Growth and energy metabolism in Atlantic salmon (*Salmo salar*), with possible positive effects of starvation at elevated temperature

Master in Nutrition of Aquatic Organisms in Aquaculture

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Abstract

Due to global warming, the sea water temperature will probably increase, which may affect cold-water species like Atlantic salmon. It is therefore of great importance to see how elevated temperature will affect the growth and energy metabolism of Atlantic salmon, and investigate if starvation at elevated temperature can have positive effects on fish performance. Adult immature Atlantic salmon (700 g), were used. The fish was divided into three experimental groups, one fed *ad libitum* at 13°C , one fed *ad libitum* at 19°C , and one starved at 19°C . The trial period lasted for 60 days, prior to a 49 day long recovery period where all fish were fed *ad libitum* at 13°C. During the trial period feed intake was significantly higher at 19°C fed fish, compared to 13°C fed fish. However there was a lower level of IGFBP1b in plasma and down regulated *igfbp1a* in muscle, as well as higher plasma glucose concentrations in 19°C fed fish. Fish fed at 19°C also had a higher feed conversion ratio (FCR), lower nutrient retention and lower growth and mean body weight. Condition factor (CF), stored nutrient composition and organ somatic indexes did not change due to temperature. After recovery fish that were previously fed at 19°C had a lower feed intake than 13°C fed fish, and plasma IGFBP1b was still lower. There were still tendencies to higher FCR, and significant lower nutrient retention in fish previously fed at19°C. Weight and growth was therefore lower in fish fed at 19°C also during recovery. CF, stored nutrient composition and somatic indexes were normal during recovery. To investigate if starvation at elevated temperature can provide positive effects on growth regulation, 19°C starved fish was compared to 19°C fed fish. In 19°C starved fish during the trial period plasma GH levels and muscle *ghr1* and *ghr2* increased, and plasma IGF1 as well as muscle *igf1* and *igfbp1a* decreased. *Igf2* expression in muscle did not change in starved fish. Weight and growth decreased, while there were still seen a minor length growth. CF, stored lipid and energy, as well as HSI and VSI decreased. Stored protein concentration and CSI did not change due to starvation. During recovery fish previous starved at 19°C, showed higher feed intake (%BM), compared to 19°C fed fish. Growth regulating Hormones hormonal parameters showed normal concentrations and expression levels. There were seen tendencies to lower FCR, as well as significantly higher nutrient retention in fish previously starved. However, weight and growth did not completely recover. Also CF as well as stored energy and lipid were lower in fish previous starved, while somatic indexes were at normal levels.

In summary, these results indicate that 19°C is to high temperature for optimal growth for Atlantic salmon. The low nutrient retention at 19°C indicates higher energy expenditure. However there was not seen mobilization of endogenous energy sources due to elevated temperature, and thereby no significant increase in catabolic activity. 49 days recovery period was not long enough for fish previously fed at 19°C to fully recover. Starvation at 19°C led to weight losses and increased catabolic activity, showed both by high levels of GH, low levels of IGF1 and use of endogenous lipid stores. Unchanged protein stores indicated that the starvation was not severe enough to deplete the lipid stores. Neither starved fish experienced full compensation growth during recovery, however there were seen compensatory tendencies in feed intake and nutrient retention.

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Abbrevations

ADP	-	Adenosine diphosphate
ATP	-	Adenosine triphosphate
ACTβ	-	Bovine actin β
BM	-	Body mass
cDNA	-	Complementary DNA
CF	-	Condition factor
CSI	-	Cardio somatic index
Da	-	Dopamine
ddH_2O	-	Distilled water
DELFIA	-	Dissosiation enhanced lanthanidefluorescent immunoassay
DNA	-	Deoxyribonucleic acid
EF1ab	-	Elongation factor 1ab
ELISA	-	Enzyme-linked immunosorbent assay
EPV	-	Energy productive value
FCR	-	Feed conversion ratio
FTP	-	Final temperature preferendum
GDH	-	Glutamate dehydrogenase
GDP	-	Guanosine diphosphate
GH	-	Growth hormone
GHBP	-	Growth hormone binding protein
GHR1	-	Growth hormone receptor 1
GHR2	-	Growth hormone receptor 2
GTP	-	Guanosine triphosphate
GLUT4	-	Glucose transporter 4 (Facultative Na^+ independent glucose transporter)
HSI	-	Hepato somatic index
ILT	-	Incipiental lethal temperature
IGF1	-	Insulin-like growth factor 1
IGF2	-	Insulin-like growth factor 2
IGFBP1	-	Insulin-like growth factor binding protein 1
IGF1R	-	Insulin-like growth 1 factor receptor
LPV	-	Lipid productive value
MNE	-	Mean normalized expression
mRNA	-	Messenger RNA
PCR	-	Polymarase chain reaction
PPV	-	Protein productive value
qPCR	-	Quantitative PCR
RNA	-	Ribonucleic acid
RPL13	-	Ribosomal protein L13
RT-PCR	-	Real- time PCR
SE	-	Standard error
SGLT	-	Na^+ dependent glucose co-transporter
SGR	-	Somatic growth rate
SRIF	-	Somatostatin
TRF	-	Time resolved fluorescence
ULT	-	Ultimate lethal temperature
VLDL	-	Very low density lipoproteins
VSI	-	Viscera somatic index

1.0 Introduction

1.1 Climate change

It is likely that there will be an increase in mean temperature at the earth's surface due to climate change in the next decades. According to (IPCC, 2013) the average sea surface temperature may increase with more than 2°C by the end of this century. The arctic regions will warm more rapidly than the global average (IPCC, 2013, Pinet, 2009). Also the temperature in the Atlantic ocean will increase more than the Pacific- and Indian ocean (Levitus et al., 2005). Also the average sea temperature increase around the Norwegian cost may be 2°C in the next 50-100 years. There will be a greater increase in fjords and costal area then in the open ocean (Øivind Bergh, 2007). Today the mean summer temperatures in southern Norway (Kristiansand), is around 17°C (July-august). At the western coast the summer temperature can reach 16°C (seatemperature.org, 2014), figure 1.1. Therefore, the forecast temperature predictions suggests that there will be seen longer periods with water temperatures above the growth optimum for Atlantic Salmon (> 17°C), at the western- and Southern cost of Norway in the future. However due to lower mean summer temperature in Northern Norway today (figure 1.1), it is likely that there will be a better climate for salmon production year round in Northern Norway.

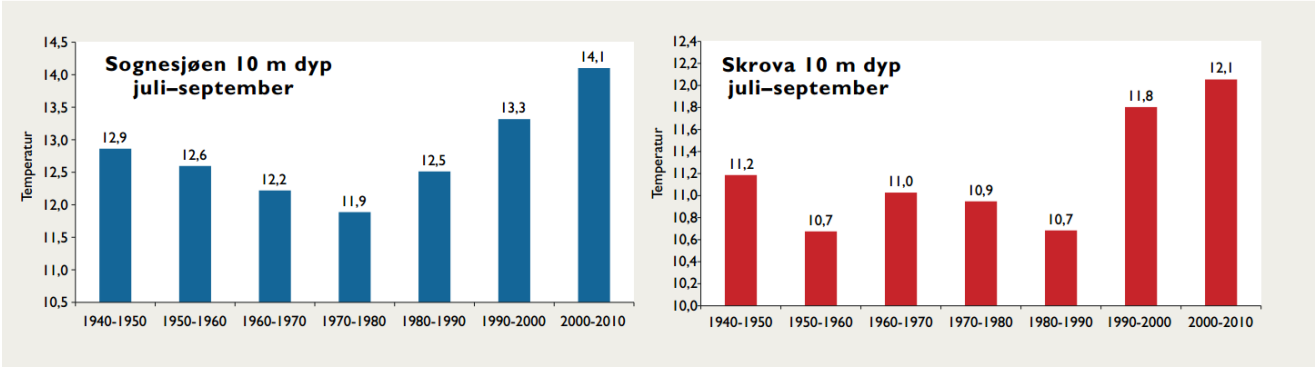

Figure 1.1: 10 years mean of summer temperature at the surface layers (10 meter), of Sognesjøen at the western coasts of Norway, and Skrova, in Northern Norway from 1940 to 2010 (IMR, 2012).

1.2 Temperature

Temperature is one of the most pervasive environmental factors affecting picilothermic animals, by that they are not able to maintain body temperatures by physiological means, and body temperature therefore fluctuate according to environmental temperature. Fish exchanges heat through the body, mostly through the body wall. When water temperature changes, there has to occur thermal equilibrium between fishes and the water, however there is some time lag before equilibrium is reached, which increases with fish size. Therefore large fish are not so much affected by short fluctuations in water temperature (Elliott and Elliott, 2010). However neither large nor small fish does necessary tolerate higher mean temperature year round, as will be the case with global warming. Fish can tolerate temperatures within the borders of the upper and lower lethal temperatures (Jobling, 1994d). The incipiental lethal temperature (ILT), is what 50 % of the fish sample can tolerate for a longer period (usually 7 days), but not survive indefinitely. The ultimate

lethal temperature (ULT), is the temperature which the fish cannot tolerate even for a short time, and is often called the critical thermal maximum/minimum (Elliott and Elliott, 2010). The different temperature zones for juvenile Atlantic salmon is shown in figure 1.2. The lethal temperature within a fish species will vary from individual to individual, according to their previous thermal history. Fish previously acclimatized to high temperatures, will have a higher tolerance for high temperatures compared to individuals acclimatized to lower temperatures. Both abiotic factors such as season, photoperiod and salinity as well as biotic factors such as nutritional status, growth, life cycle and disease, will interact with the temperature tolerance, and therefore ULT is not necessary a fixed temperature under all conditions. When a fish is moved from low temperature to higher temperature, it will gradually lose the tolerance for low temperature, and improve tolerance for high temperatures, and thereby gradually get acclimatized to the new environment. Generally fish will acclimate/compensate more rapidly to the new conditions when there is an increase in temperature, compared to when the fish has to acclimatize to lower temperatures, mostly due to that the metabolic rate is faster at higher temperatures. Even though the fish may tolerate a wide range of temperature; they will if possible, choose to spend most of the time within a certain temperature, called the final temperature preferendum (FTP). This temperature is not dependent on previous thermal history, but is close to the optimal temperature for growth. The FTP is not a single fixed temperature but a temperature zone. Like thermal tolerance FTP is also affected by many abiotic and biotic factors, among that age and size affects FTP, with juvenile fish often prefer higher temperatures than adult fish (Jobling, 1994d).

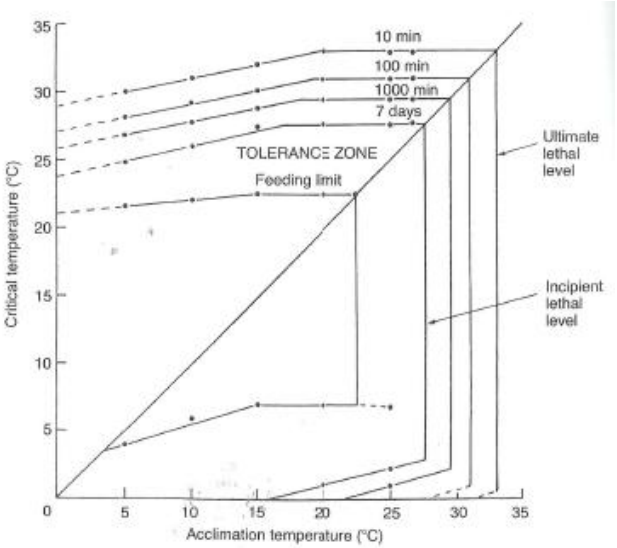

Figure 1.2 Thermal tolerance polygon for juvenile Atlantic salmon. (McCarthy and Houlihan, 1997)

The optimal temperature for growth of Atlantic salmon smolt (40-60 g) is 13 -14°C in seawater (Handeland et al., 2003), and 14°C for 150-300 g smolt (Handeland et al., 2008). However they grow in temperatures up to 19-20°C, with high growth rate at 18°C, even though mortality is higher at 18°C compared to lower temperatures (≤14°C) in post smolt (Handeland et al., 2003, Handeland et al., 2008). Also reproductive growth and development are restricted to lower temperatures (Jobling, 1997). Many physiological processes and endocrine signals affecting food intake and growth are affected by acclimation to 18°C, indicating that this temperature is too high for long-term growth in post-smolt (Kullgren et al., 2013). The optimal feed conversion rate (FCR), is found to be at approximately 3°C lower than the optimal growth temperature, and thereby around 10°C for post-smolt (Handeland et al., 2003, Handeland et al., 2008). According to (Hevrøy et al., 2012), adult Atlantic salmon (1.6 kg) prefer temperatures below 17°C, with optimum growth ≤ 13°C (Hevrøy et al., 2013). Temperature above 22°C can be lethal for Atlantic salmon (Monahan, 1993, Elliott and Elliott, 2010).

1.3 Growth

1.3.1 Growth and bioenergetics at high temperature

Somatic growth is a very complex regulated process, and is dependent on many different behavioral and physiological factors. It takes place when the difference between anabolic and catabolic processes is positive (anabolic). What makes growth even more complex is that the growth is not constant. Periods of length growth and fast growth alternates with weight gain and low growth rate (Mommsen and Moon, 2001). Growth is related to weight gain, which is considered synonymous to an increase in stored energy. How much of the ingested energy that is available for growth can be calculated by the energy balance equation used in bioenergetics, which is simplified written as:

$$E(G) = E(In)-E(Out)$$

Where E (G) is energy available for growth, E (In) is ingested energy and E (Out) is energy loss due to metabolism and faecal losses. An abiotic factor which affects either metabolism or food consumption will therefore have a profound impact on fish growth. Temperature is such a factor and influences both ingestion and metabolism, and thereby growth rate. When there is sufficient food supply, an increase in temperature will increase ingestion rate until the temperature reaches the upper thermal tolerance for the species, where there is a decline in ingestion rate (figure 1.3 a). The reason for decreased ingestion rates around the upper thermal tolerance may be due to limitations in the capacity of the circulating and respiratory systems to deliver oxygen to the tissues under conditions of high oxygen demands and little solubility of oxygen at high temperatures (Jobling, 1997). The metabolic rate (amount energy used pr unit of time), also increases with increasing temperature, (increase with 1.65-2.7 fold for every 10°C (Jobling, 1994c). However metabolic rate does not show the same decline as ingestion rate when temperature is approaching the upper thermal limit (figure 1.3 a) (Jobling, 1994b). Since metabolic rate is increasing and ingestion rate declining near the upper thermal limit, energy available for growth and other non-basal activities will approach zero as temperature approaches the upper thermal limit. Figure 1.3 b, shows that growth increases with increasing temperature, peaks and then declines with increasing temperature. The temperature where resources available for growth are at maximum is the optimum temperature for growth (which is close to FTP). This temperature is a few degrees lower than the temperature where

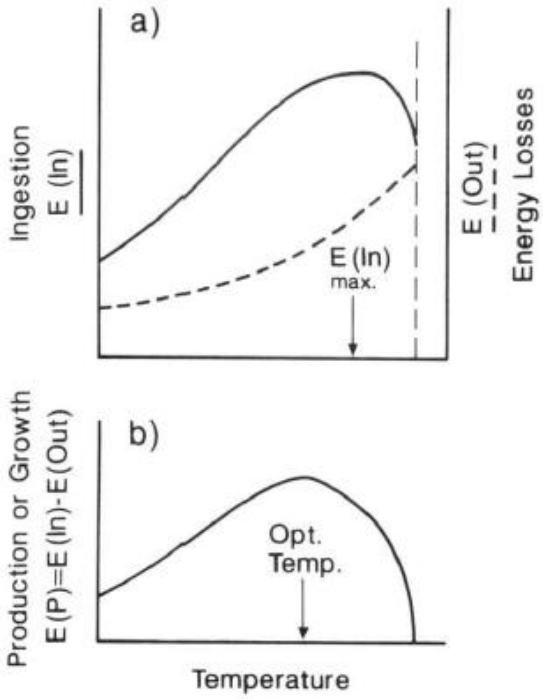

Figure 1.3: a) The effects of temperature on ingestion and metabolism rates. Dashed line shows metabolism rate, and solid line shows food intake. **b)** Shows the resources available for growth after metabolism is deducted from ingestion rate in figure a. Optimal temperature for growth is seen where there is most energy available for growth (Jobling, 1994b)

ingestion rate is highest (figure 1.3) (Jobling, 1994b). When food supply decreases, the best growth rate is seen at lower temperatures. This is because at lower temperature less energy is needed to maintain basal metabolism (which increase with temperature), and thereby more energy will be available for growth (Jobling, 1994b). When the fish is fed *ad libitum*, 25-50% of the energy will be available for growth and other non-basal activities (Jobling, 2001). When temperature is approaching the thermal maximum the energy requirements to the fish exceeds the aerobic capacity. Therefore anaerobic metabolism has to contribute increasingly to meet the energy requirements of the fish. Therefore beyond thermal maximum fish cannot survive for a long time since anaerobic metabolism is energetically expensive and leads to the production of potentially damaging metabolic byproducts like lactic acid (DFO, 2012)

1.3.2 Compensatory growth

Teleost fishes may exhibit extraordinary fast growth rates after periods of food shortages and starvation. By doing so they may achieve the same size and weight as fish that has been reared at optimum conditions (control fish), after a recovery period. Compensatory growth is thereby significantly higher than the growth rate of fish that has not experienced growth depression. Eventually this accelerated growth declines to growth rates similar to control fish. There are different levels of compensation (see figure 1.4). In full compensation, the starved fish may eventually achieve the same size as the control fish. Partial compensation means that the starved fish fails to achieve the same size at the same age as control fish, however they show a relatively fast growth rate. Over compensation is when the starved fish achieves a bigger size at the same age than non-starved fishes, due to extremely strong compensation growth. However over compensation is seldom happening. In fish compensatory growth normally describes increase in growth rate in whole body length or weight. However compensatory growth shows a diversity of forms. It may sometimes only restore lost energy reserves, or only length/weight growth, or a combination of restored energy reserves, length growth and weight growth. Sometimes full compensation of energy storages independent of length or mass growth may occur. There are many factors contributing to the compensatory growth. Especially hyperphagia is of great importance. Hyperphagia is when the rate of food consumption is higher than normal, (higher than fish that has been reared at normal/optimum conditions). By managing to have a higher feed intake, and thereby higher energy intake, the starved fish can manage to reach the size of the non-starved fish. (Ali et al., 2003).

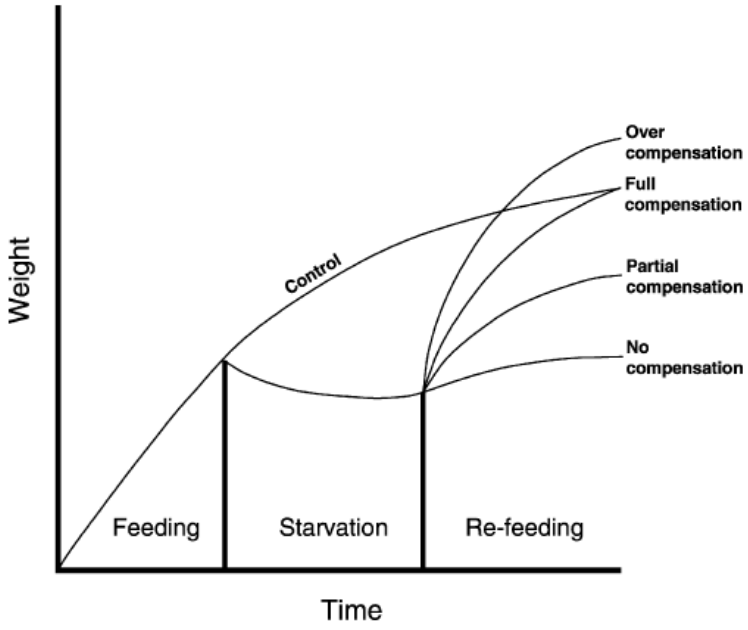

Figure 1.4: Different levels of compensatory growth in teleost fishes, (Ali et al., 2003)

1.3.3 Energy storages

Most of the energy fish needs come from the three main nutrients glucose, lipids and proteins.

