The effect of temperature on feed intake and growth in Atlantic salmon (*Salmo salar*) – Is ghrelin involved as a hunger hormone?

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Abstract

Efficient feeding regimes for Atlantic salmon farming rely on knowledge and understanding fish feeding biology, as well as the physiological mechanisms that control appetite and feed intake. Atlantic salmon is an ectotherm animal, and temperature is one of the predominant environmental determinants that influence feed intake, digestion rates and how efficiently the fish utilize feed. Similarly, to other vertebrates, teleosts appetite and feed consumption are complex processes regulated by central and peripheral signals. However, little is known about how temperature affects the dynamics of the gastro-intestinal tract (GIT) transit and how this relates to the signalling factors involved in digestive processes. The GIT digests and absorbs the ingested feed under the control of neural and hormonal factors, many of which also regulate appetite via signalling pathways that stimulate (orexigenic) or inhibit (anorexigenic) hunger. Of these, ghrelin is commonly presumed to be an orexigenic hormone in teleosts. Ghrelin is mainly produced in enteroendocrine (EEC) cells in the stomach and requires the enzyme membrane-bound O-acyltransferase 4 (*mboat4*) to be activated. The role of ghrelin is well-described in mammals, however, knowledge of the regulatory effect of ghrelin on appetite and feed intake in Atlantic salmon is still limited. This study investigated potential involvement of ghrelin on regulation of feed intake, gut-appetite signalling and growth in Atlantic salmon post-smolt of approximately 200 g reared at 8°C (low-temperature), 12°C (control) or 15°C (high-temperature) for two months. Fish were fed once a day for 2 h using automatic feeders, and feed intake was assessed by collecting and quantifying uneaten feed. At the end of the two months, 10 fish from each temperature group were collected 2 h post-feeding, followed by sampling every 4th hour for 24 hours. Biometry data for growth and somatic indexes, plasma, GIT compartmental content, and stomach tissue were collected. Assessment of the temporal changes in stomach filling, gut transit, Ghrl plasma levels, and gene expression of *ghrl-I, ghrl-II* and *mboat4* in stomach tissue was performed. Temperature had a significant impact on feed intake, gut-appetite signalling and growth. Growth and feed intake increased with temperature, where the 8°C group had a generally weaker performance than the 12°C and 15°C groups. The gut transit rate was highest in the 15°C group, with a more rapid evacuation rate and digestion, reaching almost complete stomach evacuation 24 hours post-feeding. However, there was no correlation between stomach fullness and ghrelin gene expression or Ghrl plasma levels. The expression of *ghrl-I* was significantly affected by temperature, however, both *ghrl-II* and

mboat4 remained unaffected. Ghrl plasma levels exhibited distinct differences in temporal trends between the temperature groups. The 8°C group showed elevated levels before the normal mealtime, whereas the 12°C and 15°C groups showed depressed levels. No correlation was observed between the gene expression of *ghrl-I* or *ghrl-II* and the Ghrl plasma levels in any of the temperature groups. The lower growth performance in the 8°C was expected as temperature has a significantly effect on metabolic processes and growth, however, the greater growth performance in observed in the 12°C group compared to the 15°C was unexpected. At the last sampling, some males in the 15°C group were starting to or were already mature. This negatively affected the growth and energy allocation, resulting in lower growth in the 15°C male group than anticipated. This may have impacted the feed intake, as the amount of feed provided vas determined by biomass of the group, however, the 15°C and 12°C groups revealed similar feed intake and FCR. The absence of a correlation between stomach fullness, mRNA expression of ghrelin in stomach, and Ghrl plasma levels indicate that ghrelin does not have the same orexigenic function in Atlantic salmon as previously anticipated.

List of abbreviations

ANOVA	Analysis of Variance
AGRP	Aguouti-related Peptide
CCK	Cholecystokinin
cDNA	complementary Deoxyribonucleic Acid
CNS	Central Nervous System
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
EEC	Enteroendocrine Cells
ELISA	Enzyme Linked Immunosorbent Assay
EtOH	Ethanol
FCR	Feed Conversion Ratio
GBI	Gallbladder Index
GHRL-I	Ghrelin 1
GHRL-II	Ghrelin 2
GHS-R	Growth Hormone Secretagogue Receptor
GIT	Gastrointestinal Tract
GSI	Gonadosomatic Index
GLP	Glucagon-like Peptide
GOAT	Ghrelin O-acyltransferase
HFI	Hindgut Fullness Index
HIS	Hepatosomatic Index
K-factor	Condition factor
MBOAT	Membrane-bound O- Acyltransferases
MFI	Midgut Fullness Index
NPY	Neuropeptide-Y
PCR	Polymerase Chain Reaction
PIT	Passive Integrated Transponders
PYY	Peptide YY
qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic Acid
SMR	Standard metabolic rate
SFI	Stomach Fullness Index

