months with little or no food (Navarro et al. 1992). However, in contrast to the results of Paper I, other studies on fasting in fish have shown an almost complete depletion of mesenteric fat stores before lipid mobilization in liver and muscle commences (Navarro and Gutiérrez 1995). Differences among species or life stages of the fish used may possibly explain such discrepancies, as may differences in temperatures. However, it is also likely that the initially high CF of the rainbow trout fasted in Paper I suggests that they initially had relatively large fat stores which might explain why the mesenteric fat was not depleted by a 4-week fast. Adult salmonids such as rainbow trout can store large amounts of fat in their muscle (up to about 20% is not uncommon), and as muscle is the largest tissue in fish, muscle fat can be a substantial source of energy during fasting, whereas the relatively small liver may be expected to contribute less. However, although total lipid content in

the liver was unaltered in the fasted fish, the NMR analyses revealed that the total FA concentrations had decreased, indicating a shift of lipid metabolism in the liver. Fasting specifically decreased levels of 18:1 (*n*-9) FA in muscle, suggesting an induced use of FA oxidation for energy in muscles during fasting, as 18:1 (*n*-9) is one of the main energy sources in teleosts (Tocher 2003). This agrees with a previous study on fasted rainbow trout, where both 18:1 (*n*-9) and 20:1 (*n*-9) were mobilized from liver and muscle (Jezierska et al. 1982). In paper I, fasting also induced an increase in another FA; the 18:2 (*n*-2) FA in muscle and liver. Together, these results demonstrate tissue-specific patterns of lipid mobilization which were elucidated by NMR-based metabolomics.

Continued length growth even during catabolic conditions by redistributing resources from lipid accumulation and muscle synthesis is an important strategy for fish subjected to large natural variations in food availability (Metcalfe et al. 2002; Nicieza and Metcalfe 1997; Hurst and Conover 2003). Mass growth is easier to regain by increasing muscle and fat stores through compensatory growth mechanisms (Nicieza and Metcalfe 1997) than skeletal growth. The coordinated response of energy reallocation might be mediated by GH-signaling, as GH alters the distribution of lipid accumulation (Kling et al. 2011) and stimulates length growth even during restricted food intake in rainbow trout (Johnsson and Björnsson 1994).

Catabolic processes in muscle, such as during sockeye salmon spawning migration (Mommsen et al. 1980) and fasting (Paper I), can be reflected in elevated alanine levels in muscle. Alanine can be used for gluconeogenesis (Canals et al. 1992; Pereira et al. 1995; Sheridan and Mommsen 1991; French et al. 1983) or energy production (Pereira et al. 1995). This may explain the decreased alanine and elevated liver glycogen in fasted fish suggesting that gluconeogenesis and/or other processes are initiated to replenish glycogen stores which usually are depleted quite rapidly during fasting. Lactate is a preferred substrate for gluconeogenesis and may contribute to maintaining or even increasing liver glycogen stores during fasting in sockeye salmon (French et al. 1983). Plasma levels of lactate decreased in the fasted fish (Paper I) suggesting that is was used up to a larger extent by the food-deprived fish. This indicates that it may be important for gluconeogenesis during fasting also in rainbow trout (Paper I). Glycogen stores are rapidly depleted during the more acute phases of fasting (days) in fish (Navarro et al. 1992; Sheridan and Mommsen 1991), but the stores seem to be very dynamic and may be replenished during prolonged fasting conditions (French et al. 1983), which is further supported by Paper I. The glycogen conserving effect may be regulated by GH (Aas-Hansen et al. 2005). It can be speculated that mechanisms that act to preserve glycogen stores in the liver may be important for the fish to cope with low food availability.