Glucose

Glucose from feed is transported to the liver where it can be used as energy, stored as glycogen, transformed to storage lipids or used in synthesis of other substances like amino acids or pentoses. The gills are the tissue with highest glucose turnover, followed by heart, red muscle and liver, with lowest turnover in white muscle. In gonadal tissues, eyes, red blood cells and nerve cells, glucose is essential as energy substrate. Therefore during periods with no carbohydrate intake and starvation, glycogenic amino acids, intermediates from the Krebs cycle or glycerol (from stored lipids), is used as substrate for making glucose. This process is called gluconeogenesis. Gluconeogenesis is a very active process in fish, and the most common substrate is the amino acid alanine. The glycogenic amino acids can be transformed to glucose through pyruvate or through the Krebs cycle. The highest gluconeogenesis activity is found in the liver and kidney. The process of making energy from glucose is called glycolysis. In this process glucose is transformed into pyruvate, and under aerobic condition further transformed to acetyl-coenzyme A (acetyl-CoA) and used in the Krebs- and respiratory cycles. However under anaerobic conditions, pyruvate is transformed to lactic acid, which not enters the Krebs cycle, and gives less energy. Most fish have limited ability to use glucose as a substrate for energy production, among other due to low activity of glucokinase, which is the first regulatory enzyme in glucose turnover. The activity of glucokinase is positive affected by insulin, and inhibited by glucagon. Excess glucose can be stored as lipids by *de novo* fatty acid synthesis through acetyl-CoA. Most of the excess glucose is stored as glycogen in liver and muscle. The glycogen storages in muscle are used locally during escape and stressful conditions. In many teleosts the glycogen storages in the liver is transformed to glucose during periods of food shortage, and transferred to the blood as glucose for use in other tissues when necessary (Hemre, 2001, Hemre et al., 2002).

Glucose is in free form in the plasma, and plays a vital role to maintain the osmotic pressure in the blood. If glucose concentrations not are withheld within the normal levels (3-6 mM), it can lead to problems with the water balance. Too high glucose levels in the blood, leads to extraction of water from surrounding tissues, which dry out the tissues. The blood glucose levels are regulated by hormones, among those insulin. The β-cells in the pancreatic tissue respond to increased amounts of blood glucose by increasing the excretion of insulin into the blood stream. However this response is very weak in Atlantic salmon. Insulin is an anabolic hormone binding to receptors on the target cells surface, leading to uptake of glucose into the cells. This receptor binding is necessary for glucose uptake in all tissues except the liver. When blood glucose is decreasing, the α-cells in pancreas excrete glucagon, which is a catabolic hormone, and stimulates breakdown of glycogen storages and release of glucose into the blood. Stress hormones like adrenaline and cortisol will also affect the turnover of glucose, by increasing muscle- and liver glycolysis, and from the liver glucose is released to the blood stream for use in other organs (Hemre, 2001). Therefore increased plasma glucose levels are used as indicator for the secondary stress response (Fox et al., 2006).

Lipid

Some fatty acids are being synthesized *de novo* in the fish, and are called endogenic fatty acids, while others has to come from the food, and are called exogenic fatty acids, and are essential fatty acids. Prolonged periods with lack of essential fatty acids, will lead to decreased growth and feed utilization. From the intestine, lipids are transported towards the liver as chylomicrones, and on the

way, some lipids are directly taken up by surrounding tissue. The rest of the chylomicrones are transported to the liver for storages, metabolism and modification. Lipids are excreted from the liver as VLDL (very low density lipoproteins), and in the blood the enzyme lipoprotein lipase will break down the lipoprotein-triglycerides into free fatty acids, which is taken up by the tissues. It is believed that the fatty acid metabolism is mainly done in the liver, but some is also done in red and white muscle. *De novo* synthesis in fish is believed to be similar as mammalian *de novo* synthesis. During *de novo* synthesis fatty acids are made from non-fatty acid molecules (amino acids or glucose). This synthesis and following storage as triglyceride is called lipogenesis. The process is hormonally controlled, and insulin is the main stimulator. The mobilization and break down of lipids into glycerol and free fatty acids are called lipolysis. The main degeneration way of fatty acids in fish is through β-oxidation. The β-oxidation does occur in the cells mitochondria and peroxisomes. Some organs like liver, heart and kidney has higher mitochondria density compared to white muscle, and has therefore a higher oxidation rate. Surplus energy is normally stored as triglycerides in fish. The lipid storages can be in the liver, viscera or muscle tissue. Storage of energy as lipids is more effective than glycogen or proteins, due to that lipids do not bind up water (Torstensen et al., 2001).

Protein

Together with lipid and carbohydrates, proteins constitute the main energy source in fish, and gives energy when oxidized. Proteins also contribute as building blocks in the tissues. The proteins from food are broken down to mono- de- and tri peptides in the intestine, and transported by the blood stream to the liver. From the liver the amino acids are sent through the blood stream to tissues needing amino acids. Protein synthesis occurs at the same time as there is protein degradation. Together these two process are called protein turnover (Espe et al., 2001). The highest protein synthesis is seen near the optimal temperature for growth (McCarthy and Houlihan, 1997). Protein synthesis is increasing with increased protein intake, and due to the size of the muscle (60-65% of body weight), 1/3 of the total protein synthesis is occurring in the muscle. 50-70% of synthesized protein is also placed in the white muscle. The process where proteins are synthesized is called translation or ribosome cycle. The rate of protein synthesis in eukaryotic cells can be regulated by the number of ribosomes present in the cell, which will determine the maximum rate of protein synthesis possible, or by the supply of free amino acids which is used as substrate during the translation. Ribosomes increase in number when water temperature is decreasing, and the quantity and specificity of elongation factors may also be affected by temperature, as a compensatory response. Protein degradation starts with that proteins are degraded down to amino acids by proteases. Thereafter most of the amino acids are deaminated into ammonium and α-keto acid. The deamination is done by glutamate dehydrogenase (GDH). This enzyme is inhibited by high energy levels (ATP and GTP), and is stimulated by low levels of energy (ADP and GDP). Other deamination processes like the purine nucleotide cycle are also degrading proteins, and producing substrates that can be used in the Krebs cycle (Espe et al., 2001).

1.4 Endocrinology

There are many hormones that affect growth and development in fish. The GH/insulin like growth factor system is one of the main regulators of somatic growth and energy metabolism in fish (Peterson and Waldbieser, 2009), and includes Growth hormone (GH), GH receptors, Insulin-like growth factor 1 and 2, (IGF1 and 2), IGF receptors and IGF binding proteins (IGFBP).

1.4.1 Growth hormone

Growth hormone is produced and released in the somatotropic cells in anterior pituitary, and is an essential regulator of growth and metabolic functions. There is found two types of GH hormones in Atlantic salmon (GH-I and GH-II). One should therefore be aware that there can be differences between these two genes. However analyses shows that coding regions of GH-I and GH-II is 95 % equal, and so far no significant functional differences has been revealed between them (Björnsson, 1997, Schalburg et al., 2008). GH has a growth promoting effect in salmonids (both length and weight growth), and is the principal regulator of somatic growth. As for mammals this is probably mostly done through the "dual-effector" mechanism, where GH stimulates secretion of IGF1 and increase tissue sensitivity to IGF1. In fact GH has so strong stimulatory effect on skeletal (length), growth, that it induces length growth also during starvation. When feed is available GH increase weight growth by increasing appetite and feed conversion (Björnsson, 1997). GH also plays a major role in the energy metabolism in fish, and there are at least two major metabolic effects of GH in salmonoids. This is the stimulating of lipid mobilization and protein accretion. The catabolic lipolytic effect of GH is seen through release of fatty acids and glycerol from the liver. The lipolytic effect of GH is mediated through increased triglycerol lipase activity (Björnsson, 1997). By decreasing the lipid stores, protein accretition and carbohydrate sparing are possible. It is not sure whether all GH dependent lipolytic effects in muscle tissue are mediated direct through GH, or indirect by locally produced IGF1. However, indications in mammals are that GH can regulate adipose metabolism directly and not through IGF1. (Mommsen and Moon, 2001). The GH mediated increase in whole body protein accretion is due to an increase in protein synthesis in some organs like heart, liver, gills, and stomach. GH does not decrease protein degradation but increase the rate of protein synthesis (Björnsson, 1997). Most tissues, in particular muscle, needs amino acids as building blocks, and GH accelerate amino acid uptake from the gut and plasma into the muscle tissue, and thereby growth promotion in muscle (Mommsen and Moon, 2001). GH excretion is regulated by many environmental factors, with photoperiod as the most important factor (Gabillard et al., 2005). Regulation of GH is under hypothalamic control. The major regulator of GH secretion from pituitary is somatostatin (SRIF), which is found in the hypothalamus and pituitary. It inhibits GH release *in vitro* and lowers circulating GH levels *in vivo.* Circulating insulin-like growth factor 1 (IGF1), and circulating GH are also shown to inhibit GH secretion. This is done through a negative feed-back control on GH secretion. GH releasing factors (GHRH) and dopamine (DA) stimulate secretion of GH in pituitary cells, and DA may also reverse the inhibitory effects of SRIF. Also ghrelin may stimulate GH release. In mammals when GH is released into the blood stream, nearly 60% of the circulating GH is bound to high affinity GH binding proteins (GHBPs), which modify the GH activity in two ways; by protecting GH from degradation and thereby increasing their biological half-life, as well as competing with GH receptors (GHR) for GH binding (Björnsson et al., 2002). In the target tissues there is found GHRs with high affinity and low capacity. There is believed that the biological actions of GH is mediated through these receptors, similar to mammals (Björnsson, 1997).

1.4.2 Growth hormone receptor

Growth hormone receptor (GHR) is a transmembrane receptor belonging to the cytokine receptor super family, and found in at least nine teleosts species, including Atlantic salmon. In Coho salmon two isoforms have been found (GHR1 and GHR2), (Björnsson et al., 2002). The physiological actions of GH are mediated through GHRs, which are located at the cell surface of the target tissues. In fish GHR is expressed in almost all tissues, with the highest expression in liver in tilapia (*Oreochromis*

mossambicus) (Fox et al., 2006), indicating that the liver is the major target for GH. However due to the size of muscle tissue, also the muscle is of great importance (Björnsson et al., 2002). In mammals both high levels of insulin and IGF1 can work as negative feedback on expression and function of GHR, and thereby inhibit GHR expression (Ji et al., 1999).

1.4.3 Insulin-like growth factor 1

Insulin like growth factor 1 (IGF1), belongs to the insulin super family and is a peptide. IGF1 is similar to IGF2 and insulin in amino acid sequence, receptor binding and biological functions. IGF are one of the main players in muscle growth and development in teleost fish. It works as a powerful stimulator for many anabolic processes in muscle tissue, including increased DNA and protein synthesis, amino acid uptake, cell proliferation and activation of mitogenesis as well as decreasing protein degradation rate (Mommsen and Moon, 2001). Even though the metabolic actions of IGF1 and GH is quite similar, there are some differences on their effects on carbohydrate metabolism, which makes IGF1 slightly more anabolic than GH (Norbeck et al., 2007). IGF1 physiological actions are largely mediated through specific IGF1 receptors. Plasma concentrations of IGF1 fluctuate according to the nutritional state of the fish, and are very dependent on the concentration of other hormones. Normally plasma concentrations are around 25 ng/ml in fish, but only 0.1% of this is in a biological active form (free IGF1), and not bound to binding proteins. (Mommsen and Moon, 2001). Both IGF1 and IGF2 plasma levels are correlated to liver mRNA levels, showing that liver is the main source for circulating IGF (Gabillard et al., 2003a). Especially IGF1 transcription in liver and gills shows high response to activation of GH. Liver produce IGF1 for systemic functions, while other organs like muscle produce IGF1 for autocrine and paracrine actions, and may not be regulated by GH in the same degree. This is because *Igf1* expression in muscle is controlled by a lot of hormonal factors, among those insulin and IGF1, leading to an autocrine feed-forward activation of *igf1* gene expression. Also other factors like environment and nutritional status directly regulates IGF1 in muscle (Hevrøy et al., 2013, Kullgren et al., 2013).

1.4.4 Insulin-like growth factor 2

Also insulin-like growth factor 2 (IGF2), is a peptide belonging to the insulin super family, and has many of the same functions as IGF1 in fish. Not only IGF1, but also IGF2 is related to local paracrine/autocrine regulation of at least muscle growth in adult teleosts (Hevrøy et al., 2007, Hevrøy et al., 2011, Mommsen and Moon, 2001). Unlike in mammals IGF2 also play a vital role in growth and development of adult fish, and not only during embryogenesis (Vong et al., 2003). It is discussed if the expression of *igf2* is controlled by GH or not. Like for IGF1 the expression is highest in liver, but also other organs express *igf2*. There is often a higher expression of *igf2* than *igf1* in non-hepatic tissues. Like IGF1, its bioavailability is controlled by IGF binding proteins, and its actions are largely mediated through specific receptors. In mammals IGF2 is shown to bind to a broader range of receptors (different types of insulin, IGF1 and IGF2 receptors), than IGF1, and may therefore have a broader range of biological functions than IGF1 (Chao and D'Amore, 2008).

1.4.5 IGF1 binding proteins

There are documented six IGF-binding proteins with isoforms in teleosts (Kevin et al., 2006, Shimizu et al., 2011) . In Atlantic salmon, these are 23- 28 and 43 kDA IGFBP. 43 kDA is called *igfbp2* and 23 kDA is called *igfbp1.* (Hevrøy et al., 2011). IGFBP1 has probably a major role to regulate the metabolic actions of IGF1 in teleosts. Fish IGFBP1 is very similar to mammalian IGFBP1 (Hevrøy et al., 2013, Kajimura and Duan, 2007), but also has some similarities with mammalian IGFBP4 (Kajimura and

Duan, 2007). The circulating IGFBP limits the access of IGF to specific tissues and receptors. The IGFBP increase the half-life of IGF with many hours, by avoiding attack from proteolytic enzymes. When the IGF is released from IGFBP it is biological active, living the circulation and entering target tissues (Le Roith, 1997). By making IGF1 biological active or inactive, IGFBP can tune IGF actions during many catabolic and stressful conditions. Under normal environmental conditions, the inhibitory effect that IGFBP1 has on IGF, is turned off, and thereby favoring fast growth and development. When the conditions not are favorable, IGFBP1 is "turned on" to restrict IGF signaling by binding up free IGF. The binding of IGF to IGFBP has higher affinity than binding to IGF receptors, and thereby IGFBP prevents IGF from binding to IGF receptors. In plasma 95% of IGF1 is bound to IGFBPs. IGF is released from IGFBP by proteolysis of IGFBP, which decreases the binding affinity and releases IGF to IGF receptors (Kajimura and Duan, 2007). Like for IGF, IGFBPs are mostly produced in the liver, but also other organs produces these molecules for autocrine/paracrine use (Le Roith, 1997). IGFBP1 is induced by many different catabolic conditions, like starvation, malnutrition, protein restriction and hypoxia. In mammals circulating IGFBP1 is also affected by insulin level, showing its involvement in glucose metabolism (Kajimura and Duan, 2007). Insulin inhibits transcription of *igfbp1*, and is the primary determinant of *igfbp1* expression. Also IGF1 and IGF2 have an inhibitory effect upon expression of *igfbp1*. Somatotropin release inhibitory factor (SRIF), which inhibits release and synthesis of GH, up regulates the expression of IGFBP, and therefore lowers the bioavailability of IGF1 (Mommsen and Moon, 2001).

1.4.6 IGF1 receptor:

IGF1 receptor is a transmembrane tyrosine kinase receptor, and activated by IGF or insulin (Blakesley et al., 1999). In fish IGF1 receptor is found in many tissues, among those liver, gill and muscle (Mommsen and Moon, 2001). The physiological actions of IGF1 and IGF2 are mediated mostly through IGF1 receptors, located at the cell surface of the target tissues. In mammals they bind IGF1 with high affinity and IGF2 and insulin with slightly lower affinity. IGF1 receptors are involved in cell proliferation and growth, by mediating the biological action of the IGFs into the cells (Planas et al., 2000). IGF1 receptor seems to be very similar to mammalian IGF1 receptor, both structurally and functionally (Moriyama et al., 2000).

1.4.7 Glucose transporter 4:

Carnivorous fish have a poor biochemical competence to use glucose as fuel, and often shows insulin resistance-like metabolic behavior. The reason is probably poor peripheral utilization of glucose, which is related to the ability for glucose to transfer across plasma membranes, and the cells to metabolize it. In mammals two protein families are used to transport glucose across membranes. The Na^+ dependent glucose co-transporters (SGLT), and the facultative Na^+ independent glucose transporters (GLUT). Some of these GLUTs have also been identified in fishes. GLUT4 transporter in fish shows similarities with mammalian GLUT1 and GLUT4 (Mommsen and Moon, 2001). In fish the lower ability to clear glucose from the blood, can be due to low of levels of GLUT (Capilla et al., 2002), or GLUTs with lower affinity compared to mammals (Capilla et al., 2002, Capilla et al., 2004). In mammalian the stimuli of glucose uptake by insulin is mediated mostly by GLUT4. When stimulated by insulin GLUT4 will rapidly move from intracellular storages unto the plasma membrane (Capilla et al., 2002, Capilla et al., 2004). There is still some uncertainty of how GLUT4 is functions in fish. But it is mainly expressed in insulin sensitive tissues, like white muscle, adipose tissue, (Menoyo et al., 2006), kidney, gills and heart (Capilla et al., 2004). In fish it looks like insulin regulation of *glut4*

mRNA expression is tissue specific, and red muscle is affected by plasma glucose levels, while white muscle is not (Capilla et al., 2002).

1.4.8 GH/IGF regulation by temperature

Not only photoperiod affects the plasma levels of GH. Also temperature regulates plasma levels of GH and IGF1, high temperatures increases plasma levels. However temperature does not increase GH mRNA in pituitary, only secretion into plasma (Gabillard et al., 2003b). The temperature mediated plasma GH increase will enhance IGF1 expression in liver and plasma. However autocrine/paracrine expressions of IGF1 or IGF2, nor plasma IGF2, are not up regulated due to temperature. The growth promoting effects of temperature is therefore mediated through increased plasma IGF1 levels. However there is only seen increase in plasma IGF1 due to temperature mediated increase of plasma GH when there is optimal nutritional conditions. The reason why GH does not increase plasma IGF2 levels is likely due to that IGF2 is not affected by temperature itself but by nutritional status of the fish (Gabillard et al., 2005).

1.5 Starvation

Fish can survive for a long time without food, and fasting periods are part of the natural life cycle of many fish species. For salmon winter months and spawning migration are examples of such periods. Many fish species can therefore starve for many months and thereafter fully recover after re-feeding. Such fish species are well adapted to mobilize their metabolic reserves to survive during starvation periods. The effects of starvation on metabolism is dependent on many factors, among those fish species and which tissue has metabolic storages (Navarro and Gutiérrez, 1995). Normally adult Atlantic salmon lose most weight the first weeks of starvation, thereafter minor changes are seen. This is probably due to a combination of emptying of intestinal track as well as the energy requirement during starvation decrease, leading to lower metabolic rate (Christiansen, 1996). In fact in some species the metabolic rate is decreased by 30-40% after a few days starvation (Ali et al., 2003). Even though fish lose weight during starvation, they often continue to have length and skeletal growth (Christiansen, 1996). This is done by using the body reserves to relocate energy into skeletal growth, which is initiated by growth hormone (Kullgren et al., 2013). Fish with high condition factor before starvation often increase in length faster than fish that have low condition factor. Due to the length growth during starvation, compensation growth will easily increase weight when feed is available (Christiansen, 1996).

1.5.1 Phases during starvation

It is possible to divide starvation in fish into three different phases: **Phase 1; Hormonal and physiological changes, Phase 2; Mobilization of lipids and Phase 3; Mobilizing of proteins** (Bar and Volkoff, 2012, Einen et al., 1998).

Phase 1: Hormonal and physiological changes: At start of the starvation period many different hormones changes in concentration. Leading to that energy is diverted away from weight growth, to sustain essential metabolic processes. This growth retardation is associated with increased plasma GH as well as reduced GH sensitivity in some tissues, among those the liver. This leads to lower synthesis rate of IGF1 in those tissues, and thereby less IGF1 in plasma (from liver). However in the viscera, there is seen a higher sensitivity for GH during starvation, leading to increased lipid depletion here. The increased sensitivity in adipose tissues enhances the lipolytic actions of GH (Norbeck et al., 2007). Also during starvation increased GH will likely stimulate the mobilization of energy reserves

like glycerol and fatty acids from other tissues (Björnsson, 1997). There is often seen a reduced GHR expression during starvation, and the GH resistance during starvation has been linked to GHR damages; however it may also be damages in the *igf1* synthesis process. Anyway the reduced GHR expression can be a mechanism to reduce circulating IGF1 and growth during starvation (Fox et al., 2006). Like for GH, also IGF1 sensitivity is altered in some growth sensitive organs like the gills during starvation. This is seen as reduced expression of IGF1 receptors in those tissues. However in some organs there is not seen a reduced IGF1 sensitivity, showing that important protein stores are spared during starvation. This is especially true for vital organs like the heart (Norbeck et al., 2007). Also plasma IGF1 concentrations are depressed during starvation (Mommsen and Moon, 2001). Therefore low levels of plasma IGF1 is generally associated with starvation or malnutrition, even thou there are no differences in circulating GH levels (Fox et al., 2006). Like for *igf1* expression, also *igf2* expression is decreasing during starvation in Atlantic salmon. Expression of *igfbp1b* increases during starvation, due to higher catabolic activity in starved fish. Therefore *igfbp1* is a good molecular marker for catabolic activity in fish (Hevrøy et al., 2011).