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1. Introduction

1.1 General background

Over the last four decades, the global aquaculture industry has grown at an average annual growth rate of 8% per year (FAO, 2020). As a result, aquaculture has become one of the fastest-growing animal food-producing sectors, with salmonid farming significantly contributing to the global supply of high-value fish. In 2021, the total global production of Atlantic salmon (*Salmo salar*) was 2.7 million tonnes (Shahbandeh, 2020), Norway being the leading producer with 1.33 million tons (FAO, 2020). The Norwegian commercial salmon farming has had exponential growth with larger production units, capacity, and improved resource allocation, due to an increasing demand for animal protein globally (FAO, 2016). New technologies for more efficient and sustainable production have been developed and implemented, facilitating greater production and handling of larger quantities of fish (Aas *et al*., 2019; Moe Føre *et al.,* 2022). Given the surge in production and increasing volumes of fish, it is crucial to implement efficient logistical models for handling all aspects of the production, especially feed. Feed is the most important input factor in commercial salmon farming (Aas *et al.,* 2022), as it represents more than half of the operational expenses in intensive salmon production (SSB, 2012). Feed is a critical input component in aquaculture and a limiting element for future expansion, due to a scarcity of marine raw materials and possible limitations of alternative sustainable ingredients. Efficient utilization of the feed is essential due to its impact on sustainability, the environment, and economic profitability (Aas *et al.,* 2019). Taken together it is therefore, necessary to minimise feed waste and optimise feeding regimes to maximise feed conversion and growth to achieve satisfactory economic results (Aas *et al.,* 2019). As of 2023, the feeding of Atlantic salmon in traditional net pens at sea relies on observing fish behaviour and appetite through underwater cameras, occasionally supplied with echo sounders to set feeding intensity and meal duration. It allows producers to control feeding remotely, and this practice is a tight interaction between farmers which are on-site, and feeders stationed at the feeding centres (Føre *et al.,* 2018). This feeding method typically yields a feed conversion ratio (FCR) of 1.2 to 1.4 (Aas *et al.,* 2022), implying that there could be instances of overfeeding during certain stages in production. FCR is the ratio between feed consumed (kg) and how much fish is produced (kg) and is commonly used in aquaculture to assess resource utilization (Aas *et al.,* 2022; Ytrestøyl *et al.,*2015). Overfeeding significantly reduces production profitability, and results in a higher efflux of waste, which can adversely affect the environment as less nutrients are taken up by the fish and instead released into the environment (White, 2013; Taranger *et al.,* 2014). On the

other hand, underfeeding can result in reduced growth performance and negatively affect production efficiency. Hence, it is of great value to improve feed utilization to enhance industry sustainability, reduce environmental impacts, and maintain competitiveness in the global market (Ytrestøyl *et al.*, 2015). New feeding technologies are constantly being developed and implemented in the aquaculture sector; however, to efficiently use the new technologies, one must rely on reliable feeding models to achieve a stable high-feed intake. To develop efficient feeding models, the industry requires more biological knowledge of appetite regulation in Atlantic salmon. Therefore, gaining more knowledge about biological mechanisms that regulate appetite, food intake, gut transit, and growth is vital to utilise food effectively.

1.2 Appetite control in teleosts

In all vertebrates, appetite regulation is a complex process that involves interactions between peripheral signals and the brain. Appetite is strongly modulated by short-term factors via the GIT (gastrointestinal tract) and long-term factors linked to metabolic and energetic status (Volkoff, 2016; Rønnestad *et al.*, 2017). Key elements for short-term regulation of feed intake include sensory- and mechanical stimuli, as well as the production and secretion of hormones by specialized enteroendocrine (EEC) cells. (Blundell *et al.*, 2015). Long-term control of feeding is somewhat controversial, both in mammals and in fish, particularly since fish can survive long periods without feed (Volkoff & Rønnestad, 2020). However, for all vertebrates, including fish, the control of appetite mainly takes place in the hypothalamus (Klockars *et al.*, 2019; Rønnestad *et al.*, 2017).