There was a pronounced increase in plasma VLDL, the lipid transport proteins that is responsible for distributing TG from the liver to other tissues, during fasting. The increased plasma VLDL may arise from decreased uptake by LPL in lipid storing tissues as a part of a mobilizing state (Black and Skinner 1986). Elevated plasma or extracellular levels of FAs may signify a state of lipid mobilization and a signal to decrease LPL activity and perhaps increase lipolysis in adipose or other lipid storing tissues. This hypothesis is supported by the responses indicated in Paper IV where adipocyte LPL activity was inhibited directly by FAs.

Elevated temperature has adverse effects on the metabolism (Papers II & IV)

When subjected to temperatures slightly above the optimal for three months, Atlantic salmon post-smolts have reduced food intake and FCE, leading to impaired growth (Paper II). This is consistent with previous results showing that the optimal temperature for FCE is between 11 and 13.4°C for Atlantic salmon, depending on fish size (Handeland et al. 2008). However, the results of Paper II show that protein digestibility was improved in the highest temperature, and thus nutrient uptake appears unlikely to be the sole explanation for the lower FCE and growth rate. Instead, decreased plasma levels of tyrosine, glutamine and phenylalanine suggest increased protein turn-over, which might contribute to less efficient conversion of food into growth. Tyrosine may protect against skeletal deformations and effects of temperature stress in white sea bream (*Diplodus sargus*) larvae (Saavedra et al. 2010) suggesting an important function for tyrosine in fish growth. The lipid metabolism of fish at 18°C reflects an energy mobilizing state, with increased levels of plasma VLDL and lipids containing unsaturated fatty acids, as well as decreased HDL and choline. Similar effects on plasma metabolite levels were observed in food deprived rainbow trout (Paper I) indicating that catabolic and/or lipid mobilizing processes are present. It may be hypothesized that this change in metabolic profile is a general feature during a negative energy balance in salmonids. Choline is important in lipid metabolism, both as a component in membranes (Hazel 1984), for energy production and growth (Rumsey 1991). Lower plasma levels of metabolites related to energy metabolism, *e.g.* creatine and glucose, further suggest that the elevated temperature induce a shift towards mobilizing or catabolic processes (Paper II).

The reduced food intake was accompanied by elevated plasma leptin levels indicating a connection between leptin, food intake and temperature that has not been shown previously. Plasma leptin levels were higher in fish acclimated to the highest temperature after 3 months, with a tendency towards a similar response already after 1 month (Paper II). This effect was concurrent with a decreased food intake and FCE, as well as changes in several metabolite levels. Although speculative, it may be possible that leptin induces reduced food intake during elevated temperatures. Anorexigenic effects of leptin have been demonstrated in salmonids (Murashita et al. 2011; Murashita et al. 2008). As fasting or restricted feeding has been shown to elevate plasma leptin levels (Kling et al. 2009; Johnsen et al. 2011; Trombley et al. 2011) it is hard to say if leptin has a negative effect on food intake or if the elevated plasma leptin in Paper II is a consequence of decreased food intake.

The fish in Paper II were kept at different temperatures in circular tanks and were sampled after one and three months (Figure 7). After one month in the highest temperature fish showed no adverse effects on growth. The responses after one and three months, temperature acclimation clearly differ and long-term effects may provide more ecological relevant conclusions.

Temperature acclimation of the fish affected the lipid uptake in adipocytes and the lipolysis in hepatocytes (Paper IV). Fish had been kept in either 10°C or 15°C for 11 to 13 days before isolation of hepatocytes and adipocytes. Freshly isolated cells directly incubated for a few hours are considered to reflect the metabolic status of the cell in the live fish at that moment (Albalat et al. 2005b) and can hence be used to study the acute response and sensitivity of the cells to a treatment after the fish has been subjected to an environmental stimuli. The stimulatory effect of FAs on LPL activity was only apparent at 10°C in adipocytes, whereas the inhibitory effect of FAs on glycerol release (lipid breakdown) was observed at 15°C in hepatocytes (Paper IV). This indicates that there may be a complex metabolic-nutrient-temperature interaction in liver and adipose tissue of fish. The mechanisms for the temperature effects are unknown, but they are probably caused by the increased metabolism and energy demand during acclimation to the higher temperature that has been shown *in vivo* and discussed above (Paper II).