The physiological changes at this stage include increased catabolism, mostly by the use of glycogen, but also of protein and lipids (Bar and Volkoff, 2012, Einen et al., 1998). Muscle glycogen is used for muscular activities; therefore the variations in muscle glycogen during starvation are probably more related to changes in muscle activity than starvation (Hemre et al., 2002, Navarro and Gutiérrez, 1995). However while muscle glycogen not is so affected by starvation, liver glycogen is more affected. And there is normally a significant decrease in liver glycogen during starvation in teleosts, but the overall contribution to the total energy expenditure is relatively small when looking on the limited weight of liver, HSI normally lower than 3% (Hemre et al., 2002),(Navarro and Gutiérrez, 1995). Plasma glucose level is normally maintained at a more or less steady state during long periods of starvation, and comes largely from the liver glycogen, at least during the initial stages of fasting (Navarro and Gutiérrez, 1995)

Phase 2 Mobilization of lipids: During this phase hormonal concentrations stays similar to phase 1, or continue to increase. When glycogen stores are getting depleted, lipid stores are mobilized. In this phase the mobilization of proteins are declining, and lipids are the main source for energy (Bar and Volkoff, 2012). This phase lasts often for long periods, until lipid stores reach a critical threshold. There is also seen a reduction in mobilization of proteins as energy source in this period (Einen et al., 1998), this probably due to high levels of circulating growth hormone. For most teleost fish, the main lipid store is the intestinal/viscera lipids, and for many species, this is the first place where lipids are mobilized from. (Navarro and Gutiérrez, 1995). In Atlantic salmon intestinal mass decreases with 20-50% during the first days of starvation, but thereafter there are little decrease in intestinal mass (Einen et al., 1998). This can be due to that in the beginning of starvation, viscera lipids are more important than muscle lipids (Christiansen, 1996), while later muscle lipids is contributing more. However in adult Atlantic salmon (2-5 kg), studies indicate that there is no preferential use of visceral lipids compared to muscle lipids, (Einen et al., 1998). Generally fish that store a lot of lipid in the muscle tissue, mobilize this lipids as soon the starvation starts, and almost 60% of the lipid storages is found in muscle tissue in Atlantic salmon (Christiansen, 1996). Lipid levels in liver decrease faster than lipid in muscle and viscera. However the lipid loss from liver is quantitatively much lower than for muscle and viscera. (Einen et al., 1998).

Phase 3, Mobilizing of proteins: When lipid stores are getting depleted (less than 2 % of body mass in Atlantic salmon), protein tissue start to degrade more rapidly (Bar and Volkoff, 2012) (Einen et al., 1998). This phase is recognized by a markedly increase in ammonia products in the blood, due to increased protein mobilization (Bar and Volkoff, 2012).One of the main reason for that fish can withstand long periods of starvation is because the muscle has a lot of proteolytic enzymes that can mobilize the tissue proteins for fuel when required (Christiansen, 1996). Increase in proteolytic activity therefore increase with starvation period. In fact during prolonged starvation, muscle protein is the main energy source in fish. (Navarro and Gutiérrez, 1995). White muscle is normally the tissue that is most sensitive to starvation, and responds to starvation by reducing the rate of protein synthesis. Protein synthesis of other tissues like liver and gills are little affected by starvation, showing their importance for the function of these organs. Like white muscle also viscera lose a lot of protein during starvation in adult Atlantic salmon (Christiansen, 1996, Einen et al., 1998).

1.6 Aim and hypothesis for this study

Studies indicates that water temperatures around 19°C, is above the optimal temperature for growth in Atlantic salmon (Handeland et al., 2003, Handeland et al., 2008, Hevrøy et al., 2012, Hevrøy et al., 2013, Kullgren et al., 2013), and too high to sustain long time growth (Kullgren et al., 2013). However, little research has been focusing on possible positive effects of starvation during periods with high temperature.

The overall aim of this master thesis was to examine the endocrine growth regulation, feed utilization and energy metabolism in Atlantic salmon during a period of high sea temperature and starvation, as well as possible compensatory responses to re-feeding at optimal water temperature. It is hypothesized that elevated sea temperatures affect growth, energy metabolism and feed utilization in Atlantic salmon.

To answer this aim it will be:

- Investigated if seawater temperatures above the optimum for growth reduce growth and feed intake.
- Examined if high temperature will increase energy consumption and favoring catabolic actions.
- Investigated how the endocrine regulation of muscle growth during starvation and elevated temperature works.
- Determined possible positive effects of starvation during re-feeding after periods of elevated temperature

2.0 Materials and methods

2.1 Experimental design and sampling

2.1.1 Experimental design

The experiment was conducted at Matre Aquaculture Research Station, Institute of Marine Research, Matredal, Norway (N61°). 13 April 2011, 600 large immature Atlantic salmon (0+, NLA strain), ~700g, where transferred from sea cages to 9, 3m x 3m indoor tanks, with 50 individually pit tagged fish in each tank (figure 2.1). Thereafter, two weeks of adaption to the indoor tank system followed. The fish were fed three times a day (08:15-10.00, 11:30-12:30 and 14:00-15:00), using a automatic feeding system and waste feed collectors which collected feed 15 min after feeding ceased. Both out feeding in g/day and feed collecting g/day was measured. Feed intake in g/day, as well as feed intake % BM was calculated. The feed used was an experimental feed recipe produced by Skretting AS, Stavanger, Norway. The feed nutrient composition was analyzed and contained 30% lipid, 45% protein, 5.1% ash and 5% moisture, with energy content of 25.1 MJ/kg. The inlet water was oxygenated to keep 90% saturation in all tanks during the whole experiment. A 18:6 light regime was used. After two weeks acclimatization (29 April), the trial period started. The 9 tanks were divided into three different treatments with triplicate tanks for each treatment.

Treatment 1: The fish was kept at 13°C and fed *ad libitum* during the trial period (60 days).

Treatment 2: The fish was kept at 19°C with no feeding during the trial period

Treatment 3: The fish was kept at 19°C and fed *ad libitum* during the trial period

After the trial period (60 days), all groups were fed in a recovery period for 49 days at 13°C with feeding according to their appetite (*ad libitum*).

For both water temperature increases and decreases, the temperature was changed by 1°C/day until reaching desired temperature.

2.1.2 Experimental fish

At start of experiment the fish had a condition factor on 1.14 ± 0.008 (SE). The whole body composition of the fish at start was as follow: 870 ± 23 KJ/100 g ww energy, 11.3 g ±0.7g/100g ww lipid and 18.9 ± 0.2 g/100 g ww protein. The somatic indexes of the fish were as follow: HSI (1 ±0.05), VSI (6.82 ± 0.14) and CSI (0.10 ± 0.003).

2.1.3 Sampling regimes

There were three samplings;

- **Sampling 1:** Start sampling (28. April 2011)
- **Sampling 2:** Sampling after trial period (27. June 2011)
- **Sampling 3:** Sampling after recovery period (15. august 2011).

All sampling were taken four hours after feeding to ensure comparable post-prandial levels of free amino acids and hormones (Hevrøy et al., 2007). All fish where sedated using ISO-eugenol in the tanks, and then anaesthetized with Tricaine Methanesulfonate, and killed with a blow to the head. Thereafter length and weight was registered, and tissues and organs sampled. Three Fish where randomly sampled from each tank. From sampling 1, nine fish were sampled for organ analyses, and nine fish sampled for whole fish analysis. From sampling 2, totally nine fish was sampled from each treatment (three from each tank), for whole fish analysis (totally 27 fish). Similarly, 27 fish were sampled for organ analysis. The sampling of fish at sampling 3, was equal to sampling 2. Under all samplings the fork length was measured to the nearest 0.5 cm, whole fish weight to the nearest gram. Heart, liver and viscera were measured to the nearest 0.01 g. White muscle, liver and blood were collected for further analysis. The blood was collected from the caudal veins by using a syringe, and put into heparinized tubes. Plasma was collected after centrifugation at 1250 g for 10 minutes. Organ samples were frozen in liquid nitrogen (- 80°C), during sampling and kept at -80°C until analysis. The whole fish were frozen at -20°C until further processing.

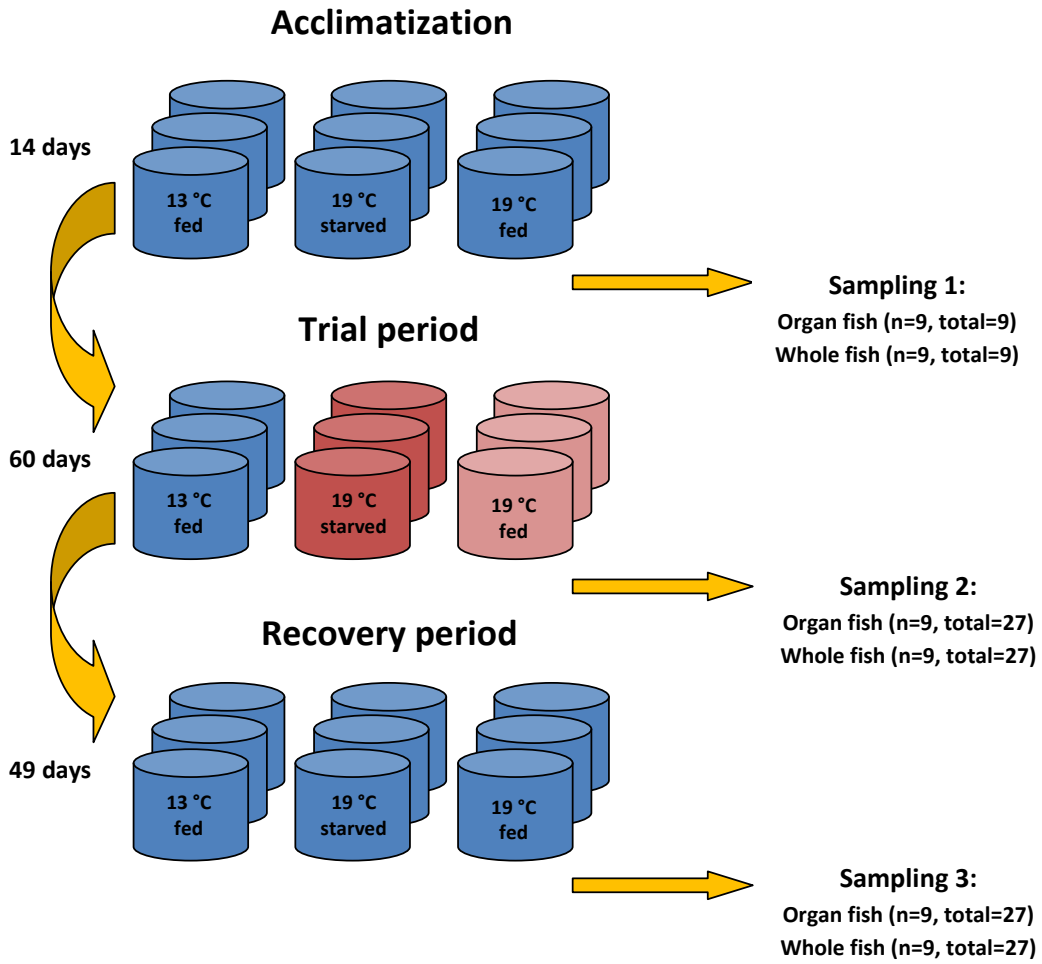

Figure 2.1: Experimental design. All fishes were initially kept at 13°C for 14 days acclimatization. After sampling 1, the temperature was increased to 19°C for two groups (19 starved and 19 fed) during a 60 days trial period. During trial period 13°C fed and 19°C fed was fed *ad libitum*. After sampling 2 a 49 days long recovery period followed, where all groups were hold at 13 13°C and fed *ad libitum.* After 49 days of recovery sampling 3 was conducted.

2.2 Analytical methods

2.2.1 Gene expression

RNA isolation

RNA was isolated according to NIFES protocol: "Met.MOL.01-57, 281-RNA rensing og RNA kvalitet". The RNA was isolated from white muscle in Atlantic salmon.

Before starting the procedure, the bench and equipment was cleaned by using Sigma RNase Zap™ . The samples were taken directly from -80°C freezer and kept on dry ice until cutting. Six sample tubes were prepared in each round, by using the BioRobotEZ1 DSP (Qiagen N.V., Hilden, Germany). First sample was cut and weighted to 0.150 g, and the other samples were cut into similar size by visual estimate to avoid thawing. The 0.150 g pieces were homogenized in QIASOL using zirconium beads (4 mm), in Precellys 24 homogenizer (Bertin Technologies, Aix En Provence, France). After incubating for 5 min chloroform was added to separate RNA from protein and DNA. To achieve phase separation the samples were centrifuged for 15 min at 12000g and 4°C in an Eppendorf centrifuge 5415R (Eppendorf, Hamburg, Germany).

When centrifugation was finished, >350 µl of the upper blank supernatant was transferred from each tube into 2 ml sample tubes, which was loaded into the EZ1 robot (Qiagen N.V., Hilden, Germany) . The following program was used at EZ1 (table 2.1):

Table 2.1: The program used on the EZ1 robot

EZ1 Program	
Gene expression:	Total RNA
Mini- or Universal tissue	Universal tissue
Including DNase	Yes
Elution volume	50 µl
Total volume	300 µl

RNA concentration and purity

The RNA concentration and purity was measured using NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, USA). The measurement was done according to standard procedure at NIFES (MET.MOL.01-57, NanoDrop ND-1000).

The tubes were kept on ice during the testing. The spectrophotometer probe was washed with ddH_2O before use, and wiped clean with a clean towel between each measurement. 2 µl of each sample was loaded to the probe to measure concentration and purity. Just before loading unto the probe, the sample tubes were vortexed for a few seconds to ensure accurate measurements. To ensure that no magnetic beads were brought unto the spectrophotometer, a magnet holder (Invitrogen DYNAL bead separation, Carlsbad CA, USA), was used to hold the sample tubes after vortexing.

The 260/280 and 260/230 absorbance ratio was used as indicators for sample purity.

RNA quality

The quality was tested by using a RNA Integrity Number algorithm (RIN algorithm), on the Agilent 2100 Bioanalyser (Agilent technologies, USA) together with RNA 6000 Nano and RNA 6000 Pico LabChip kit (Agilent Technologies, Santa Clara, CA, USA). 12 random samples, including samples from each isolation round, were tested. The measurements were done according to the manufacturers guidelines. Before adding RNA samples to the wells, the RNA samples were diluted with ddH_2O to a final concentration of 100-500 ng/µl. 2 µl of this dilution was then added to the wells. The RNA fragments were analyzed by capillary electrophoresis.

Reverse transcription-PCR

The procedure was done as described in NIFES standard procedure "MET.MOL.01-55, 279 - RT REAKSJON".

The cleaned RNA was diluted with ddH_2O in new tubes to a concentration of 50 ng/µl ± 5%. Nanodrop ND-1000 (Saveen Werner, Malmö, Sweden) was used to get right concentration.

The standard curve was made by mixing together 5 µl from each sample and measure at the spectrophotometer. Then six serial dilutions (100 – 3.13 ±5% ng/µl) was made from this pool. The standard dilutions were made in triplicates on the 96 well plate, except dilutions 6.3 and 3.1 ng/µl, which were duplicates. The RT reaction mix was made using TaqMan reverse transcription reagent kit (Applied Biosystems, Foster City, CA, USA). The final concentration of RT mix was: TaqMan RT (1X), magnesium chloride (5.5 mM), 1deoxyNTPs Mixture (500 µM per dNTP), RNase inhibitor (0.4 u/µl), Oligo $d(T)_{16}$ (2.5 µM), Multiscribe reverse transcriptase (1.67 u/µl). In each well 20 µl of the RT reaction mix was added, plus 10 µl of RNA from the respective samples tubes. All samples were made in duplicates. Also two negative control wells were made. One non-amplification control (nac), containing RNA from the RNA pool used for making standards and RT reaction mix without the enzymes. The other control was a non-template control (ntc), containing RT reaction mix and 10 µl RNase free water instead of RNA. 30 µl RT-PCR where preformed by using the program shown in table 2.2, using the PCR system GeneAmp PCR 9700 (Applied Biosystems, Foster City, CA, USA).

Table 2.2: Showing the program used during RT-PCR of cDNA

Step	**Incubating**	**RT**	**Reverse Transcriptase inactivation**	**End**
	HOLD	HOLD	HOLD	HOLD
Temp (°C)	25	48	95	4
Time (min)	10	60	5	∞
Volume (µl)	30			

Primers

The primers used in this experiment were provided by Dr. Ernst Morten Hevrøy. The primers for *ghr1, igf1, igf2, igf1ra and the housekeeping genes ef1ab* and *actβ*, where previously presented in (Hevrøy et al., 2013). *Glut4* were presented in (Menoyo et al., 2006), were it is named *glut-i2. Igfbp1a* are presented in (Hevrøy et al., 2014), (table 2.3).

Table 2.3: Showing the different primers used for qPCR. EF1ab, RPL13 and actb are the housekeeping genes.

Target gene	Primer sequence	Amplicon size (bp)	Accession no.
Ef1ab	For 5' -CCCCTCCAGGACGTTTACAAA	57	AF321836
	Rev 5' -CACACGGCCCACAGGTACA		
actb	For 5' -CCAAAGCCAACAGGGAGAA	102	BG933897
	Rev 5' -AGGGACAACACTGCCTGGAT		
Rpl13	For 5' - CCAATGTACAGCGCCTGAAA	110	NM_001141291
	Rev 5' - CGTGGCCATCTTGAGTTCCT		
Ghr1	For 5' -TGGACACCCAGTGCTTGATG	70	AF403539
	Rev 5' -TCCCTGAAGCCAATGGTGAT		
Ghr2	For 5' - TCGGAACATTCCAGAACCTC	164	NM_001123594.1
	Rev 5' -GGTCATCCAGACCTTCGTGT		
Glut4 II	For 5' -CGCTTCCTCTACATCATCCGTTC	268	AY566722.1
	Rev 5' - AGCATATCCCCCACTTCCTGT		
Igfbp1a	For 5' - GGTCCCTGTCATGTGGAGTT	184	KC 122927
	Rev 5' - TTCCAGAAGGACACACACCA		
Igf1	For 5' -TGACTTCGGCGGCAACA	119	M81904
	Rev 5' -GCCATAGCCCGTTGGTTTACT		
Igf2	For 5' -TGCCAAACCTGCCAAGTCA	66	AY049955
	Rev 5' -GGCACCATGGGAATGATCTG		
Igf1ra	For 5' -TGCACAACTCCATCTTCACC	132	EU861008
	Rev 5' -GGGGCTCTCCTTCTGTCCTA		

Quantitative PCR (qPCR)

The cDNA plates made for RT-PCR where thawed on ice and 30 µl RNase free water was added into each well, and spun down for 1 min at 700 rpm (Eppendorf centrifuge 5810R, Hamburg, Germany), before shaken for 5 min at 1500 rpm (Eppendorf Mixmate PCR 96, Hamburg, Germany). The procedure used was the NIFES standard MET.MOL.01-56, 280- RealTime PCR.

A SYBRgreen mastermix (Roche Applied Sciences, Basel, Switzerland), containing the specific primers (table 2.4), where prepared at a RNA/DNA free room (table 2.4). By using a pipetting robot (Biomek®

3000, BeckmanCoulter, Brea, Ca, USA), 8 µl of SYBRgreen mastermiks and 2 µl of RNA was transferred to each well on a 384 well plate. The plate was spun down at 1500 rpm for 2 minutes (Eppendorf sentrifuge 5810R, Hamburg, Germany). Thereafter it was inserted into the Light Cycler 480 Real-Time PCR system (Roche Applied Sciences, Basel, Switzerland). In addition to the normal qPCR, a melting curve analysis was preformed to ensure only one gene sequence was produced during each PCR analysis. Table 2.5 is showing the program settings used during qPCR.