1.2.1 Central control of appetite

In the brain, the hypothalamus plays a critical role in appetite regulation (Timper & Brüning, 2017). The physiological mechanisms that control appetite have been investigated in mammals and other vertebrates (Klockars *et al.*, 2019), but the neuroendocrine regulation of appetite in fish has been less explored (Rønnestad *et al.*, 2017; Volkoff, 2019). Nevertheless, it has been suggested that the key roles of neuropeptides and hormones that regulate appetite in mammals and fish have been evolutionary conserved (Volkoff *et al.*, 2005; Rønnestad *et al.*, 2017). Appetite involves the sensations of hunger and satiety conveyed to the hypothalamus's feeding centre (Timper & Brüning, 2017). In the hypothalamic circuit in mammals, the arcuate nucleus (ARC) plays a major role in integrating peripheral signals that regulate appetite (Klockars *et al.*, 2019). Within the ARC, there are two sets of neurons that have distinct functions in appetite

regulation. One group consist of neurons that co-express appetite-stimulating (orexigenic) neuropeptide Y (NPY) and Agouti-related protein (AGRP). The second group of neurons co-express appetite inhibitors (anorexigenics) pro-opiomelanocortin (POMC)/ cocaine- and amphetamine-regulated transcripts (CART) (Klockars *et al.,* 2019; Rønnestad *et al.,* 2017). Hunger results in increased energy expenditure which further promote foraging behaviour, in contrast, satiety allocates energy for somatic growth (Timper & Brüning, 2017). Peripheral signals regarding feed intake, metabolic status and digestion are integrated in the hypothalamus. The integration of signals of peripheral signals promotes production of orexigenic and anorexigenic factors. (Klockars *et al.,* 2019; Volkoff, 2016; Rønnestad *et al*, 2017)

1.2.2 Peripheral control of appetite by the GIT

The peripheral endocrine signals that originate in the GIT serve an important role to control the initiation and termination of a meal (Rønnestad *et al*, 2017; Volkoff, 2016). The communication system between the GIT and the brain is called the gut-brain axis and is a bi-directional signal axis central for maintaining metabolic homeostasis and transforming sensory information between the GIT via neural or hormonal signalling pathways and the CNS (Bauer *et al.,* 2015; Sam *et al.,* 2012). The GIT is the largest endocrine organ in vertebrates (Sam *et al.,* 2012). Within the mucosal layer of the GIT, there are specialized cells with endocrine functions which are referred to as EEC cells (Latorre *et al.,* 2016). EEC in the GIT release endocrine signals in response to luminal content and mucosal stretching, where their primary function is to act on the GIT itself to regulate digestion and uptake of nutrients (Mendieta-Zerón *et al.,* 2008; Rønnestad *et al.,* 2017; Timper & Brüning, 2017;). In addition, the GIT produces neuropeptides and hormones that are communicated to the brain and interact with the central nervous system (CNS) (Calo *et al*,.2021; Holmgren & Olsson, 2009; Krogdahl, 2001). This communication is important for regulating appetite and feed intake, as they stimulate and suppress appetite-regulating neuropeptides in the brain (Krogdahl, 2001; Sam *et al.,* 2012; Timper & Brüning, 2017). EECs can sense luminal content via apical nutrient sensors, which triggers a cascade of intracellular signals, resulting in the production and release of signal molecules into the bloodstream (Calo *et al*., 2021). This can influence a wide range of physiological processes, including feed intake, intestinal motility, and gastric and intestinal secretion of digestive enzymes and hormones (Mayer, 2011; Raybould, 2010). EECs will release several peptide hormones, including orexigenic such as ghrelin with an appetite-stimulating effect, and anorexigenics such as cholecystokinin (CCK), peptide tyrosine (PYY), and glucagon-like peptide (GLP) (Calo *et al*., 2021; Rønnestad *et al.,* 2017). For higher vertebrates,

cholecystokinin (CKK) is an important gastrointestinal hormone which plays an important role in digestion. The hormone is produced in the midgut and secreted into the bloodstream in response feed ingestion. When this hormone is released, it stimulates pancreatic enzyme secretion, induces gallbladder contraction and further the release of bile (Little *et al.,* 2005; Rønnestad *et al.,* 2007). When fish is starved, bile is stored in the gall bladder which results in the gallbladder progressively getting fuller over time (Talbot & Higgins, 1982).