Elucidating the endocrine regulation of lipid metabolism (Papers II, III & IV)

An increase in the environmental temperature from 12°C to 18°C, which is above the growth optimum as discussed above, was also reflected in changes in GH and leptin endocrine signaling (Paper II). The metabolic changes are described above and involved stimulated lipid mobilization and increased protein turn-over at the highest temperature. The most evident change was a shift towards increased lipid mobilization at 18°C. Given that GH is generally thought to be lipolytic, and to stimulate lipid mobilization during fasting, this result is possibly linked to GH, which tended to increase with temperature. GH and IGF-I are influenced by seasonal variations in environmental temperatures and photoperiod (Gabillard et al. 2005; Picha et al. 2008). Temperature alters the distribution pattern of lipid accumulation among tissues (Paper II) and similar reallocation of fat resources is demonstrated in GH-treated rainbow trout (Kling et al. 2011). Despite its close relation with GH, which acts as an IGF-I secretagogue, IGF-I plasma levels did not change with temperature. Hepatic desensitization may prevent GH from eliciting an elevation of circulating IGF-I, *e.g.* during fasting (Pierce et al. 2005). IGF-I can also be regulated by nutritional status, independent of GH (Picha et al. 2008; Beckman 2011). Nutritional status may also modulate the responsiveness to hormones (Aas-Hansen et al. 2005). Negative feedback mechanisms are often present, acting at the levels of plasma hormones keeping them stable.

Ghrelin is involved in lipid metabolism, favoring energy storage, and changes food intake, in fish (Picha et al. 2009; Riley et al. 2005; Kang et al. 2011). Although there were clearly both metabolic and food intake differences between the temperature groups in Paper II, this was not reflected in plasma ghrelin levels. Also here, there may be mechanisms acting that keep plasma ghrelin levels stable. However, more studies on the biology of ghrelin in fish, in particular its metabolic actions, are needed to elucidate its relationship with energy homeostatic mechanisms. These should include more components of the system (gene expression, secretion rates and receptor levels) and aim to resolve its potential interaction with nutritional and environmental factors.

Figure 7. Top: Atlantic salmon in an experimental tank. Bottom: Members from the Fish Endocrinology Laboratory during sampling. Photos: Fredrik Jutfelt.

In Paper III, the expression of three different genes; HSL which is lipolytic, LPL which stimulates tissue lipid uptake, and FAS which is lipogenic, were analyzed in rainbow trout after 1-6 weeks of GH treatment. Based on previous studies (Sheridan 1986b; O'Connor et al. 1993) the working hypothesis was that if GH acts as a lipolytic hormone, it would mainly suppress lipogenic pathways, while stimulate lipolytic actions, by affecting expression of the genes chosen. Four tissues were analyzed revealing a more complex picture. GH-induced lipolysis by phosphorylation of HSL has previously been characterized in salmonids (O'Connor et al. 1993; Michelsen et al. 1994), but to our

knowledge, the effect of GH on HSL transcription was investigated for the first time in salmonids (Paper III). The results show that HSL mRNA levels were not elevated by GH treatment in any of the tissues, and it is therefore likely that the lipolytic effect of GH is executed through activation of HSL at the protein level and/or via other lipases. This is similar to mammalian data, where GH stimulates HSL activity in adipose tissue without affecting HSL gene expression (Slavin et al. 1994; Richelsen et al. 2000). Thus, a posttranslational, rather than a translational mechanism may be a well conserved mode of action of GH on HSL. The increased lipolysis observed during fasting has been suggested to be stimulated by GH (Leatherland and Nuti 1981; Sheridan 1986a). However, the present results argue against GH as being directly responsible for the fasting-induced changes in HSL expression in mesenteric fat, muscle and liver observed in rainbow trout (Kittilson et al. 2010). Interaction between GH and nutritional status might result in tissue-specific alterations in sensitivity to GH (Norbeck et al. 2007; Norrelund 2005). Two subtypes of HSL are differentially regulated in mesenteric fat, muscle and liver during fasting in rainbow trout (Kittilson et al. 2010). As GH receptors (GHR) 1 and 2 are also differentially regulated in mesenteric adipose tissue and liver during similar conditions (Norbeck et al. 2007) this could be an underlying mechanism causing HSL expression to increase during fasting, but not as a direct response to GH. In mammals, a different TG lipase, the adipose triglyceride lipase (ATGL) (Zimmermann et al. 2004), has been identified, but its existence in fish has not been investigated.