Table 2.4: Reagents in the SYBRgreen master mix

Reagents	Volume (µl)	Final concentration
ddH_2O	2.8	
Forward primer (50 µM)	0.1	0.625 µM
Reverse primer (50 µM)	0.1	0.625 µM
TaqMan universal PCR Master Mix (2X)	5	

Table 2.5: The program used during qPCR

Step	Pre-incubating	Amplifying			Melt curve analysis			Cooling
Temp (°C)	95	95	60	72	95	65	97	40
Time	5 min	10 sec	10 sec	10 sec	5 sec	1 min		10 sec
Cycles	1	45			1			

The software Lightcycler® 480 software, version 1.5.0.39 (Roche Applied Sciences, Basel, Switzerland), was used to determine cyclic time where fluorescent reached a threshold level (Ct value), calculating standard curves, sample error and efficiency of the reactions. The error should be below 0.04 and the efficiency between 1.8 and 2.2.

2.2.2 DELFIA® time-resolved fluorescence assay

DELFIA technology (Dissociation-Enhanced Lanthanide Fluorescent Immunoassay), from PerkinElmer Life Sciences (Waltham, MA, USA), is a high-performance and robust immunoassay, which has many benefits compared to traditional ELISA Immunoassays. Among other it is more sensitive than ELISA and therefore better to use when there is little sample available (PerkinElmer, 2013).

In current experiment sample molecule (in this case plasma GH), competed with a labeled biomolecule (in this experiment, biotin labeled GH, containing europium) for the binding sites in the wells of the immunoassay plate (figure 2.2). The lower concentration with sample GH, the more biotin GH will bind to the antibodies in the well, giving a stronger count (illumination), compared when most antibodies are occupied by sample GH, which will not illuminate. However, the labeled GH is also almost non-fluorescent. Therefore after the binding reactions, DELFIA Enhancement Solution (Waltham, MA, USA,) is added. The low pH of the enhancement solution will dissociate the Europium from the labeled GH. The free Eu^{3+} then rapidly forms a new highly fluorescent chelate, which is protected by a micelle formed by the components of the enhancement solution. In this way the enhancement solution will increase the fluorescence of the lanthanide chelates. The fluorescence lifetime of the lanthanides is several orders of magnitude longer than the non-specific background. This makes it possible to measure the label after the background has decayed, which will minimize the noise ratio. (PerkinElmer™).

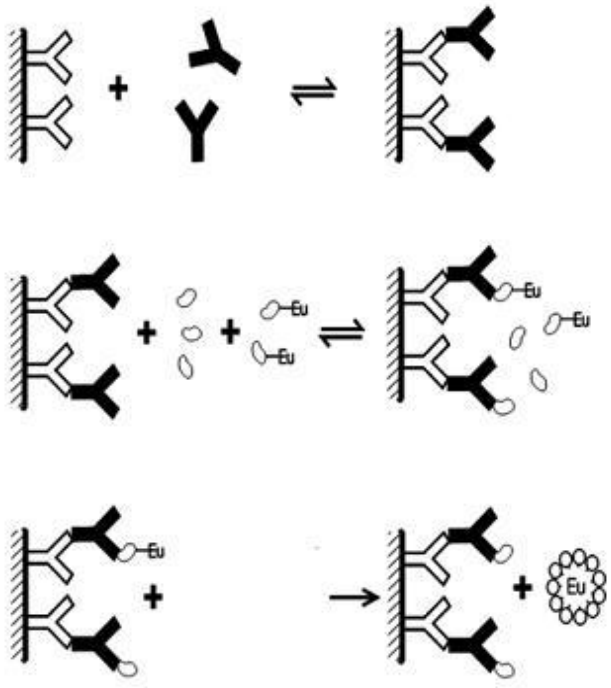

Figure 2.2 Illustration example of competitive immunoassay (PerkinElmer™)

Plasma-GH TRF Immunoassay

Part of the master thesis was to participating in developing this analysis at NIFES. The main protocol used was developed by Professor Munetaka Shimizu (Hokkaido University), and Dr Ernst Morten Hevrøy (Ewos AS), with MsC Nobuto Kaneko (Hokkaido University), taking part in developing the practical implementation of the method. In this experiment the analysis was conducted as described by Nobuto Kaneko, (TR-FIA for GH, 18.03.2013).

Finding suitable concentrations of antibody, label and GH-standard

Before doing immunoassay analysis on plasma GH from sample fish, the most suited concentrations of antibody (Anti-Salmon Growth Hormon, Rabbit 5 µl lyophilized, Lot: AJI-PAN1, Gro Pep, Adeilade SA, Australia), Biotin labeled GH and GH standard (Lot: DAB-GHB1, Salmon/Trout growth hormone, 20 µg, Gro Pep, Adeilade SA, Australia) had to be found. First the antibody-label combination was found, using only antibody and biotin-labeled GH. Afterwards the most suitable antibody-label-standard GH combination was found, using the protocol (TR-FIA for GH, 18.03.2013).
Following antibody-label-standard combinations where tested:

a) Ab 1:8000, Label 50 ng/ml, standard 0.8-100 ng/well
b) Ab 1:8000, Label 70 ng/ml, standard 0.006-1.6 ng/well
c) Ab 1:8000, Label 50 ng/ml, standard 0.05-6.4 ng/well

There were concluded with that combination b) was the most suited one. This both because it showed a very smooth standard curve, with an R^2 value of 0.97. And also due to previous studies (Pottinger et al., 2003, Shimizu et al., 2009), it was believed that the plasma GH concentration would be within the range of standard curve b), which would be (0.3 – 80 ng/ml).

Measuring sample plasma GH

The immunoassay with sample plasma could now be run, using the protocol written by Kaneko. The calculations and amounts of the different substances used can be seen in appendix 2.1. All samples were made in duplicates.

The DELFIA Anti-Rabbit IgG coated 96 well Microtitration plate, was put on ice and washed with 200 µl DELFIA wash buffer (1x) (Turku, Finland). The DELFIA wash buffer where made by diluting 25X DELFIA wash buffer with ddH_2O. The plate was tamped upside down on paper towels to get rid of wash buffer. Thereafter 100 µl DELFIA Assay buffer (Turku, Finland) to all wells except BG. Nine serial dilution was made of the standard GH (0.006 – 1.6 ng/well), in duplicates. Thereafter 20 µl control plasma was added to control well in duplicate. There after 20 µl sample plasma was added to appropriate sample wells, in duplicates. Two empty wells were also used as background wells (BG), to control back ground count. BG was kept empty until day 3, when enhancement solution was added. Thereafter 1:8000 antibody was added to all wells except BG and the plate was stored at 4°C 600 rpm (MS 1 Minishaker, IKA – Werke GmbH & Co, Staufen im Breisgau, Germany), in dark overnight. Next day, after centrifugation at 4°C 1 min at 3000 rpm (Eppendorf sentrifuge 5810R, Hamburg, Germany), biotin-labeled GH was added. Thereafter the plate re-covered and shaken overnight (dark). The following day after centrifugation and plate wash, DELFIA Avidin-EU (Turku, Finland) was added and plate shaken at room temperature for 3 hours. After final washing, room temperate enhancement solution was added to all wells. The plate was read in VICTOR™ X5 2030

Multilabel Plate Reader (PerkinElmer, Waltham, MA, USA), using the software PerkinElmer 2030 Workstation, Europium protocol.

2.2.3 Proximate analysis

These analyses where done by technical staff at NIFES, except Immunoassay of IGF1 and IGFBP1b and plasma measuring of glucose. Immunoassay of IGF1 and measuring plasma glucose was done At the Univeristy of Arakansas by Prof. Christian Tipsmark. Plasma IGFBP1b was done at Hokkaido University by MSc-student Miki Pukada under supervision of Prof. Munetaka Shimizu.

Plasma IGF1 radioimmunoassay

The immunoassay of plasma IGF1 was conducted at the University of Arkansas, USA by Professor Christian K. Tipsmark. The measurement of plasma Igf1 was conducted by using salmon Igf1 as standard and tracer. Anti-barramundi Igf1 was used as primary antibody (GroPep Bioreagents Pty Ltd, Adelaide SA, Australia). Iodination of the hormone as well as the procedure for the radioimmonoassay followed the described protocol (Tipsmark et al., 2006). The total Igf1 from each plasma sample was acid ethanol extracted followed by neutralization. Thereafter the extracts were incubated with iodinated tracer and primary antibody overnight at 4°C. Next day the bound antigen were precipitated with secondary goat antirabbit IgG antibody and polyethylene glycol for 2 h (20°C), followed by centrifugation at 3.000 g for 60 min (4°C).

Time-resolved fluorescence immunoassay of plasma IGFBP1b

The method for this analysis has not yet been published. However the analysis was conducted at Hokkaido University, Sapporo Hokkaido, Japan by Msc Miki Pukada and Professor Munetaka Shimizu.

Plasma glucose

Plasma glucose was measured at the University of Arkansas, USA by Ass. Professor Christian K. Tippsmark. The plasma glucose concentration was measured using a glucometer (Clarity Plus, Diagnostic Test Group, Boca Raton, FL, USA).

Stored energy

Energy concentration was determined by following the NIFES procedure ”MET.NÆR.01-10, 096 – Energibestemmelse ved bombekalorimeter”. Energy content in whole fish and feed was measured using a bomb calorimeter (Parr calorimeter 6300 with a water circulating system 6520A, Parr Instrument Company, IL, USA).

Stored lipid

Whole fish total lipid: This analysis followed the Norwegian standard 9402 ”Atlantic Salmon – Measurement of Color and Fat”, first edition, 1994. This method is developed at NIFES, and at NIFES described as “MET.GRU. 01-02, 091 – Fett bestemmelse, etylacetat metode”. This method was used to measure total lipid content in whole fish. Since primarily only non-polar lipids are soluble in ethyl acetate. It is likely that not all polar lipids were detected.

Feed: For this analysis an acid hydrolysis method was used. The analysis was done according to the NIFES method “MET.GRU. 01-01, 083 – Fettbestemmelse ved syrehydrolyse”. This description is closely related to the Official Journal of the European Union, L15/28, 18.1.84, method B.

Stored protein

Crude-protein in whole fish and feed was analyzed by the Dumas method as described in the NIFES protocol "MET.GRU. 01-05, 171 – Råproteinbestemmelse ved hjelp av nitrogenanalysator". The nitrogen content was measured using a Vario Macro Cube instrument (Elementar, Hanau, Germany). By multiplying the nitrogen content with 6.25, crude protein content was found.

2.3 Calculations

Mean normalized expression (MNE)

The Ct values were exported to Microsoft excel 2007, where they were used to calculate mean normalized expression (MNE), using the program Microsoft Excel geNorm version 3.2. GeNorm uses an algorithm to find the most stable reference genes, and from this the MNE for the different genes can be calculated (Jo Vandesompele, 2002).

Calculating GH concentration from immunoassay

Microsoft excel 2007 where used to do the calculation of standard curves. In exel the plate counts was transformed into logarithmic values, to get a linear standard curve. A standard curve was made by taking the average of all standard counts from all plates containing sample GH. The equation from this curve was used to calculate the GH concentration (ng/ml), in the samples.

Productive values

Retention of lipid, protein and energy were calculated as protein productive value (PPV), lipid productive value (LPV) and energy productive value (EPV). The productive values are calculated on tank level, not individual level. Formula 2.1 shows the calculation off PPV (LPV and EPV are equivalent).

2.1) $$PPV = \frac{(B2-B1)}{I}$$

Where:

B1 = Protein content in the whole fish at start
B2 = Protein content in the whole fish at the end
I = Eaten protein

Organ indexes

Organ indexes of heart (cardio somatic index (CSI), liver (hepato somatic index (HSI) and viscera (visceral somatic index (VSI)) were calculated as follow:

2.2) $$Organ\ index = \frac{weight\ of\ organ\ (g)}{total\ weight\ of\ fish\ (g)} * 100\ \%$$

Condition factor

Condition factor (K), was found as:

2.3) $$K = \frac{w}{L^3} * 100$$

Where:

w = whole fish weight (g)
L = Fork length (cm)

Specific growth rate

SGR was found by using formula 2.4:

2.4) $$\text{SGR} = \frac{\ln(w2) - \ln(w1)}{d} * 100$$Where:

w1 = weight in period 1 (sampling 1)

w2 = weight in period 2 (sampling 2)

d = days between period 1 and 2

In this experiment there were 60 days between sampling 1 and 2, and 49 days between sampling 2 and 3.

Nutritional loss during starvation

Finding protein-, lipid- and energy loss during starvation in 19°C starved fish was done as described in formula 2.5:

2.5) $$\text{Nutritional loss} = \frac{\text{N1} - \text{N2}}{w1 - w2}$$

Where:

w1 = weight (g), in period 1 (sampling 1)

w2 = weight (g), in period 2 (sampling 2)

N1 = g nutrient (protein, lipid) pr fish in period 1 (sampling 1)

N2 = g nutrient (protein, lipid) pr fish in period 2 (sampling 2)

N1 and N2 for energy was in kJ, not gram

Weight gain

Weight gain was calculated according to formula 2.6

2.6) Weight gain = weight p2 – weight p1

Where:

Weight p2 = weight (g) in period 2 (i.e. trial period)

Weight p1 = weight (g) in period 1 (i.e. start of experiment)

Length gain

Length gain was calculated according to formula 2.7

2.7) Length gain = length (p2) – length (p1)

Where:

Length (p2) = Fork length (cm), in period 2 (i.e. trial period)

Length (p1) = fork length (cm), in period 1 (i.e. start of experiment)

Feed conversion rate (FCR)

FCR was found by formula 2.8:

2.8) $$\mathrm{FCR} = \frac{\text{feed intake}}{weight\ gain}$$

Where:

Feed intake = mean feed intake in g/period (i.e trial period)

Weight gain = mean weight gain in g/period

2.4 Statistical analysis

The statistical analyses were carried out using the statistical software R 2.15.2. Graphs and plots where made in Microsoft Excel 2007/GraphPad Prism 6. All values are presented as mean ± standard error (SE). A 95% confidence interval was used for all tests. One-way ANOVA on the mean of tank level was used for all data on phenotypical parameters. For individual fish analyses (plasma hormones and muscle genes, a linear mixed effect model (lme), was used, with the tanks as random variables. For all results, the treatment (13°C fed, 19°C starved and 19°C fed), was used as predictor variables. If there were found significant differences, a general linear hypothesis model (glht), Tukeys post-hoc test was used to analyze significant differences between the group means. To check if the data had homogeneity in variance a visual plot plotting variable vs. predictor was used. Since homogeneity in variance is more important than normal distribution and the underlying distribution in theory should be normal distributed, data were only transformed if it lead to more homogeneity in variance. If necessary the data was log transformed. Following datasets were transformed:

- All gene expression in white muscle
- IGFBP1b in plasma (trial)

For correlations, GraphPad Prism 6 was used, using a non-parametric correlation (Spearman's rank correlation), with a two tailed p-value. Individual correlation was used when possible, if not mean correlation on tank level was used.

3.0 Results

3.1 Feed intake

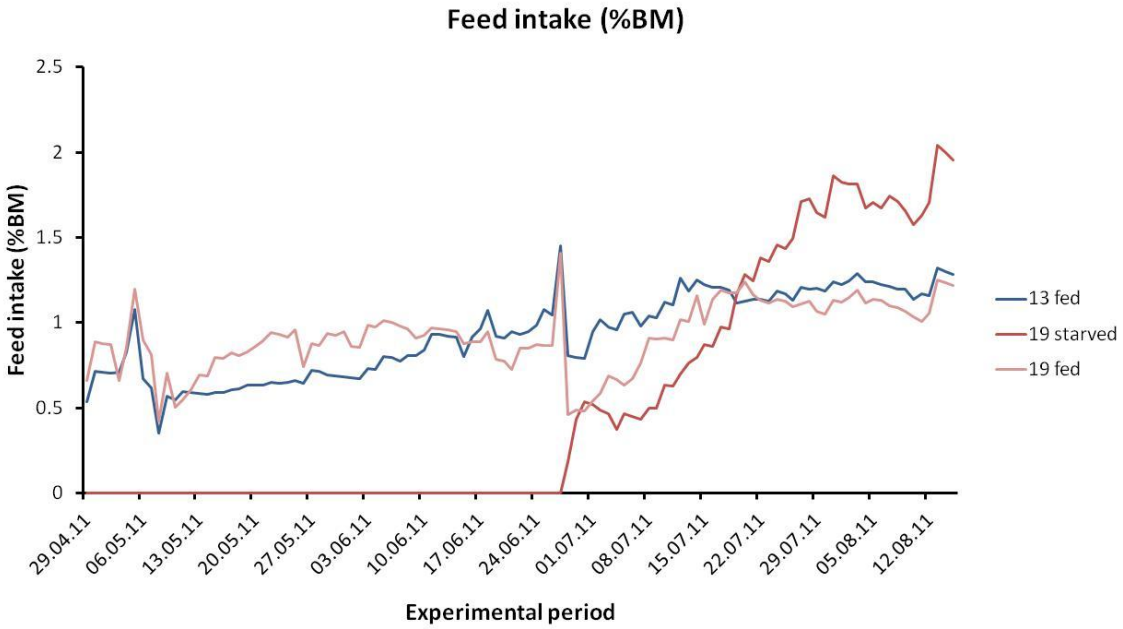

Figure 3.1: Daily feed intake shown as % of bio mass (BM), shown on treatment level (not tank level).

Table 3.1: Showing mean daily feed intake as % ± SE of body mass (BM) during trial (60 days),- recovery (49 days),- and whole experimental period (109 days). n = 3. Significant differences are indicated with lower case letters.

Period	13 fed	19 starved	19 fed
Trial period	0.76 ± 0.02^{b}	0	0.86 ± 0.02^{a}
Recovery period	1.14 ± 0.02^{a}	1.19 ± 0.08^{a}	1.00 ± 0.03^{b}
Whole period	1.9 ± 0.02^{a}	1.19 ± 0.07^{b}	1.86 ± 0.02^{a}

In feed intake as % of body mass, no differences were seen between the fed groups during whole experimental period (figure 3.1 and table 3.1). Trial period showed a significant higher intake in 19°C fed fish compared to 13°C fed ($p=0.024$), (table 3.1). During recovery period 19°C fed had significant lower feed intake than all other groups ($p<0.017$) (table 3.1).

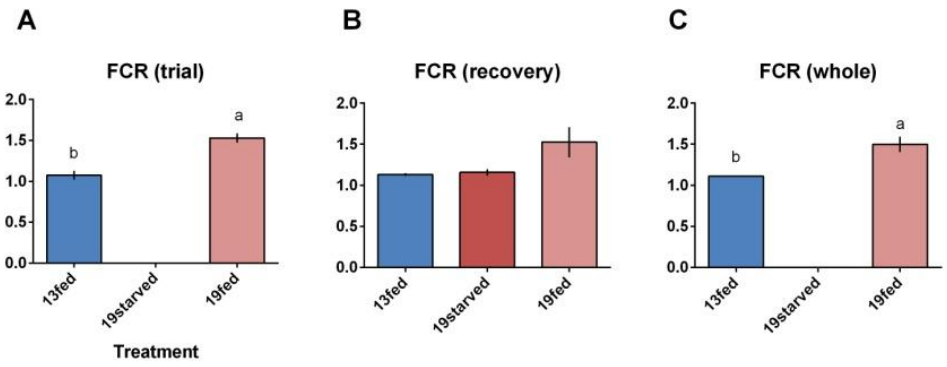

Figure 3.2: Feed conversion ratio during trial period (A), recovery (B) and during whole experimental period (C). n = 3. Data is presented as mean with ± SE. Significant differences are indicated with lower case letters.

The feed conversion ratio (FCR) during the trial period was significantly higher in 19°C fed compared to 13°C fed ($p = 0.005$) (figure 3.2 A). During recovery, 19°C fed had tendencies to higher ratio than 13°C fed ($p = 0.093$) (figure 3.2 B). FCR during whole experiment had higher ratio in 19°C fed fish compared to 13°C fed fish ($p<0.02$) (figure 3.2 C).