1.3 GIT transit and links to appetite

There is a close link between feed intake, GIT transit rates, digestion, absorption, and evacuation. All these processes are under physiological control with feedback to integrate the delivery of nutrients and energy to the tissues (Rust, 2003). Thus, how gut transit affects feed intake could be beneficial for understanding feeding behaviour in Atlantic salmon. The GIT is divided into four main compartments with different roles in digestion and with close control of the downstream passage of digesta. Feed is ingested through the mouth and enters the oesophagus, which is further transported to the stomach (Figure 1 (a)). The stomach serves as a reservoir of ingested food and is a visco- elastic muscular organ which expands when feed is ingested. Stomach filling, and capacity to stretch, are major short-term regulatory factors, and the mechanical response is highly involved in determining meal size and frequency of meals (Camilleri, 2015; Krogdahl, 2001). The enteric nervous system, a network of autonomic nerves scattered along the length of the stomach, directly control stomach functions like muscle contractions (Holmgren & Olsson, 2009; Latorre *et al.,* 2016) Digestive enzymes are secreted in the stomach, and circular muscle contractions contribute to effectively process the feed into at fluidized mass (chyme/digesta) (Grove *et al.,* 1978; Krogdahl, 2001). When the chyme reaches a suitable consistency, stomach contractions will transfer the content into the pyloric area and then through the pyloric sphincter into the intestine (Figure 1 (b & c)) (Krogdahl, 2001; Mock *et al*, 2022; Rust, 2003). The medial intestine (Figure 1 (c)), also called the midgut, is a muscular tube highly important for nutrient absorption in Atlantic salmon. In the midgut, the mucosa mainly consists of enterocytes. The enterocytes are cylindrical, and the folded apical surface creates a large surface for the absorption of nutrients (Krogdahl, 2001; Mock *et al*, 2022; Rust, 2003). The gall bladder and the secretion of bile highly influence midgut content. Bile is produced in the liver, stored in the gall bladder, and further secreted in the midgut in response to the presence of feed. Bile adds mucus, bicarbonate, bile salts and apolipoproteins to the chyme, which is important for fat digestion (Krogdahl, 2001; Mock *et al*, 2022; Rust, 2003).

After the chyme has been processed in the midgut, and most of the nutrients are absorbed, the remaining content will be transferred to the hindgut (Figure1 (d)). The enterocytes in the hindgut differ from those in the midgut, as they can absorb and transport substances from the lumen to the blood. In the hindgut, vacuoles facilitate the absorption, digestion, and transportation of bigger molecules, for instance, intact proteins (Krogdahl, 2001; Mock *et al.*, 2022). Undigested food will be evacuated as faeces through the rectum.
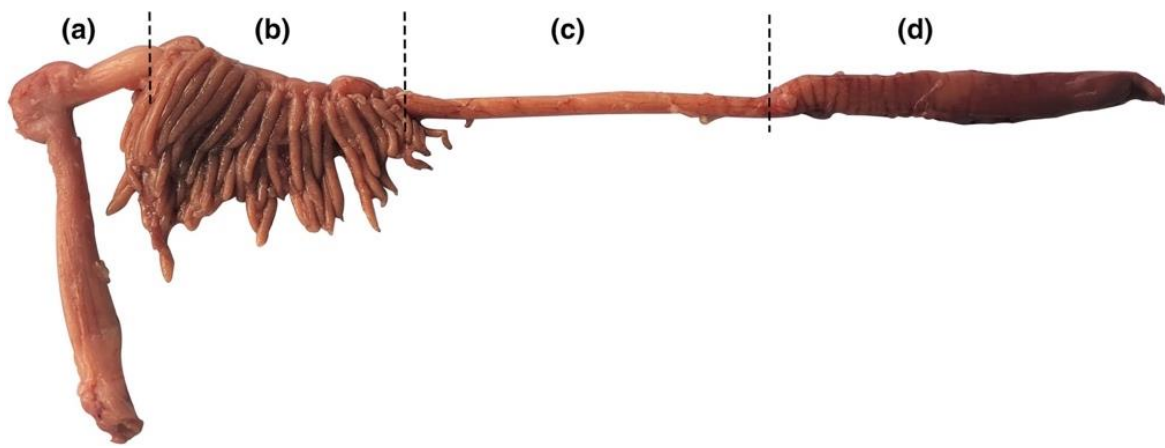
Figure 1: Gastrointestinal tract (GIT) of Atlantic salmon. Distinct regions of the GIT are labelled as; (a) stomach, (b) pyloric caeca, (c) midgut and (d) hindgut (Mock et al., 2022)