The most prominent effect of the GH-treatment in Paper III was a strong decrease in LPL gene expression in the liver during the whole treatment period, which supports the role of the liver as a site for lipid mobilization upon GH stimulation. As LPL is responsible for the uptake of TG from circulating lipoproteins, suppression of LPL transcription which will most likely result in lowered LPL protein levels, will lead to a decreased lipid deposition in the liver. This mechanism could contribute to the lower hepatic lipid content of GH-treated compared with sham-treated rainbow trout (Kling et al. 2011), brown trout (Johnsson et al. 2000) and coho salmon parr (Sheridan 1986a). Decreased hepatic lipoprotein uptake might make more VLDL available for other tissues to use as an energy source. Thus, in addition to stimulating hepatic lipid mobilization by inducing changes in enzyme activity (Sheridan 1986a; O'Connor et al. 1993) GH also appears to reduce the deposition of lipids in the liver in salmonids by acting on the level of transcription on the LPL gene.

In Paper III, there was a tendency for hepatic FAS expression to be initially stimulated by GH, but this response was later diminished. Despite being important lipid stores, neither belly flap nor mesenteric adipose showed any substantial response to GH-treatment in transcription of the investigated genes. GH has been shown to elevate muscle LPL mRNA levels *in vitro* (Cruz-Garcia et al. 2011), but *in vivo*, endocrine signaling is much more complex as it may include cross-talk with numerous other factors. This may explain why the stimulatory effect on LPL transcription in muscle seen *in vitro* was not observed *in vivo*. The belly flap is the most ventral part of salmonid body musculature (Figure 1) and has higher lipid content than other parts of the musculature. GH promoted lipid accumulation in the belly flap *in vivo* in rainbow trout (Kling et al. 2011). Therefore it is surprising that none of the genes investigated in Paper III showed any GH-regulated

transcription. The results hence suggest that GH mediates its lipogenic effects on the belly flap via post-translational regulatory mechanisms.

To summarize, it appears that on a transcriptional level, the lipid mobilizing effects of GH in salmonids are mainly mediated through decreased hepatic LPL expression. However, there is a need for caution when effects on transcription without any corresponding proteomic data, as mRNA levels not always correspond to protein levels or enzyme activity. An example of such discrepancy is the regulation of LPL by FAs in rat adipocytes, where HR-LPL activity increased, while both mRNA and protein levels of LPL decreased (Amri et al. 1996). Tissue-specific effects of GH on lipid mobilization from different tissues may be partly explained by its differential effects on lipid metabolic gene expression.

To further address the regulation of lipid metabolism in salmonids, an *in vitro* experiment on hepatocytes and adipocytes was carried out (Paper IV) with the purpose to investigate the potential involvement of FA and ghrelin. The decreased LPL activity seen after FA treatment indicates that FAs decreases lipid uptake in adipocytes by inhibiting this enzyme. This is likely a result of direct negative feedback of FAs on LPL activity as has been previously described in mammals (Amri et al. 1996; Anderson et al. 1997; Bengtsson and Olivecrona 1980). Similarly, *n*-3 FAs decrease the amount of active LPL on the cell surface of avian adipocytes (Montalto and Bensadoun 1993). This suggests that a negative influence of FAs on adipocyte lipid uptake may be a common mechanism in vertebrates. This could be an important signal *e.g.* during fasting, when energy is mobilized, resulting in elevated plasma levels of FA. These would then signal to the lipid storing tissues such as adipocytes to decrease its lipid uptake and subsequent storage so that the lipids instead are available as energy.