3.2 Whole fish analyses

3.2.1 Growth and development

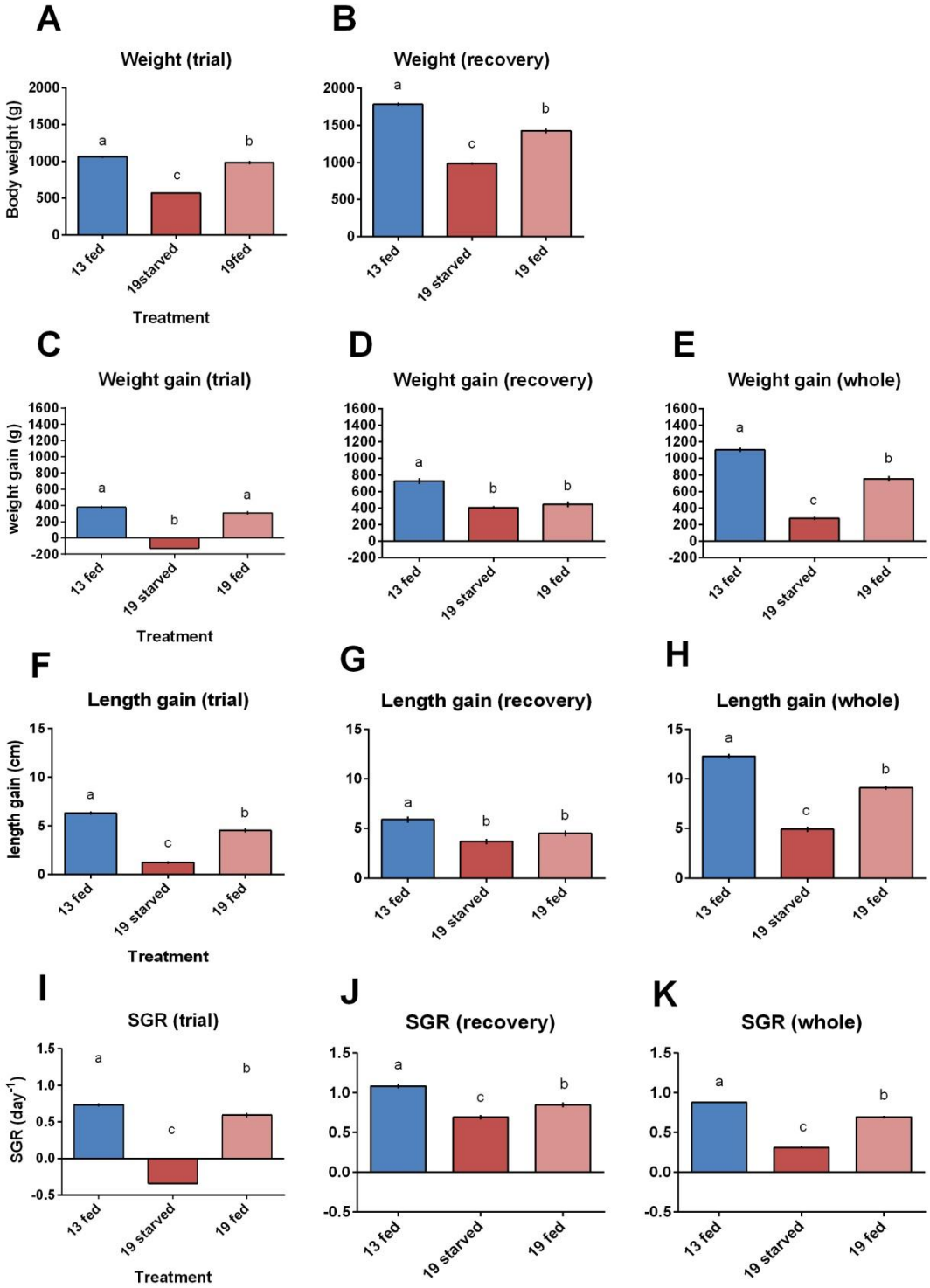

Figure 3.3: (A-B) Showing weight (g), after trial and recovery period. Weight gain during the trial (60 days) (**C**), during recovery (49 days) (**D**) and during whole experiment (109 days) (**E**). Length gain (cm), during trial (60 days) (F), recovery (49 days) (**G**), and during whole experimental period (109 days) (**H**). Mean specific growth rate (SGR) during trial (60 days) (**I**), recovery (49 days) (**J**), and during whole experimental period (109 days) (**K**). n = 3, and data is presented as mean with ± SE. Significant differences are indicated with lower case letters.

During the trial period the mean body weight was significantly reduced in the 19°C starved fish compared to 13°C fed and 19°C fed fish ($p<0.001$). The 19°C fed fish had a lower body weight than fed fish reared at 13°C ($p<0.025$) (figure 3.3 A). After the recovery period the starved fish at 19°C was significant lighter than 13°C and 19°C fed fish ($p<0.003$), (figure 3.3 B).

The weight gain was significantly higher in fed fish compared to starved fish during the trial period ($p<0.001$). Also 13°C fed fish showed tendencies to higher weight gain than 19°C fed fish ($p=0.069$) (figure 3.3 C). During recovery the 13°C fed fish had significantly higher weight gain than 19°C fed fish ($p<0.005$) (figure 3.3 D). Weight gain during the whole experiment was significantly higher in 13°C fish than 19°C fish ($p<0.005$). Also 19°C fed is higher than 19°C starved ($p=0.001$) (figure 3.3 E).

Length gain during the trial period was lower in 19°C fish compared to 13°C fish ($p<0.001$). Also 19°C fed had higher length gain than 19°C starved ($p<0.001$) (figure 3.3 F). Recovery showed higher length gain in 13 fed°C fed fish compared to 19°C fish ($p<0.006$). 19°C fed fish showed tendencies to higher gain than starved fish ($p=0.055$) (figure 3.3 G). Length gain during the whole period had lowest gain in 19°C starved fish ($p<0.001$), and 19°C fed fish lower than fish at 13°C ($p<0.001$) (figure 3.3 H).

Specific growth rate (SGR) in fish starved at 19°C was lower compared to the other during trial period (60 days) ($p<0.01$). Also, 19°C fed was significantly lower than fish at 13°C ($p<0.035$) (figure 3.3 I). During the recovery period (49 days), SGR was higher in fish at 13°C compared to 19°C ($p<0.004$). Also 19°C starved was lower than 19°C fed ($p=0.001$) (figure 3.3 J). During the whole experiment there were the same pattern, 13°C fish was significant higher than 19°C fish ($p<0.004$), and 19°C fed fish was higher than 19°C starved fish ($p<0.001$) (figure 3.3 K).

3.2.2 Metabolic state

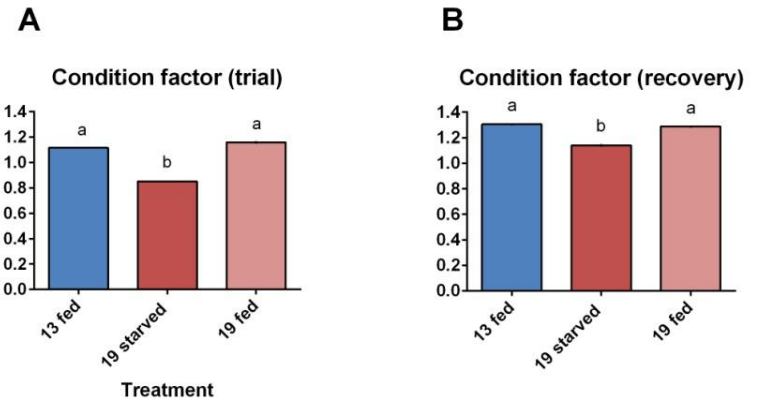

Figure 3.4 A-B Condition factor after trial period (A) and after recovery (B). Data is presented as mean with ± SE (n=3). Significant differences are indicated with lower case letters.

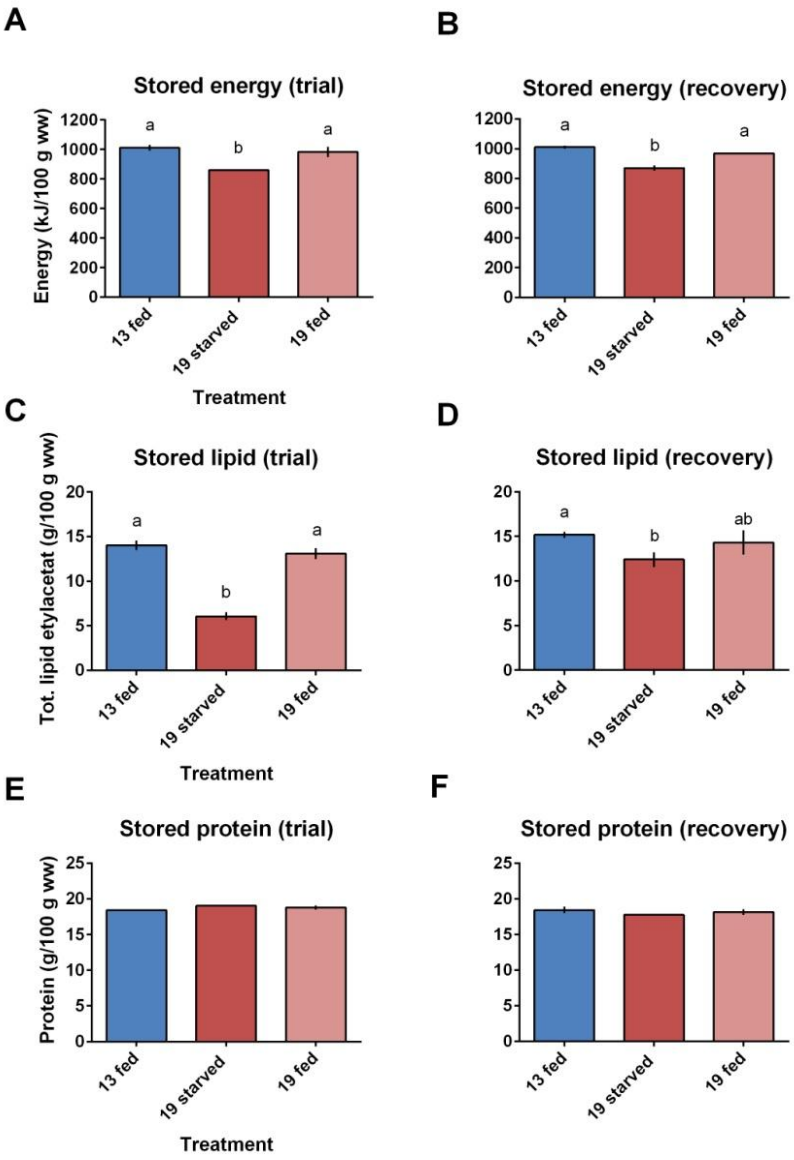

Figure 3.5: Stored energy and nutrient composition in whole fish. Energy stored in the fish after respectively trial (A), and recovery (B). Stored lipid after trial and recovery period (C and D). E (trial) and F (recovery) shows stored protein in whole fish. n = 3, and data is presented as mean with ± SE. Significant differences are indicated with lower case letters. Only whole fish analyses

Table 3.2: Showing stored energy and nutrients during trial and recovery period

NUTRIENT CONTENT			
	13 fed	19 starved	19 fed
energy kJ/ 100g ww (trial)	1011 ± 19[a]	861 ± 2[b]	983 ± 34[a]
energy kJ/ 100g ww (recovery)	1011 ± 10[a]	871 ± 17[b]	970 ± 3[a]
Lipid g/ 100g ww (trial)	14.03 ± 0.44[a]	6.07 ± 0.35[b]	13.10 ± 0.50[a]
Lipid g/ 100g ww (recovery)	15.17 ± 0.16[a]	12.40 ± 0.39[b]	14.30 ± 0.63[ab]
Protein g/ 100g ww (trial)	18.43 ± 0.09	19.03 ± 0.09	18.80 ± 0.30
Protein g/ 100g ww (recovery)	18.43 ± 0.26	17.73 ± 0.03	18.13 ± 0.24

After the trial period (figure 3.4 A), the condition factor in 19°C starved fish was significantly lower than the other groups ($p<0.001$). During the recovery period the condition factor increased in all groups, but still there was a significantly lower condition factor in 19°C starved fish compared to the other groups ($p>0.001$). The condition factor in the 13°C fed and 19°C fed fish was similar (figure 3.4 B).

Total stored energy concentration was significantly lower in fish starved at 19°C compared to the other groups after trial and recovery period (figure 3.5 A-B, table 3.2).

It was significant less stored lipid after trial period in fish starved at 19°C compared to the other groups ($p<0.01$). There was 8 g (100g ww (57%), more lipids in fish fed at 13°C than 19°C starved fish after trial (figure 3.5 C). After recovery period the 19°C starved fish was only significant lower than 13°C fed fish ($p=0.044$) (figure 3.5 D) (table 3.2).

Stored protein was not different between group neither after trial- or recovery period (figure 3.5 E-F) (table 3.2).

The hepato somatic index (HSI) was significantly lower in starved fish compared to fish fed normal (13°C and 19°C fed), $p<0.04$, during trial period (figure 3.6 A). After recovery, there were no differences between groups (figure 3.6 B).

Viscera somatic index (VSI), was significant lower in 19°C starved fish compared to other groups after trial period ($p<0.004$) (figure 3.6 C). After recovery, no differences were seen (figure 3.6 D).

Cardio-somatic index showed no significant differences between the treatment groups neither after trial or recovery period (figure 3.6 E-F).

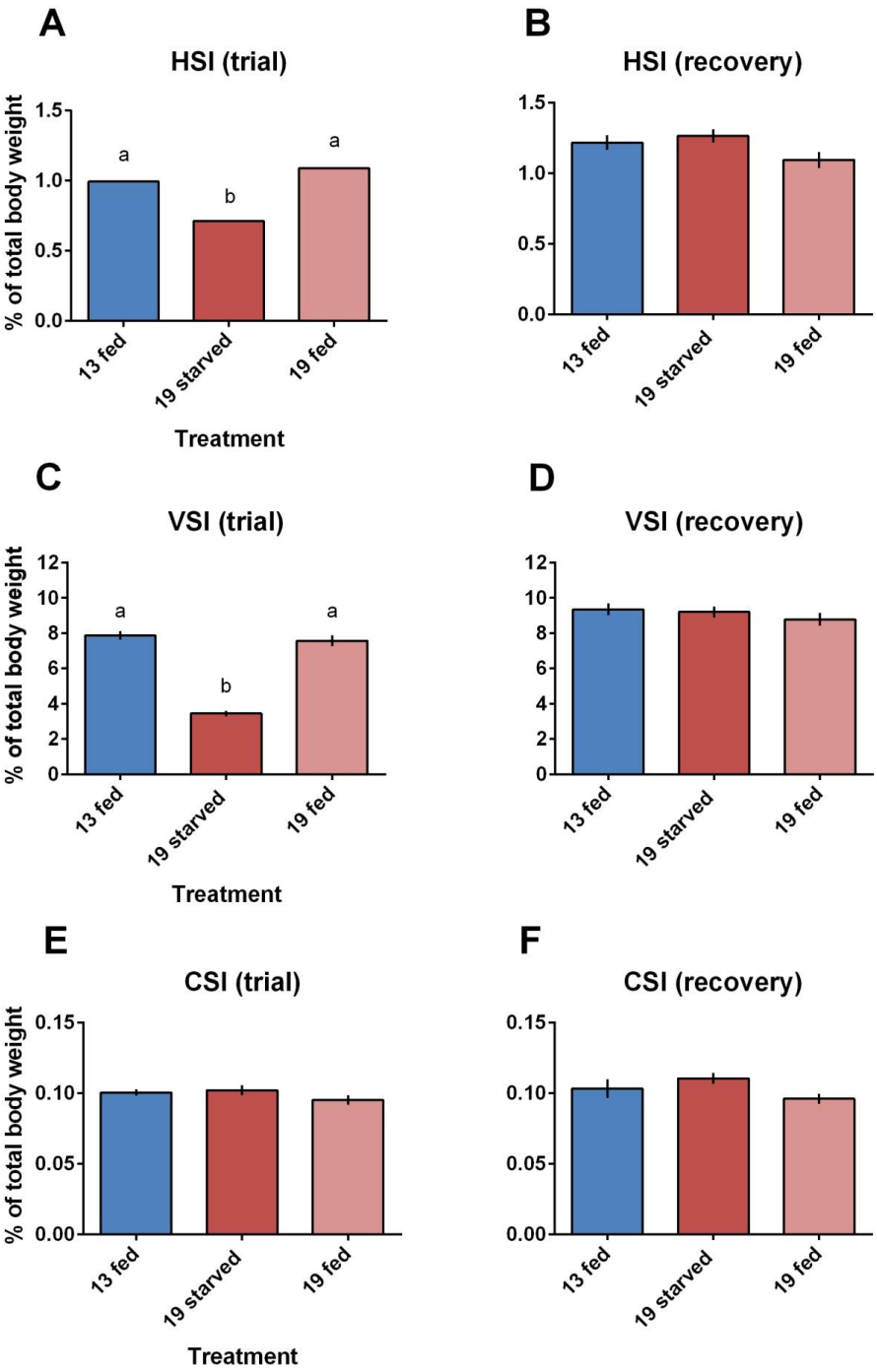

Figure 3.6: Hepato-somatic index (HSI) is seen after trial period (**A**), and after recovery period (**B**). Figure **C** and **D** shows viscera-somatic index (VSI) after respectively trial and recovery. Cardio somatic index (CSI), is presented for trial and recovery in figure **E** and **F**. n = 3, and data is presented as mean with ± SE. Significant differences are indicated with lower case letters.

3.2.3 Nutritional productivity and loss:

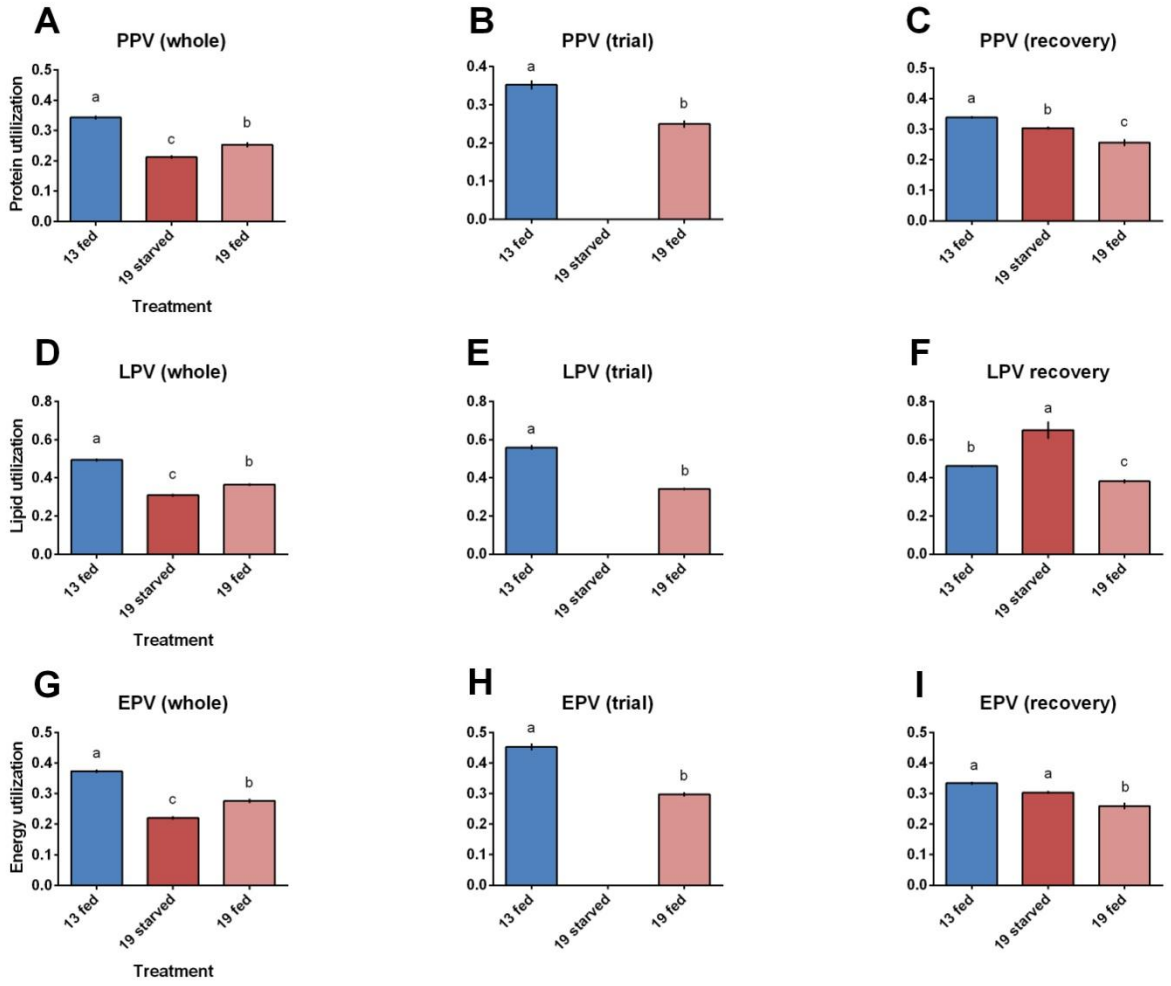

Figure 3.7 A-C: Protein productive values (PPV), during whole experiment, trial and recovery periods. **D-F**: Lipid productive values (LPV) during whole experiment, trial and recovery periods. **G-I**: Energy productive values (EPV), during whole experiment, trial and recovery period. n = 3. Significant differences are indicated with lower case letters.