The GIT, especially the stomach, is critical in regulating the gut transit rate (Mock *et al.,* 2022; Rust, 2003). Gut transit in Atlantic salmon refers to the passage of feed through the gastrointestinal tract from ingestion to evacuation (Rust, 2003). Several factors, including external factors such as feed composition, pellet size and water temperature, influence gut transit time. Fish has a generally higher feed intake and digestion rate in warmer water, resulting in a shorter gut transit time, this also accounts for Atlantic salmon (Aas *et al*., 2017; Miegel *et al.,* 2010; Mock *et al.,* 2022; Volkoff & Rønnestad, 2020). As well as environmental factors, it is suggested that meal size and feeding frequency may influence feed transit and gut evacuation (Gomes *et al.,* 2023; Handeland *et al.,* 2008; Navarro-Guillén *et al.,* 2023). The gastric emptying curve is close to exponential with time, and in several fish including sockeye salmon (*Oncorhynchus nerka*) and rainbow trout (*Oncorhynchus mykiss*) there is a strong correlation between the required time for emptying and the return of appetite (Brett & Higgs,1970; Grove *et al.,* 1978). Amount of feed required to reach satiation is dependent on gastric emptying time,

fish size and temperature. A model for gastric emptying time has been developed by Grove *et al.* (1978), suggesting that gastric emptying time decreases with higher temperatures.

1.4 Temperature effects on growth and metabolism

The appetite and feed intake of Atlantic salmon are strongly affected by various external factors, including photoperiod, oxygen saturation, food availability, stress, and temperature (Volkoff & Rønnestad, 2020). While these factors have been studied in relation to growth, there is very limited understanding of their impact on appetite regulation and the hormonal and neural signalling pathways involved. Atlantic salmon is a poikilothermic animal, meaning that the environmental temperature determines its body temperature. As a result, water temperature affects all its physiological processes, including metabolic rates, energy balance, growth, behaviour, appetite, and feed intake (Brett, 1971; Brett, 1979; Prosser & Nelson, 1981; Aas *et al.,* 2017). Standard metabolic rates (SMR) rise in poikilothermic fish with increased temperatures, which is caused by increased biochemical rates (Neubauer & Anderson, 2019; Volkoff & Rønnestad, 2020). The increase in energy expenditure leads to a greater demand for feed for survival (Volkoff & Rønnestad,2020). "*The impacts of temperature on feeding vary depending on species, but usually, voluntary food intake increases with moderate temperature increases and decreases when temperatures are outside the fish's optimal temperature range*" (Volkoff & Rønnestad, 2020). Feed intake and feeding behaviour are closely related, and temperature can affect several of the processes for feeding behaviour, such as olfaction, the sensitivity of sensory systems, locomotion performance, and swimming performance (Volkoff & Rønnestad,2020). Temperature may, therefore, both directly and indirectly affect appetite and feed intake. Knowledge of how seasonal changes in environmental conditions, such as temperature, affect the dynamics in the GIT transit and the expression of orexigenic factors (e.g., ghrelin) is still limited in Atlantic salmon.

1.5 Ghrelin

Ghrelin is categorized as a peptide hormone and was first isolated from rat stomach tissue. It was firstly identified as a growth stimulant to bind to the growth hormone secretagogue receptor (GHS-R) in the brain (Kojima *et al.,* 1999). Ghrelin was further discovered in humans, which resulted in the identification of ghrelin in a wide range of vertebrates (Kojima *et al.,* 1999; Kojima & Kangawa, 2005) including Atlantic salmon (Murashita *et al.,* 2009; Hevrøy *et al.,* 2011; Volkoff *et al.,* 2017). The length of the peptide hormone varies depending on species, where ghrelin in Atlantic salmon comprises 12-26 amino acids, distinct from human ghrelin, with a length of 28 amino acids (Kojima *et al.,* 2007; Murashita *et al.,* 2009). In fish multiple forms of deacylated ghrelin exist, with variations in amino acid length and acyl modifications (Kojima & Kangawa, 2005). The modification of the third amino acid (serine 3) is responsible for the activation of ghrelin's biological function and is required for ghrelin to bind to its receptor (GHS-R) in the hypothalamus (Kojima *et al.,* 1999). When acylated ghrelin binds to GHS-R, it stimulates the release of growth hormone and appetite-stimulating peptides (Kojima *et al.,* 1999; Kojima & Kangawa, 2005; Müller *et al.,* 2015). Studies indicate that ghrelin is the only peripheral orexigenic hormone in mammals and functions as a key hormone in the endocrine control of energy balance (Figure 2) (Kojima & Kangawa, 2005; Murashita *et al.,* 2009). Energy balance and appetite is a complex process, where research on ghrelin has been important to understand endocrine control. In mammals, the ghrelin expression tends to be at its highest right before expected meals and increases in response to fasting (Cummings *et al.*, 2001; Stengel & Taché, 2012). This upregulation of ghrelin in response to fasting indicates that the hormone is synthesised and secreted as a response to hunger and plays an important role in short-term appetite regulation (Cummings *et al.,* 2001; Nonogaki, 2007; Rønnestad *et al.,* 2017). Other studies indicate that ghrelin plays critical roles in biological functions other than appetite regulations (Delhanty *et al.,* 2012) but these are outside the scope for this thesis.