The elevated glycerol release from hepatocytes after FA treatment (Paper IV) shows that FAs can increase lipolysis. It can be speculated that this is via elevated activity of HSL, the main lipase acting on intracellular TG stores (Frayn et al. 1995). The rationale behind increased lipolysis as a response to elevated extracellular FA levels remains unknown, but might be linked to a lipid mobilization state, where plasma FAs increase, as discussed previously. On the other hand, long-term dietary supplement of unsaturated FAs has a lipogenic effect in fish liver by increasing transcription and/or activity of LPL (Ji et al. 2011; Kleveland et al. 2006; Liang et al. 2002). Together, these results suggest differences in acute *in vitro* and long-term *in vivo* responses to FAs on liver lipid metabolism in fish.

Ghrelin did not affect lipid uptake, via LPL, or lipolytic activity in rainbow trout adipocytes or hepatocytes (Paper IV). The responses to ghrelin in fish seem to be species-specific as hepatic lipid accumulation is stimulated by ghrelin in goldfish (Kang et al. 2011). The discrepancy may also reflect different responses to *in vivo* and *in vitro* treatments. Ghrelin stimulates several lipogenic mechanisms, including LPL activity, also in mammals (Miegueu et al. 2011; Perez-Tilve et al. 2011; Rodriguez et al. 2009).

The evaluation of NMR-based metabolomics in fish physiology research (Paper I & II)

In Paper I, NMR-based metabolomics analysis was performed on plasma as well as both lipid and aqueous extracts from liver and muscle tissue. The metabolic profiles revealed substantial changes in the metabolome and some responses seemed to be synchronized among tissues and plasma were interrelated between tissue and plasma metabolites. Thus, with NMR metabolomics it is possible to detect many changes in cellular processes that are reflected in the plasma composition of metabolites. Hence the plasma metabolome presents a snap-shot of the physiological state of the animal. This, in combination with the minimal sample preparation needed for whole-plasma samples makes the blood plasma compartment appropriate for studies where multi-tissue analyses are not possible.

Due to the large variation in concentration as well as chemical properties of the metabolites in biological tissues and biofluids, no single method can detect all compounds present. NMR is suitable for metabolomics studies as it is reproducible and robust, non-destructive, cheap on a per-sample basis and requires minimal sample preparation (Parsons et al. 2009; Viant et al. 2009). A major advantage of using metabolomics instead of more traditional biochemical methods is also that it is non-prejudiced, allowing for the discovery of new or unexpected metabolite changes.

One limitation of NMR in metabolomics is the possible risk of peak overlap which arises when compounds have similar chemical shift and/or are present in low concentrations. This may be circumvented by different methods. CPMG attenuates signals from large molecules making it easier to detect differences in smaller metabolites. In Papers I and II, the usefulness of CPMG is demonstrated and the effect is illustrated in Figures 4A and 4B. The low detection sensitivity is a limitation of NMR. This problem can be avoided by using 2D NMR, but as the acquiring of 2D-spectra is extremely time-consuming this is rarely done. Another way of improving sensitivity is to use cryogenic probes which greatly improve the signal-to-noise ratio. The results from Papers I and II are obtained using a cryogenic probe, which cools the instrument thereby reducing signal noise, on a Varian Inova 600 MHz spectrometer.

A simplified method, based on the 'two-step' protocol by Wu et al. (2008) for preparation and extraction of tissue for NMR was validated for use in fish in Paper I. The method includes automated steps and is suitable for high-throughput extraction with minimized variation. When studying small animals, plasma volumes may be small and by using 5-mm Shigemi NMR tubes (Shigemi, USA), the required volume decreases considerably. Shigemi tubes were successfully used for plasma analyses in Paper I and II.