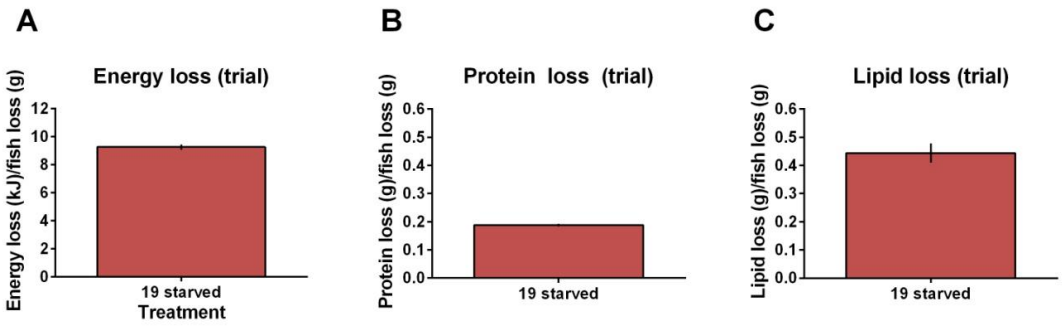

Figure 3.8: Showing energy loss (kJ/g weight loss) (**A**). Protein loss (g/g weight loss) (**B**) and lipid loss (g/g weight loss) (**C**) in 19°C starved fish during trial period. n= 3.

Protein productive values (PPV), during whole experiment, was significant higher in 13°C fish compared to 19°C fed fish ($p<0.001$). Also starved fish was significant lower than 13°C fish ($p<0.001$), and 19°C fed fish ($p=0.013$) (figure 3.7 A). PPV during trial period, was significant lower in 19°C fed fish compared to 13°C fed fish ($p=0.003$) (figure 3.7 B). In recovery period it was a significant higher productivity in 13°C fed fish compared to 19°C fed ($p<0.001$) and 19°C starved ($p=0.039$). Also 19°C starved was significant higher than 19°C fed fish ($p=0.007$) (figure 3.7 C).

Lipid productive values (LPV) during whole experiment, was a significant higher in 13°C fed fish than all other groups ($p<0.002$). 19°C fed fish was significant higher than 19°C starved fish ($p=0.003$) (figure 3.7 D). During trial period there were a significant higher productivity in fish at 13°C compared to 19°C fed fish ($p<0.001$) (figure 3.7 E).Recovery period was a significant higher in 19°C starved fish compared to other groups ($p<0.005$). Also 13°C fed fish was significant higher than 19°C fed fish ($p=0.002$) (figure 3.7 F).

Energy productivity (EPV) during whole experiment was a significantly higher in fish at 13°C compared to 19°C ($p<0.001$). 19°C fed fish had significant higher productivity than 19°C starved fish ($p=0.001$) (figure 3.7 G). During trial period there were a significant higher productivity in fish at 13°C compared to 19°C fed fish ($p<0.001$) (figure 3.7 H). There were a significant lower productivity in 19°C fed compared to other groups ($p<0.005$) during recovery (figure 3.7 I).

The energy loss during trial period in 19°C starved fish was on average 9.38 kJ for every weight gram the fish lost (3.8 A). Protein loss in the same period was on average 0.19 g for every gram fish loss, (figure 3.8 B). The lipid loss was on average of 0.44 g for every gram of fish loss, (figure 3.8 C)

3.3 Plasma

3.3.1 Hormones in plasma

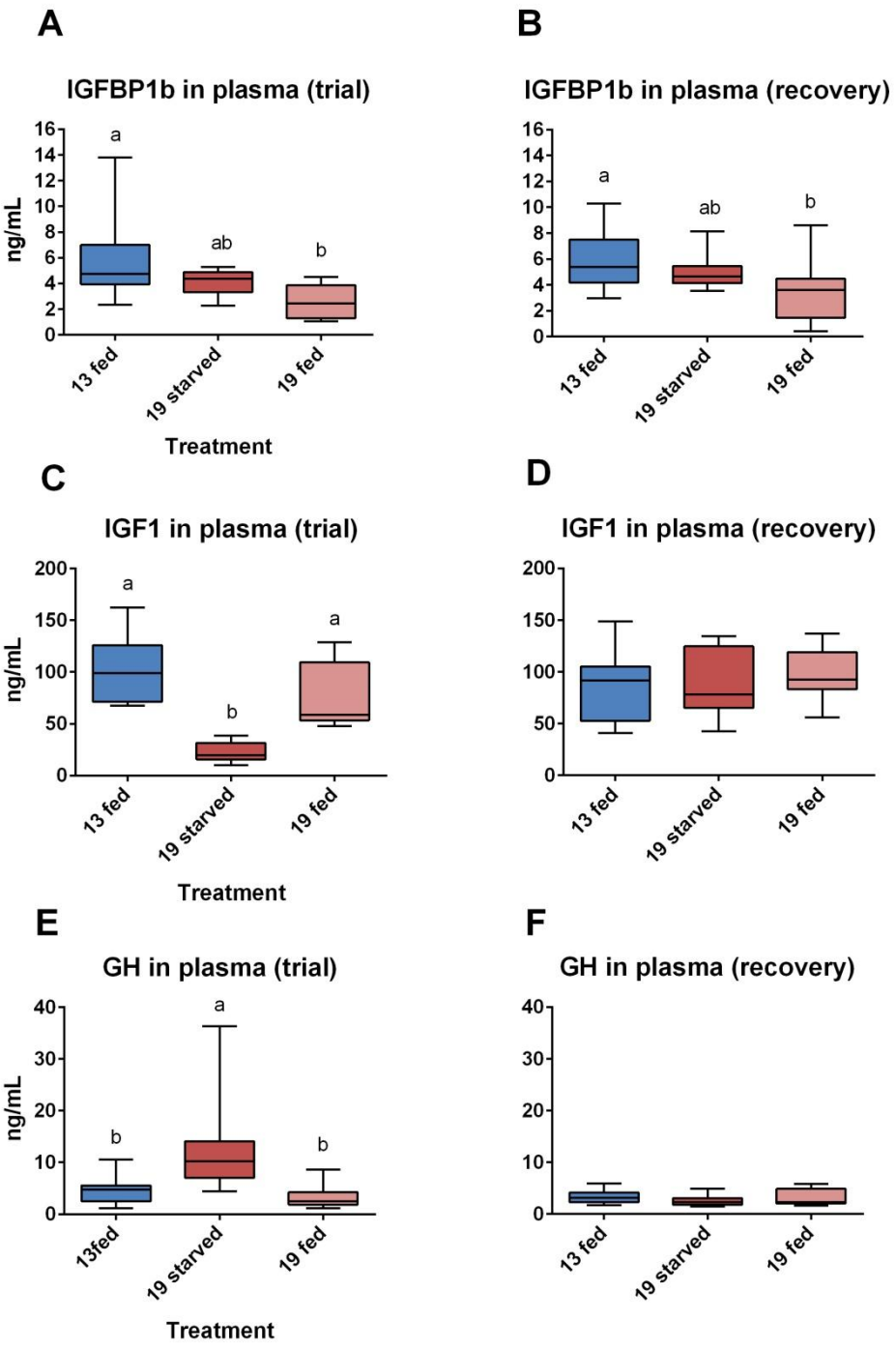

Figure 3.9: Showing hormones in plasma. Concentration of IGFBP1 is seen in figure **A** (trial), and **B** (recovery). **C-D** shows concentration of IGF1 after trial- and recovery. Figure **E-F** shows GH concentration after trial and recovery. Data is presented as range with mean (n=3). Significant differences are indicated with lower case letters

Insulin-like growth factor binding protein 1b (IGFBP1b), concentration in plasma after trial period, was significantly higher in 13°C fed fish compared to 19°C fed fish (p=0.0124), (figure 3.9 A). after recovery the IGFBP1b concentration 13°C fed fish is significant higher than 19°C fed fish (p=0.046) (figure 3.9 B).

Insulin-like growth factor 1 (IGF1), after trial period had a significant lower value in 19°C starved fish, compared to all other groups (p<0.001) (figure 3.9 C). After recovery there were no significant differences (figure 3.9 D)

Growth hormone (GH) in plasma after trial had a significant higher value in. 19°C starved fish compared to fed fish (p≤0.02) (figure 3.9 E). After recovery there were no differences between the groups (figure 3.9 F).

3.3.2 Glucose in plasma

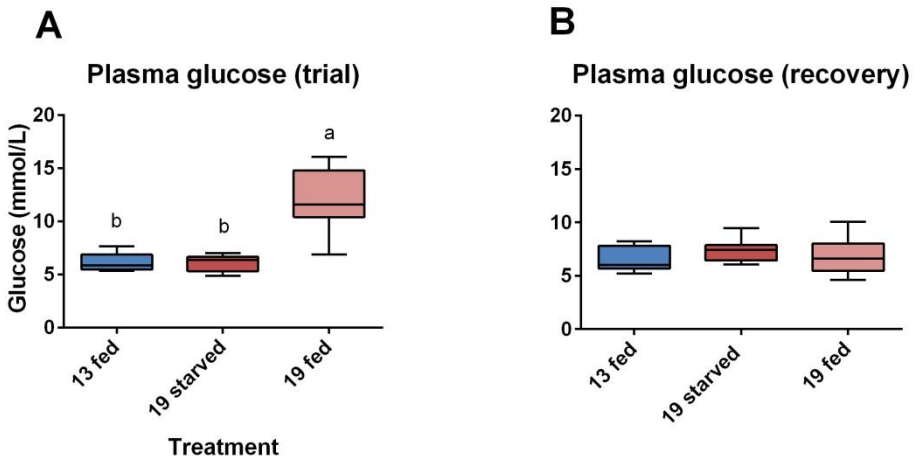

Figure 3.10: Showing plasma glucose levels after trial (**A**) and recovery (**B**) in plasma. Data is presented as range with mean (n=3). Significant differences are indicated with lower case letters.

Plasma glucose concentrations after the trial period was significantly higher in 19°C fed fish compared to all other groups (p<0.001) (figure 3.10 A). After recovery no significant differences were seen between groups (figure 3.10 B).

3.4 White muscle

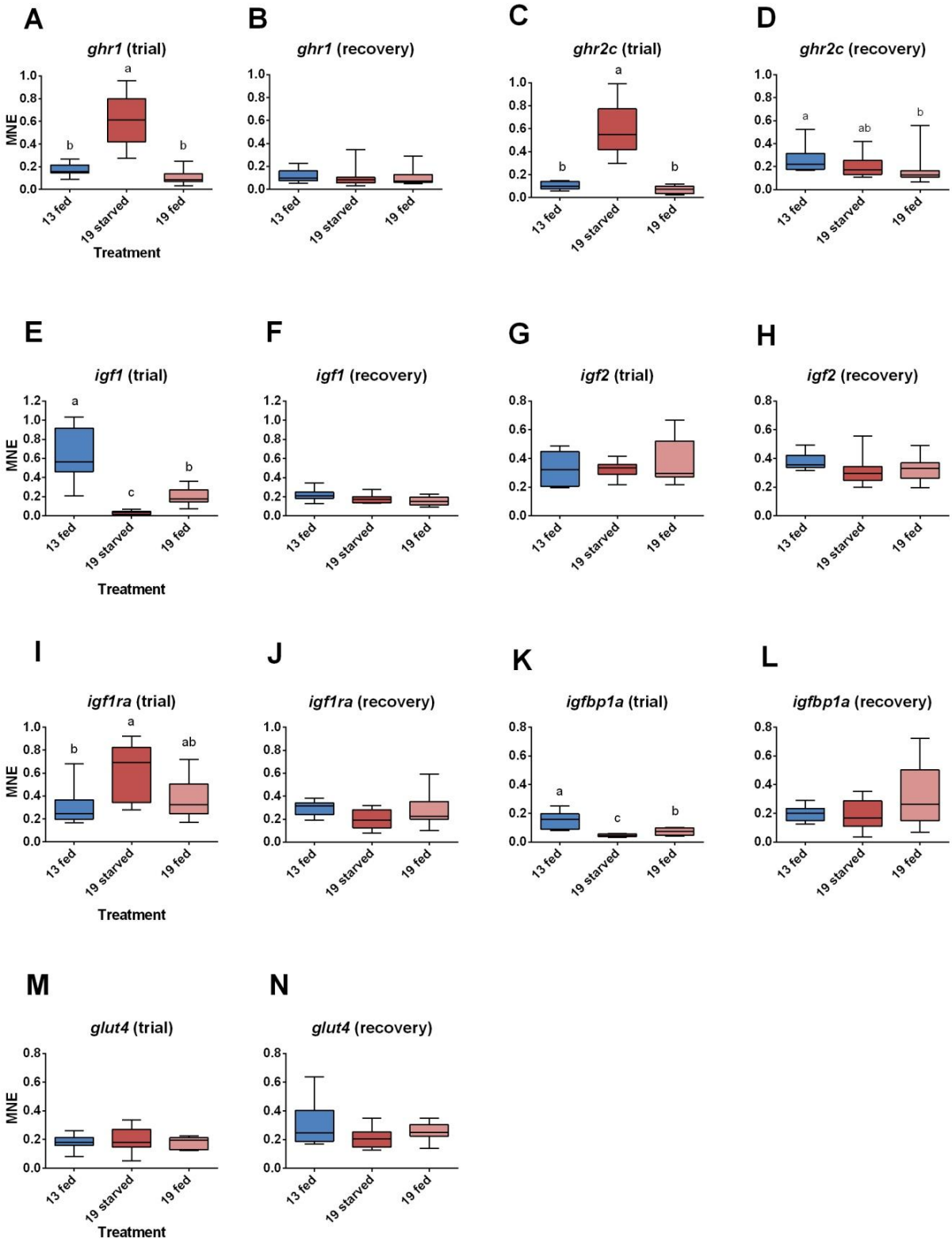

Figure 3.11 A-N: Expression of genes in white muscle after trial- and recovery period. Data is presented as range with mean (n=3). Significant differences are indicated with lower case letters.

3.4.1 Gene expression

Growth hormone receptor 1 was significantly higher expressed in 19°C starved fish compared to the two other groups during trial period ($p<0.001$). There was also tendency to higher expression in 13°C fed compared to 19°C fed ($p=0.076$) (figure 3.11 A). After the recovery period, there were no differences between groups (figure 3.11 B).

Growth hormone receptor 2c was significantly higher expressed in 19°C starved fish compared to all other groups ($p<0.001$) (figure 3.11 C). Recovery had significant higher expression in 13°C fed fish compared to 19°C fed fish ($p=0.043$) (figure 3.11 D)

After the trial period insulin-like growth factor1 *(igf1),* was significantly higher expressed in13°C fed compared to 19°C fish ($p<0.002$). 19°C starved fish was significant lower than 19°C during trial ($p<0.001$) (figure 3.11 E). After recovery there were no differences between groups (figure 3.11 F).

Insulin-like growth factor 2 (Igf2), expressed no significant differences between groups neither after trial (figure 3.11 G), nor recovery (figure 3.11 H).

After trial period *Igf1 receptor A (igf1ra)*, was significant higher expressed in 19°C starved fish compared to 13°C fed fish ($p=0.003$) (figure 3.11 I). Recovery showed no differences between groups (figure 3.11 J).

Igf binding protein 1 a (igfbp1a), had significant higher expression in 13°C fed fish compared to all other groups during trial ($p<0.009$). 19°C starved fish had significant lower expression than 19°C fed fish ($p<0.02$) (figure 3.11 K). There were no differences were seen between groups after recovery (figure 3.11 L).

Glut4 expression were not significant different between groups neither after trial (figure 3.11 M), nor after recovery (figure 3.11 N).

4.0 Discussion

4.1 Summary of results

Fish fed at 19°C had a higher feed intake than 13°C fed fish during trial period, however there were higher FCR and lower energy- and nutrient retention. The hormones had lower IGFBP1b levels, and plasma glucose was elevated. Muscle genes had down regulated *igf1* and *igfbp1a* expression. Weight and growth showed tendencies to reduction. However CF, stored energy, nutrients as well as somatic indexes remained normal during trial period. During recovery feed intake was lowered in fish previous fed at 19°C compared to 13°C fed fish. There were tendencies to higher FCR as well as lower energy and nutrient retention. IGFBP1b in plasma was still down regulated, but most of the hormones and genes had normal levels. However weight and growth rate remained low during recovery. CF, stored energy, nutrient and somatic indexes remained normal.

During trial period 19°C starved fish compared to 19°C fed fish, had up regulated plasma concentrations of GH, also *ghr1* and *ghr2* expression in muscle were up regulated. There were down regulated concentrations of plasma IGF1, as well as *igf1* expression in muscle. Also weight decreased, and there were mostly seen a negative growth rate. CF, stored energy, stored lipid, HSI and VSI were lowered, while stored protein did not change. During recovery there were a significant higher feed intake in previous starved fish compared to fish previous fed at 19°C, also there were tendencies to lower FCR and higher retention of energy and nutrients. The hormonal and genetic parameters did not significant differ from 19°C fed fish. However weight was still lower as well as SGR, but weight and length gain did not differ. CF, stored energy did neither fully recover. However stored lipid and protein as well as somatic indexes were normal.

4.2 Temperature

4.2.1 Feed intake

The total feed intake was higher in fish fed at 19°C compared to 13°C during the trial period. However, it appeared to decrease at the end of trial period. This pattern corresponds to previous studies which finds that feed intake increases the first weeks at elevated temperature in Atlantic salmon (18-19°C), but subsequently decreases (Handeland et al., 2008, Hevrøy et al., 2013, Kullgren et al., 2013). Kullgren et al., (2013), suggests that the decrease in appetite may be due to higher levels of leptin (has anorexigenic functions) at elevated temperatures. However (Hevrøy et al., 2013) suggests that since digestion itself consumes a lot of energy, it may be a survival strategy to reduce feed intake at elevated temperature. This is a likely explanation, as energy required for basal metabolism increase with temperature, and by decreasing energy used for digestion, one can save energy for non-metabolic functions like swimming and growth. In a natural environment there would have been lower amounts dissolved oxygen, and by decreasing feed intake, less oxygen is needed for digestion. This is especially important when temperature approach the upper thermal limits. Also Hevrøy et al., (2012), suggests that reduction of circulating ghrelin may be a method to reduce voluntary food intake under unfavorable conditions, to increase the aerobic scope. However, this does not explain the high feed intake during the first weeks of feeding at 19°C. Literature explains that during acclimatization to higher temperatures, two different phases are seen. First there is seen a over/undershoot in physiological possesses, thereafter a gradually stabilization. This is due to

adaptive changes in cellular enzymes (Parvatheswararao, 1968). Kullgren et al., (2013), found that many physiological and endocrine signals that affect food intake and growth are affected when post-smolt is acclimatized to 18°C. In the present study, the FCR was higher in the fish reared at 19°C. Showing a lower feed utilization at high temperature. This should not be surprising since at elevated temperatures the metabolic costs will increase, and less ingested energy will be used for growth.

During the first weeks of the recovery period, the feed intake was lower the in 19°C fed fish compared to fish at 13°C. This has likely to do with over/undershooting of physiological processes when acclimatizing to lower temperature, as also was the case when temperature increased during trial period. The fish was also under stress due to previous exposure to high temperature, and following sampling. All this stress may have lead to lower feed intake during first part of recovery period. There were also seen tendencies to higher FCR in fish previous fed at 19°C.

4.2.2 Growth and hormones

Temperature seemingly did not have any major effect on the endocrinological growth regulatory system. This is indicated by normal plasma GH and IGF1, and normal muscle expression of *igf2* and *igf1ra* in muscle. There were also seen lower levels of plasma IGFBP1b. The normal GH and IGF1 plasma levels in 19°C fed fish in this experiment was probably due to the high feed intake. This is supported by (Beckman, 2011) who found that plasma IGF1 levels are changing proportionally with feed intake. In the current study the normal endocrinological expressions should indicate normal growth pattern during trial period. However, when looking at phenotypical parameters there were seen minor decrease in weight and growth rate at 19°C. This is mainly believed to be due to that fish fed at 19°C had down regulated *igf1* expression in muscle. (Hevrøy et al., 2012, Hevrøy et al., 2013), found reduced expression of *igf1* in muscle, while normal *igf1* expression in liver and plasma at high temperature. This indicates that the energy metabolism in muscle tissue is reacting different than the liver at elevated temperatures. Due to the tendencies of decreased feed intake at the end of trial period as well as higher FCR, there may be tendencies to insufficient energy available for growth. And since the metabolic rate is increasing with increasing temperature, as explained by (Jobling, 1994c), which will require more energy, less of the ingested energy will be available for growth. There were therefore a endocrinological change as a respond to this lower available energy, which were seen by lower expression of *igf1* in fish muscle, indicating that during periods of less feed, growth of vital organs like the heart is favored before muscle growth indicated by unchanged CSI, which also documented by (Hevrøy et al., 2013). This indicates that locally produced IGF1 is more important than plasma IGF1 in regulating muscle growth. Therefore it is no surprise that the phenotypical growth parameters like SGR and weight gain, which are strongly related to muscle growth, showed minor decreases at 19°C compared to 13°C. In conclusion, there was a higher feed intake in 19°C during trial period, however the metabolic costs at 19°C were so high, that less energy was available for growth. This was seen by lower weight and length growth at 19°C, showing that 19°C is above the optimal temperature for growth in Atlantic salmon, which corresponds to the findings of (Handeland et al., 2003, Hevrøy et al., 2012, Hevrøy et al., 2013, Kullgren et al., 2013, Øivind Bergh, 2007). A summary of the endocrinological regulation at high temperature is given in figure 4.1.

During recovery, plasma GH and IGF1, as well as muscle *igf1, igf2* and *igfbp1a* had normal levels. There were also decreased plasma levels of IGFBP1b. Interestingly, there were seen a down regulation of *ghr2* during recovery in fish previous fed at 19°C. This indicates that there were some functional differences between GHR1 and GHR2 receptors, which also is confirmed by (Fuentes et al.,

2013). In fact Gabillard et al., (2006), found that GHR1 and GHR2 are differently regulated at high temperatures in rainbow trout, and GHR1 plays a more important role in the growth promoting effects of temperature, since increasing at increased temperature in muscle. In the current study, the results indicate that neither of the receptors was directly affected by temperature. However, GHR2 was more affected by the repercussions of high temperature, when the fish was trying to recover. All in all the genetic regulation indicated normal growth during recovery in fish previously fed at 19°C. However, in our experiment 49 days recovery period was not long enough time for the fish fed at 19°C, to fully gain the same length and weight as fish at 13°C. The difference between endocrine growth regulation and phenotypical growth parameters seen during recovery, is likely due to that endocrine measurements were only conducted on 9 random fishes from each treatment, which not reflected the exact growth pattern of the total sample pool, see appendix 7.1 The reason for lack of phenotypical growth compensation were probably due to that there never were seen any major growth retardation in 19°C fed fish compared to 13°C fed fish during trial period, and thereby the compensatory growth mechanisms were not activated during recovery. The reason for lower growth rate in fish previously fed at 19°C compared to fish fed at 13°C, is suggested to be a combination of lower feed intake during recovery, acclimatization to lower temperature and recovery of the stressful conditions experienced during exposure to 19°C. In conclusion, the endocrine regulation indicated strong growth during recovery period. However, there were seen lower growth rate when looking at the phenotypical parameters, this mainly due to lower feed intake in fish previous fed at 19°C. Little compensation growth was experienced mainly due to little growth retardation during trial period.

4.2.3 Energy flow and hormones

Normal levels of plasma GH, IGF1, lower levels of plasma IGFBP1b, normal muscle expression of *igf2, ghr1, ghr2, igfbp1a and igf1ra* strongly indicated anabolic conditions, and little change in energy flow between fish fed at 19°C and 13°C. This was also supported by normal levels of stored energy, lipid and protein as well as HSI, VSI and CSI and CF. All these endocrinological and phenotypical parameters indicates that 19°C did not lead to mobilization of stored energy, and thereby not increased catabolic conditions. The normal CF found in our study at elevated temperature contradicts largely literature which finds decreasing condition factor at high temperatures (18°C), in Atlantic salmon Hevrøy et al., (2013), and Kullgren et al., (2013), found lower condition factor at 19°C after 45 days. Also Hevrøy et al., (2012) and Hevrøy et al., (2013), found less stored lipid at 19°C , and therefore conclude with use of endogenous lipids during prolonged periods at elevated temperature. The reason for more catabolic conditions found in their studies is likely due to that fish at 19°C had a lower feed intake during long periods compared to fish at lower temperatures. Therefore the reason for not increased catabolic conditions in our study is due to the high feed intake during trial period. However, at the end of trial period, tendencies to decreased feed intake were seen. It is likely therefore that if the trial period had lasted longer, there would have been seen a increase in mobilization of body reserves due to reduction in feed intake, as found in (Hevrøy et al., 2013, Kullgren et al., 2013). In fact in our study there were down regulated expression of *igf1* in muscle in 19°C fed fish, indicating a slightly less anabolic state in fish muscle at elevated temperature. This together with higher FCR, lower retention of nutrients as well as elevated levels of plasma glucose indicates that the fish at 19°C, needed a lot of energy at any time to sustain basal metabolism and thereby less of the ingested energy was available for growth. This finding is supported by Hevrøy et al., (2012) and Hevrøy et al., (2013), who also found decreasing retention with increasing temperature. Abnormal high levels of plasma glucose are often related to stress (secondary stress

response), due to the stress hormones adrenaline and cortisol, which increase glycolysis in liver (Hemre, 2001, Fox et al., 2006). However in current study HSI did not change due to temperature, indicating that there were not seen a major increase in glycolysis in the liver. Due to the lower PPV seen in fish fed at 19°C, it may be that much of the amino acids from feed intake were transformed into glucose, showing the high energy demand at 19°C. Other explanations for this high level of glucose in plasma may be that at elevated temperatures, already weak glucose uptake mechanisms into the cells were getting weaker, and there were even weaker response to insulin by the cells. However studies done on catfish (*Ictalurus melas* and *Ictalurus punctatus*), found higher glucose levels in fish at high temperature, but no change in insulin production, and suggests that elevated temperatures may somehow impair the glucose metabolism in plasma (Ottolenghi et al., 1995). This is in fact a likely explanation, since in current study, there were not seen any change in muscle *glut4* expression, which could be due to impairment of glucose metabolism. However the insulin regulation of *glut4* is tissue specific, and may not be strongly regulated by plasma glucose in white muscle, as suggested by (Capilla et al., 2002). In conclusion, there were generally seen a highly anabolic state in the fish at elevated temperature. However, there were tendencies to lower feed intake at the end of trial period, indicating that the fish may have been close to lose its capability of high feed intake. It is therefore believed that there would have been a more catabolic state if the trial period had lasted longer. Already there were tendencies to less anabolism in fish muscle due to down regulated *igf1* expression.

During the recovery period plasma GH and IGF1 had normal levels, but the IGFBP1b level was down regulated. Plasma glucose did fully recover to normal levels in fish previous fed at 19°C. Also gene expression in muscle was normal, except down regulated expression of *ghr2*. This indicates normal metabolism, shifted towards anabolism. This was supported by stored energy and nutrients, VSI and CSI, which had normal levels. There were therefore no significant increase in catabolism and mobilization of stored energy compared to 13°C fed fish during recovery. However the lower feed intake, lower nutrient retention and tendencies to higher FCR, indicated that the fish previously exposed to feeding at 19°C, still had not managed to fully recover, and was using much of ingested energy to sustain non-growth processes and recover from the stressful conditions previously experienced. In conclusion, our results indicate that the fish fed at elevated temperature got enough food to sustain metabolism and growth, and thereby did not need to mobilize its own reserves. This is supported by the high feed intake, nutrient composition and somatic indexes and normal GH levels in 19°C; none of these indicates catabolic conditions.

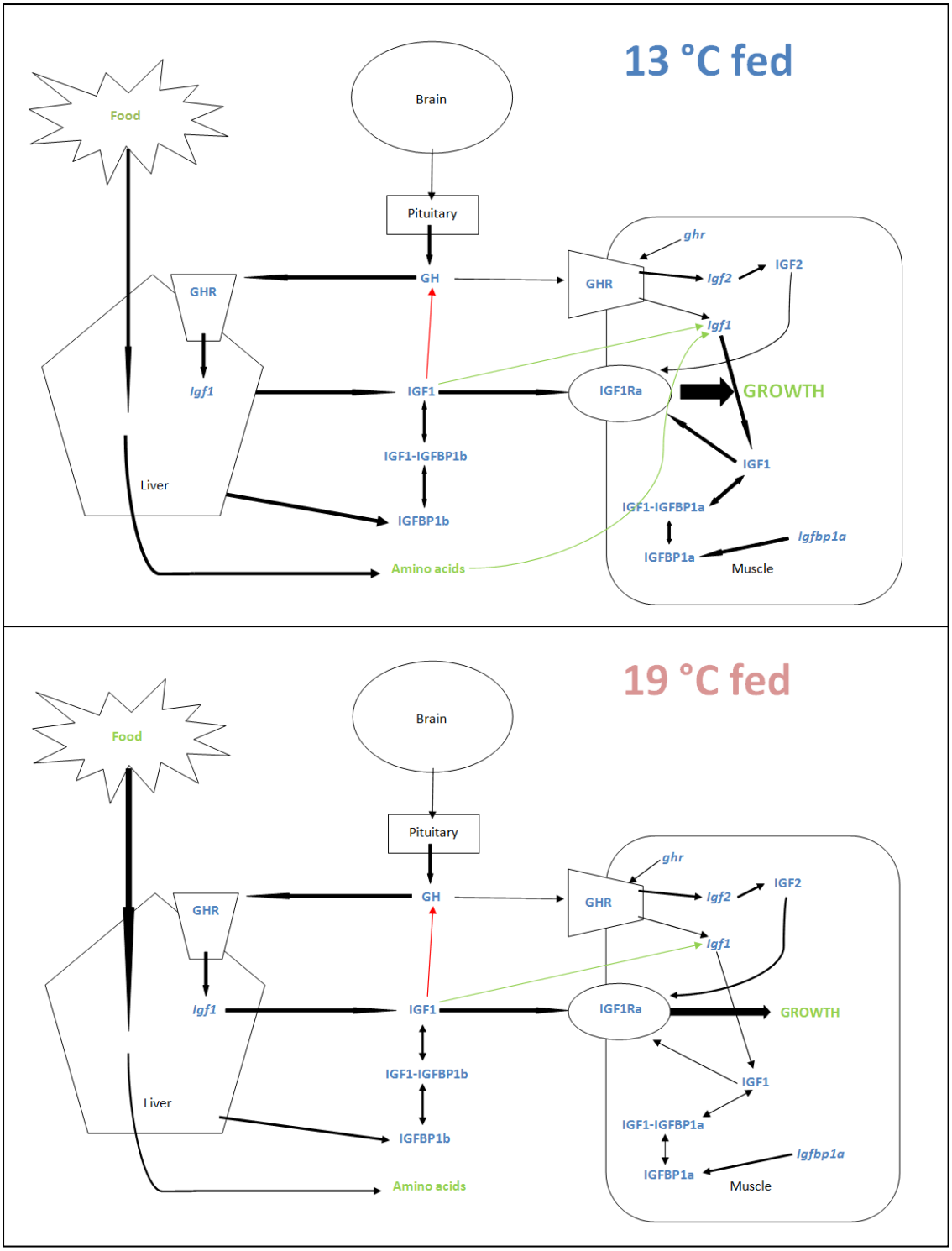

Figure 4.1: Showing the endocrine growth regulation during feeding at 13°C (top), and feeding at 19°C (bottom). Red arrows show negative feedback mechanisms, and green arrows show positive feedback. At 19°C, there was a higher feed intake, however due to the high temperature, less feed was available for growth. It is therefore believed that there was limited positive feedback from plasma amino acids on *igf1* expression in muscle, leading to less expression of *igf1* in muscle. Therefore there is seen lower expression of *igfbp1a* in muscle. The lower available energy for growth and lower expression of *igf1* in muscle at 19°C, leads to slightly lower growth compared to at 13°C.

4.3 Starvation

4.3.1 Feed intake

During trial period there was no feed intake in 19°C starved fish, thus quite different from 19°C fed fish, which had a very high feed intake during trial period.

During recovery period the starved fish showed quite impressing feed intake, and had significant higher feed intake as % BM than 19°C fed fish. However, like the 19°C fed fish, it took some weeks before the feed intake as % of BM, was really increasing. There is believed to be many reasons for this. Both acclimation to lower temperature (13°C), as well as exhaustion during exposure to high temperature, starvation and sampling. Also the digestion system had been partly degradated during starvation, and therefore during the first part of recovery the digestion system had to be rebuilt, before more food could be digested. Also the metabolism had been down regulated during starvation, and since there were some delay in down and up-regulation of metabolic rate after starvation, as explained by (Ali et al., 2003), it lead to lower feed intake in the beginning of re-feeding. After 23 days, the previously starved fish managed to get higher feed intake (%BM) than the fed group at 19°C. This is believed to be due to compensatory responses. The feed intake continued to increase during the rest of the recovery period. Also the starved fish tended to have lower FCR than the previous fed fish at 19°C.

4.3.2 Growth and hormones

During trial period the food deprivation in starved fish led to a higher excretion of GH into the plasma. This increased excretion started probably mainly due to increased ghrelin excretion into the blood from the stomach, which promoted excretion of GH from pituitary as explained by (Bar and Volkoff, 2012, Goldstein et al., 2012, Muller et al., 2002). There were lower levels of circulating plasma IGF1 as well as *igf1* expression in muscle during starvation. This may be due to that during starvation there can be a reduced GH sensitivity in some tissues, leading to lower expression of *igf1* in those tissues, as explained by (Beauloye et al., 2002, Norbeck et al., 2007). The GH mediated *igf1* expression is mediated through JAK-STAT signaling (Beauloye et al., 2002). The reduced expression of *igf1* during starvation was therefore likely due to impairment in the JAK-STAT signaling pathways when GH binding to GHR. The reduced JAK-STAT signaling may be due to that starvation will induce expression of the fibroblast growth factor 21 (FGF21), in liver, which will inhibit the STAT5 signaling, as shown in mice (Inagaki et al., 2008). IGF1 will give negative feedback on GH excretion, therefore less circulating IGF1 reduced the inhibition, and more GH was released into the plasma, leading to high levels of GH in starved fish. This theory is supported by (Björnsson, 1997, Fox et al., 2006). However there is reasons to believe that both circulating amino acids as well as IGF1 has a positive feedback on expression of *igf1* in muscle, and that GH may not be the main regulator of muscle *igf1* expression, especially when considering the high amount of truncated GHR1 compared to full length GHR1 in fish muscle (Bower and Johnston, 2010, Fuentes et al., 2013). Interestingly *igf2* expression in muscle was not down regulated during starvation, however Picha et al., (2008), suggests that muscle *igf2* expression may not be related to growth. And thereby normal *igf2* expression does not mean that there still was occurring muscle growth during starvation. Most of these hormonal and genetic regulations have also been observed in other studies during starvation (Fox et al., 2006, Inagaki et al., 2008). The reduced expression and lower circulating levels of IGF1 strongly indicated lower growth in starved fish. This was also found in the phenotypical parameters. SGR, weight and weight gain decreased during starvation, but there were still occurring length growth. This is in accordance with

Christiansen (1996) and Kullgren et al., (2013), which found that during periods of unfavorable conditions, with little feed available, length growth is preferred rather than weight growth. This length growth is mediated by relocating body reserves into length growth by the help of GH as described by (Kullgren et al., 2013). Therefore extraordinary high levels of GH may be a signal for the fish to prioritize length growth. In conclusion, there were seen increased plasma levels of GH, and severely decreased IGF1 levels and expression, leading to decreased growth rate in starved fish.

During recovery period the levels of both plasma hormones and muscle gene expression was normalized compared to 19°C fed fish. Indicating that the previously starved fish had normal growth and development at this stage. This normal gene expression was also accompanied by normal, weight and length gain. However, the SGR and weight had not yet completely recovered. Even though there were tendencies to compensation responses in feed intake and lower FCR (which may be a compensatory response), there were little compensation responses in growth, shown both at the genetic and phenotypical levels. Previous findings indicates that there has to be a combination of low HSI as well as weight loss to manage to get full compensation during recovery (Picha et al., 2008). Therefore the suggestions for that there were not seen full compensation in this experiment, was due to that HSI did not decline enough during starvation to manage to cause full growth compensation. It should be mentioned thou, that there may have been seen compensation growth during the early stages of recovery, however, no measures was conducted at that time. In conclusion there were seen normal hormonal and genetic expression after recovery, however tendencies to weaker growth compared to 19°C fed fish, and no full compensation growth were seen due to high HSI during starvation.

4.3.3 Energy flow and hormones

The high levels of circulating GH and low levels of circulating IGF1, indicates that much of the anabolic actions of GH not was functioning. Low circulating plasma levels of IGF1 is associated with starvation, even if GH levels are high (Fox et al., 2006). During starvation low plasma concentration of IGF1 was necessary as IGF1 is a strong anabolic hormone, enhancing growth (especially muscle growth). Also cronical high levels of GH has anti-insulin-like effects (Herrington and Carter-Su, 2001). However the high levels of circulating GH may probably also have a protein sparing effect as explained by (Nørrelund et al., 2001). They indicates that the protein sparing effect partly may be mediated through IGF1, which may explain the tendencies to higher expression of *igf1ra* in muscle, showing that there was not a reduced sensitivity for IGF1 in muscle tissue during starvation. This indicates that the starvation was not severe enough to degrade the muscle proteins, which also was the case, due to the unchanged stored protein content in starved fish. However other studies done on mammals, does not find any significant protein sparing effects of GH (Sakharova et al., 2008). In current study *igf2* expression did not decrease during starvation. Picha et al., (2008), suggests that muscle *igf2* expression may not be related to growth. And thereby our normal *igf2* expression does not mean that there is still occurring muscle growth during starvation. In fact there is some uncertainty of all the biological reactions mediated by IGF2 (Chao and D'Amore, 2008). One reason for the normal expression of *igf2* can be that IGF2 may have more important role in metabolism and less important role in growth enhancement compared to IGF1 during starvation. Increased levels of circulating IGFBP1b, normally indicates increased catabolic activity, it is therefore quite surprising that there was not found any increase in circulating IGFBP1b in starved fish. Hevrøy et al., (2011), found increased levels of circulating IGFBP1b in plasma of fish starved for 14 days. However the

normal levels of IGFBP1b in current study may be due to decreased expression and circulation of IGF1 in muscle and plasma in starved fish, and thereby no change in IGFBP1 was needed. It is likely that high levels of IGFBP1 is a preferable way of regulating the bioavability of IGF1 during short periods of food shortages, while during longer periods down regulation of *igf1* is more preferable (personal communication by Dr. Ernst Morten Hevrøy, Ewos AS) . In fact Hevrøy et al., (2011), did not find down regulation of *igf1* neither in liver nor muscle during 14 days starvation. In current study starvation also increased expression of *ghr1* and *ghr2* in muscle. This contradicts much of recent studies, among those of Fox et al., (2006), which studies tilapia during starvation, and found no change in liver *ghr* expression, Fukada et al., (2004), also found lower level of GHR during starvation. Hevrøy et al., (2011), found no change in *ghr1* expression in muscle tissue during 14 days starvation of Atlantic salmon. Since during starvation GH has a lipolytic effect, and diverts energy away from growth to sustain essential metabolic processes, the increase in *ghr* expression in current study may be due to that GHR play a major role in the lipolytic effects of GH in the muscle during starvation, as suggested by (Hevrøy et al., 2013). Also Mommsen and Moon (2001), suggests that GH may regulate lipid mobilization in at least visceral tissues directly. The up regulation of *ghr* may therefore be part of a "catabolic circle", which starts with that starvation is increasing ghrelin excretion, which again increases GH excretion into plasma, while there is a inhibition in synthesis of IGF, which again stimulate more circulating GH and higher expression of *ghr*, and thereby high lipolytic activity in the cells. This is supported by Picha et al., (2008), which foud elevated expression of *ghr2* in hybrid striped bass, and suggests that high levels of GH and GHR2, may facilitate mobilization of energy stores, or protein sparing. Since the GH sensitivity normally is increased in visceral tissues, as described by Norbeck et al., (2007), the high levels of GH support the theory of that most of the mobilized lipids during starvation came from the viscera, as indicated by the reduction in VSI and lipid content. There was a significant lower glucose concentration in plasma compared to fish fed at 19°C. However this due to unusual high levels of glucose in fish fed at 19°C, and not due to unusual low levels in starved fish. This is supported by that plasma glucose in starved fish did not change compared to 13°C fed fish. It also showed normal levels according to (Hemre, 2001). The normal levels of glucose in plasma in starved fish correlates well with the findings of Hevrøy et al., (2011), which not found significant higher levels in starved fish after 14 days starvation. Some studies suggests that GH is the main promoter of keeping blood glucose levels at a steady state also during starvation, as explained by Goldstein et al., (2012), but other studies does not suggests that high levels of GH is needed to sustain normal plasma glucose levels during starvation (Gahete et al., 2013). However in total the hormonal and genetic regulation clearly indicated catabolic conditions.