The use of OPLS-DA within metabolomics is relatively new, but it has been evaluated and is regarded as suitable for data mining in complex data set such as in NMR-based metabolomics (Fonville et al. 2010). Validation of OPLS-DA models can be performed by generating a large number of models using random permutations and comparing the predictive values and model fit of the random models to the original one (Lindgren et al. 1996). This validation approach was used in Papers I and II. The selection of variables (metabolites) regarded as affected by the treatment is based on their contribution to the

model which is determined by the correlation and covariance of each variable. This approach is based on the S-plot (Wiklund et al. 2008) and was employed in Papers I and II in order to determine which metabolites were affected by fasting and temperature, respectively.

CONCLUSIONS AND FUTURE PERSPECTIVES

Lipids are a major energy source for fish as other animals. Lipid metabolism is therefore also of key importance for salmonids faced with challenges such as altered environmental temperature and/or food availability, more so than protein and carbohydrate metabolism. However, there are also important links between lipid, protein and carbohydrate metabolism, such as FA synthesis from AA or carbohydrate sources. The similar responses in lipid metabolism to fasting and elevated temperature include increased plasma VLDL and unsaturated FAs concurrent with decreased HDL and choline.

During both severe nutritional conditions and at temperatures above optimal, energy stores and proteins are mobilized while glycogen stores and length growth is maintained. The prioritization of preserving length emphasizes the fundamental importance of skeletal growth over weight growth during catabolic periods, as skeletal growth is more energy demanding to attain than mass (muscle and fat) growth. The redistribution of resources may be under GH-mediated control. GH affects energy reallocation by mobilizing mainly lipid stores from several tissues, perhaps via different mechanisms. This needs to be further investigated and an *in vitro* approach might elucidate detailed, tissue specific, mechanisms under different conditions, *e.g.* nutritional status and temperature. By studying effects on several levels, from transcription to enzymatic activity, simultaneously, a more complete grasp on GH regulation of energy allocation may be obtained. It is also important to study long-term effects, *i.e.* during several months.

The importance of specific AAs in growth processes is emphasized during both fasting and acclimation to high temperature. Impaired growth may be related to decrease in plasma glutamine, tyrosine and phenylalanine. Alanine released from muscle during catabolism can be used as substrate for energy or synthesis of glucose or FAs.

NMR has been successfully used in metabolic profiling and yielded data on changes in abundance of metabolites in tissues and plasma. Further, the application of NMR in quantification of specific compounds such as lipids, with the extra benefit of obtaining detailed information on lipid classes/FA, has several advantages over gravimetrical and colorimetrical methods. Apart from in metabolomics, NMR can also be used to quantify specific compounds in biological samples. Quantification and characterization of fish lipoproteins is a promising future direction where NMR could be advantageous.

Temperatures slightly above the optimal affect the metabolism resulting in less available energy and impaired growth which in the long term may compromise reproductive success and survival. As food derivation also elicits similar responses, the added strain of limited food availability and/or quality will very likely have detrimental effects.

This thesis highlights the importance of the integrated view, studying several key tissues, using both *in vivo* and *in vitro* approaches. The thesis also cautions against conclusion on physiological functions drawn solely based on analyses at the gene expression level, as protein levels and thus *e.g.* enzymatic functions or hormone levels, do not always correlate to the corresponding mRNA levels. Another important aspect is the duration of temperature acclimation and/or treatments. It is becoming quite clear that prolonged effects differ from more short-term responses. Although one month is often considered sufficient, it is concluded that longer time periods should be used to elucidate ecological relevant results. The results may contribute to better understand lipid deposition patterns in farmed fish and potential effects of climate change on salmonids in the wild and in aquaculture.

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