Also the phenotypical parameters during trial period showed catabolic conditions. This was among other seen by reduced CF in 19°C starved fish, indicating that the fish had to mobilize its own reserves to sustain metabolic activity. According to literature Einen et al., (1998), Navarro and Gutiérrez (1995), the mobilized energy was likely taken from glycogen reserves during the first days of starvation. Some of the mobilized glycogen could have been taken from liver. This may partly explain the decreased HSI during trial period in starved fish, supported by Ali et al., (2003), who studied carp. As soon as the glycogen storages were depleted in starved fish, most of the energy was taken from stored lipids, and therefore reduced amount of stored lipids in whole fish (g/100 g ww) The mobilized lipids were replaced with water, as described by (Navarro and Gutiérrez, 1995). The viscera accumulates most of the surplus lipids ingested by the food, and generally in teleosts this is the place where lipids are mobilized from during starvation Jezierska et al., (1982), Navarro and Gutiérrez (1995), which is seen in this experiment by the tremendous decrease in VSI during

starvation. Even thou viscera looks to be the most important lipid mobilization tissue during starvation, some mobilized lipid were also coming from the muscle and liver, as described by (Christiansen, 1996, Einen et al., 1998, Navarro and Gutiérrez, 1995). Therefore some of the reduction in HSI during starvation may have been due to mobilization of lipids. The starved fish, did not show any significant change in stored protein (g/100 g ww), indicating that the protein stores were spared during starvation, which indicates that high levels of GH may have protein protecting effects, and that the starvation did not last long enough to deplete the lipid storages, as described by (Navarro and Gutiérrez, 1995). Even thou there were not seen any significant decrease in stored protein, 19% of the lost body weight came from protein loss, however 44% of the weight loss came from lipids. In conclusion, both the hormonal, genetic and phenotypical parameters clearly showed that the metabolism was shifted strongly towards catabolism, also in vital organs like the liver. However at the same time there was seen protein sparing during starvation at high temperatures.

During recovery all the hormonal and genetic factors recovered to normal levels compared to fish fed at 19°C. This should indicate shifting of the metabolic processes back to normal metabolism. However the tendencies to compensation feed intake (%BM) and tendencies to lower FCR in starved fish, indicates that more energy should be available, which could indicate an even more anabolic condition than the fed group, when defining anabolism as a organ building process. This is also supported by the higher retention values of nutrients where LPV was much higher than in fed fish, also EPV and PPV was higher in fish previously starved. The high LPV shows that most of the ingested energy were retained as lipids. Even thou there were seen a very high energy intake, as well as full compensation of lipid storages, there were not seen full compensation in stored energy, SGR nor weight, indicating that the fish had not yet fully recovered from the stressful conditions previous experienced. Looking at the feed intake hormonal and genetic regulation it may be suggested that if the recovery period had lasted longer, there would have been seen an even smaller difference between starved and fed fish. In conclusion there were not seen any major change in the energy flow after recovery between fed and starved fish and the previous starved. In fact there were seen a tendencies to more anabolic metabolism in fish previous starved, but no full compensation in weight or growth.

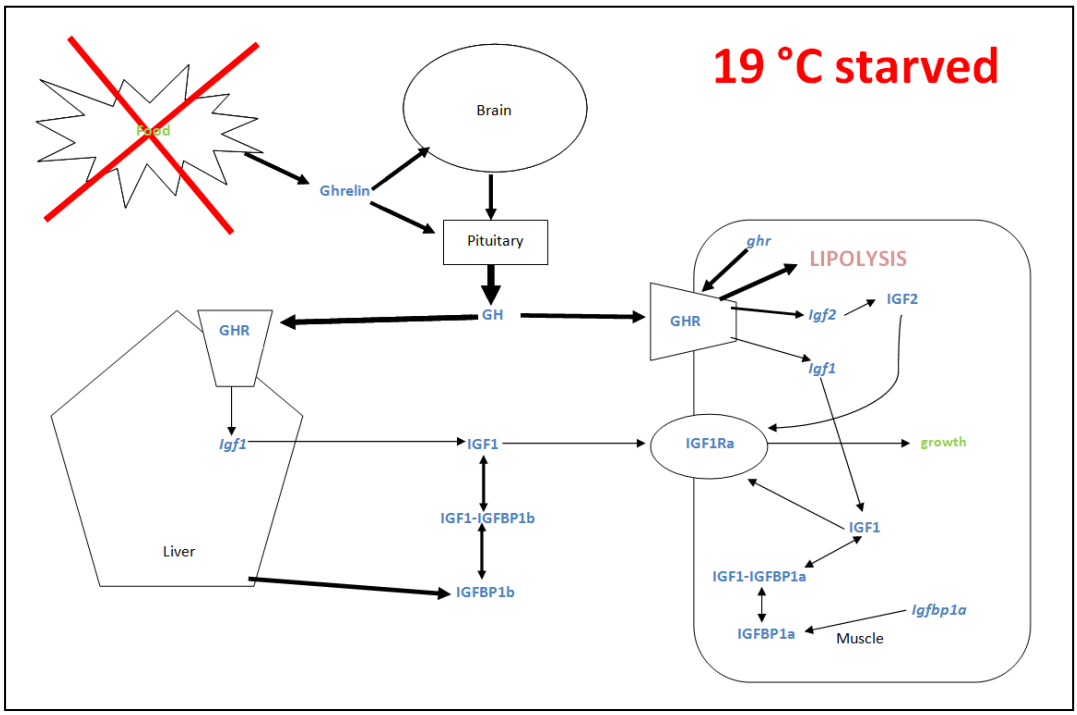

Figure 4.2: Showing genetic growth regulation during starvation at 19°C. Compared to 19°C fed, following is seen: Due to no feed intake, ghrelin is excretion from stomach is increased and transported to the brain and pituitary which stimulates more release of GH. However, the GH mediated synthesis of *igf1* is very limited during starvation, leading to that very little IGF1 is excreted into the plasma. Also normal levels of IGFBP1b in plasma leads to very little bioavalible IGF1 in plasma. The low level of circulating IGF1 and amino acids leads to now positive feedback on muscle expression of *igf1*. Low levels of IGF1 in plasma also decrease the negative feedback IGF1 has on GH, leading to even higher excretion of GH into plasma. There is also seen higher expression of *ghr* in muscle, but very low expression of *igf1*. The low expression of *igf1* in muscle is due to both inhibition of GH mediated *igf1* synthesis, as well as lack of positive feedback from circulating IGF1 and amino acids. However, GH mediates lipolysis through GHR. Due to low levels of IGF1, there is seen no, or little growth during starvation.

4.4 Hormonal and genetic markers for growth

Our results indicate that GH is not positive growth regulating hormone when feed intake is varying. However, both plasma IGF1 and *igf1* expression in muscle is much more related to growth during varying environmental and nutritional conditions. There are also seen stronger correlations between IGF1 and growth compared to GH and growth, as seen in figure 4.3. Our findings of strong relationship between growth and IGF1, corresponds well with other studies (Mommsen and Moon, 2001, Fox et al., 2006). Our results also indicate that plasma IGF1 and muscle *igf1* expression may be used as a endocrine growth marker also at temperatures above optimal temperature for growth. In all this indicates that IGF1 is a major regulator of growth, regardless of environmental conditions.

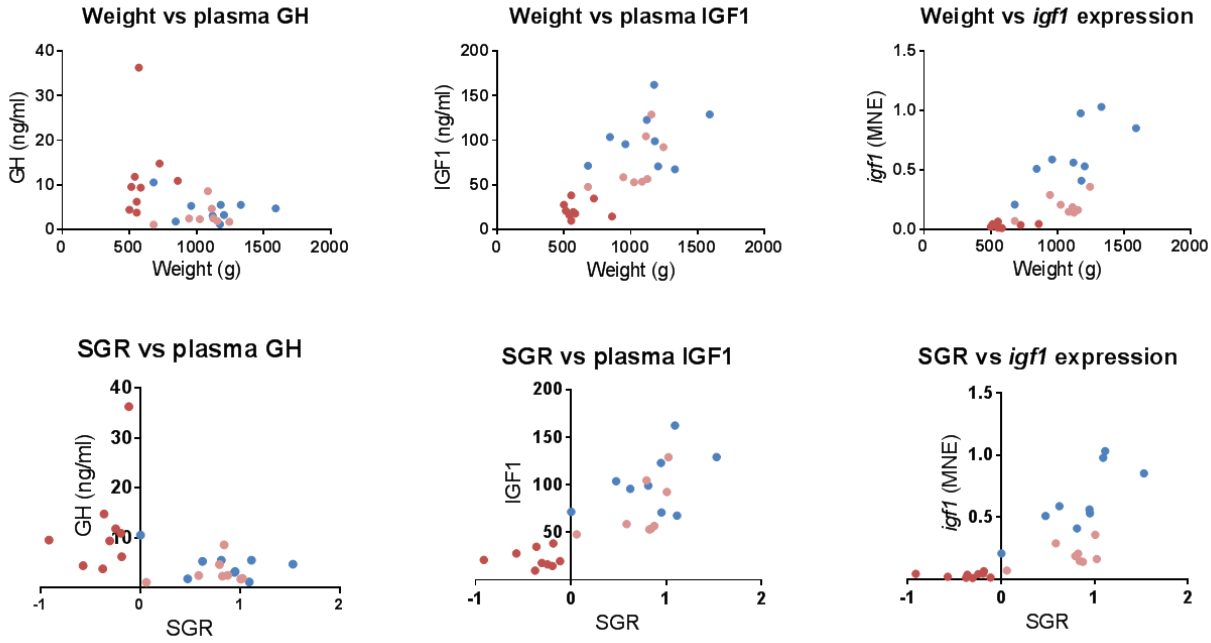

Figure 4.3: Showing individual correlation during trial period between weight and plasma GH (K = -0.41, p = 0.035), weight vs. plasma IGF1 (K = 0.74, p < 0.001) and weight vs. *igf1* expression in muscle (K = 0.79, p < 0.001). SGR vs. plasma GH (K = -0.51, p = 0.008), SGR vs. plasma IGF1 (K = 0.79, p < 0.001) and SGR vs. *igf1* expression in muscle (K = 0.82, p < 0.001). n = 9.

5.0 Conclusion

- In current experiment the feed intake was higher in Atlantic salmon reared at 19°C compared to salmon reared at 13°C. However the growth rate was significant lower at 19°C, suggesting that less nutrients was available for growth. This corresponds to the previous findings that 19°C is above the optimal temperature for growth in Atlantic salmon. At the end of the trial period feed intake decreased, indicating that Atlantic salmon may manage to maintain high feed intake when exposed to short periods of high temperatures, but not over long periods

- The energy consumption was higher in 19°C than in 13°C. However there were no indications of increased catabolism in fish fed at 19°C compared to fish fed at 13°C.

- When fish was fed at 19°C, the endocrine GH/IGF regulation of muscle growth was not severely affected by exposure to 19°C. There were down regulation of *igf1* expression in muscle, but in total the endocrine growth regulatory system showed normal anabolic growth regulation. Temperature in itself did therefore apparently not have any major effect on the genetic growth regulation

- Starvation at 19°C did have a more significant effect on the endocrine GH/IGF growth regulatory system. Elevated plasma GH, and low levels IGF1 indicated catabolic state with increased GH mediated lipolysis in muscle.

- In total there were little positive effects of starving fish at 19°C compared to *ad libitum* feeding the fish at 19°C. However starved fish had better feed intake and nutrient retention during recovery. Further research should therefore be done to investigate the possible positive effects of doing restricted feeding at high temperatures.

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7.0 Appendix

7.1 Performance of individually sampled fish

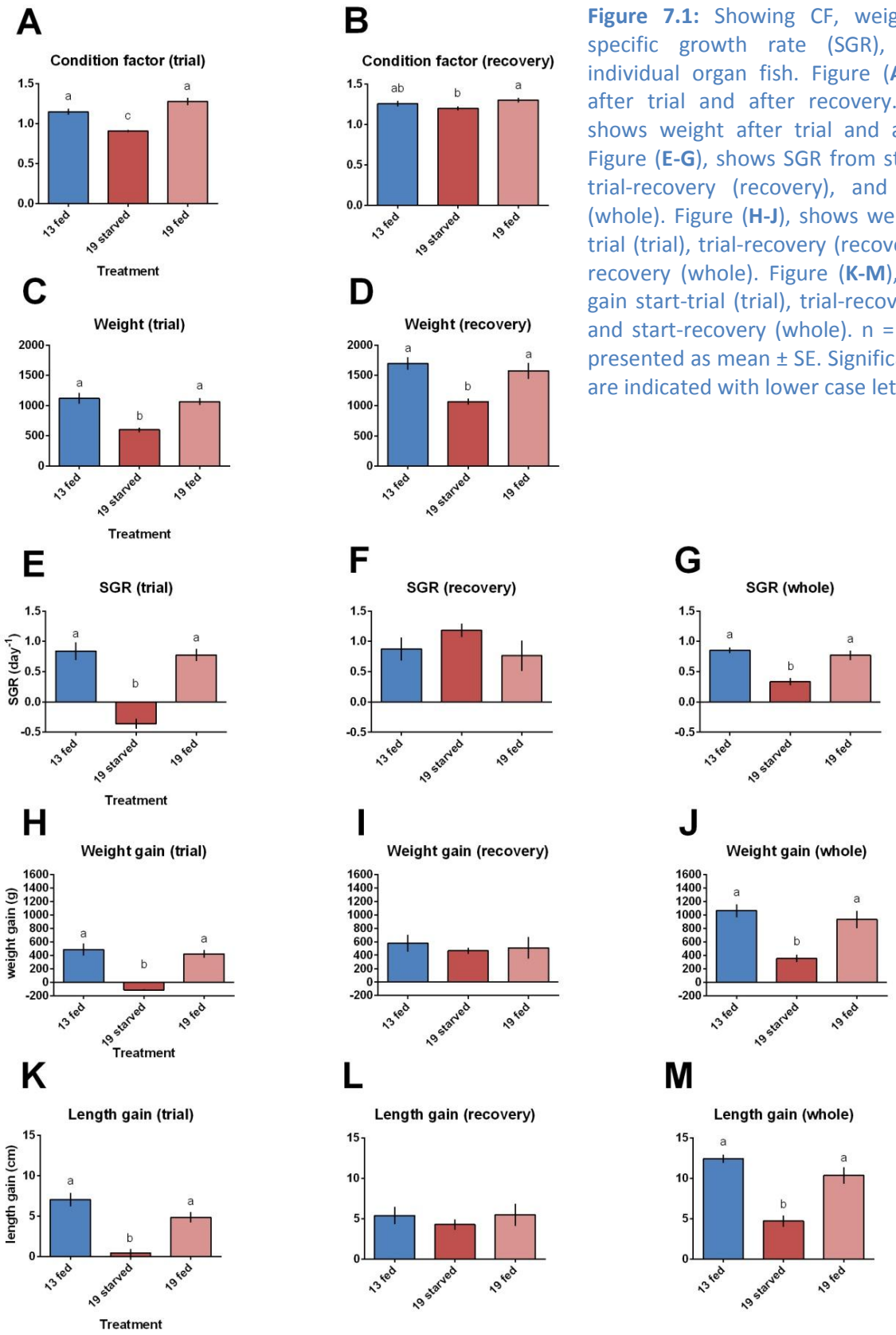

Figure 7.1: Showing CF, weight and mean specific growth rate (SGR), of only the individual organ fish. Figure (**A-B**) shows CF after trial and after recovery. Figure (**C-D**), shows weight after trial and after recovery. Figure (**E-G**), shows SGR from start-trial (trial), trial-recovery (recovery), and start-recovery (whole). Figure (**H-J**), shows weight gain start-trial (trial), trial-recovery (recovery), and start-recovery (whole). Figure (**K-M**), shows length gain start-trial (trial), trial-recovery (recovery), and start-recovery (whole). n = 3, and data is presented as mean ± SE. Significant differences are indicated with lower case letters.

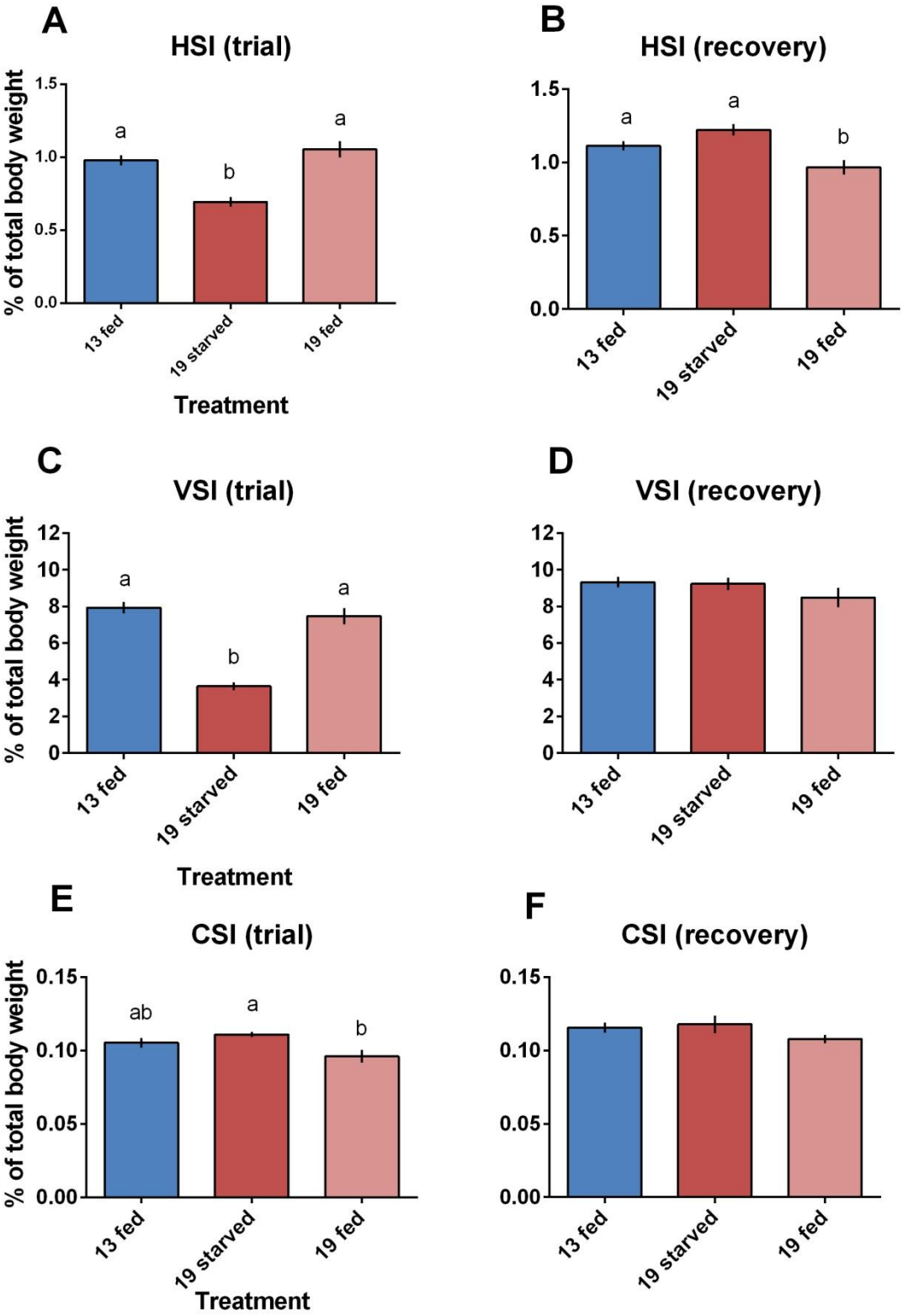

Figure 7.2: Showing somatic indexes (SI), of only the individual organ fish. Figure (**A-B**) shows hepato-somatic index (HSI) after trial and after recovery. Figure (**C-D**), shows visceral somatic index (VSI), after trial and after recovery. Figure (**E-F**), shows cardio somatic index (CSI), after trial and after recovery. n = 3, and data is presented as mean ± SE. Significant differences are indicated with lower case letters.