Ghrelin is produced in the X/A cells, which is in the mucosal epithelium in the stomach, these cells have been renamed into "*ghrelin cells*" (Date *et al.,* 2000; Nonogaki, 2007; Stengel & Taché, 2009). In all vertebrate species, ghrelin-producing cells are predominantly located in the stomach but are also found scattered in all regions in the gastrointestinal tract (Kojima & Kangawa, 2005; Stengel and Taché, 2009). Expression of ghrelin cells in the GIT can vary within the teleost family (anterior to posterior), however, available data shows that ghrelin is expressed along the whole length of the stomach wall of Atlantic salmon (Mangersnes, 2020; Murashita *et al.,* 2009). Ghrelin-producing cells in the stomach can be open and closed,

implying that ghrelin secretion is determined by a combination of absorbed nutrients, nerves, and hormonal regulation (Stengel and Taché, 2009). Production of ghrelin will increase during fasting stages which further promotes feed intake, whereas production will fall rapidly after feed consumption (Cumming *et al*., 2001). When produced and secreted, it can function both locally in the stomach, or it can be secreted from the gastrointestinal organs into the bloodstream and function as a peripheral signal for the CNS to stimulate feeding (Kojima & Kangawa, 2005). Ghrelin-producing neurons have been identified in the hypothalamus which indicate that ghrelin has an important role in controlling feed intake (Kojima & Kangawa, 2005). The majority of circulating GHRL is derived from the stomach, and is influenced by feeding state, however, little is known about circulating GHLR in humans, other than levels increase before meals and are reduced by feeding (Ariyasu *et al.,* 2001; Cummings *et al.,* 2001; Sugino *et al.,* 2002; Tschöp *et al.,* 2000). Research suggest that circulating GHRL is a meal-initiation factor due to its orexigenic effect (Cummings *et al.,* 2001). Research on plasma Ghrl levels in teleost have been conducted, however, there are contradictions in the findings. A study on Ghrl plasma levels by Pankhurst et al. (2008) showed elevated plasma levels 24 hours post-feeding for rainbow trout (*Oncorhynchus mykiss*), suggesting that ghrelin is a possible orexigenic in fish (Nonogaki 2007; Pankhurst *et al.,* 2008; Vikeså *et al.,* 2017). Hevrøy *et al.* (2011) found an upregulation of *ghrl* in starved salmon, which supports Pankhurst's findings.

Ghrelin has been identified in several teleost families, including Atlantic salmon, but its function is less explored than in mammals (Rønnestad *et al.,* 2017). In Atlantic salmon, ghrelin is present in two different splice variants, *ghrl-I* (GeneBank Accession No. AB443431) and *ghrl-II* (GeneBank Accession No. AB443432). The *ghrl-I* variant is called acylated ghrelin, with an acyl group attached to the serine 3 residual. The *ghrl – II* variant, des-acyl ghrelin does not have the ability to activate the GHS-R and can reduce biological activity in contrast to acylated ghrelin (Kojima *et al.,* 1999; Müller *et al.,* 2015; Chen *et al.,* 2009). The two splice variants have shown different expression patterns in Atlantic salmon; however, both forms exhibit the highest expression in stomach tissue (Murashita *et al.,* 2009). Combining results from Hevrøy *et al.* (2011) and Murashita´s results in a study from 2009, suggests that expression of *ghrl-I* does not play a significant role in long-time starvation (14 days). *Ghrl-II* does not seem to affect appetite regulation in Atlantic Salmon, as short- and long-term starvation does not affect gene expression (Murashita *et al.,* 2009; Hevrøy *et al.,* 2011).
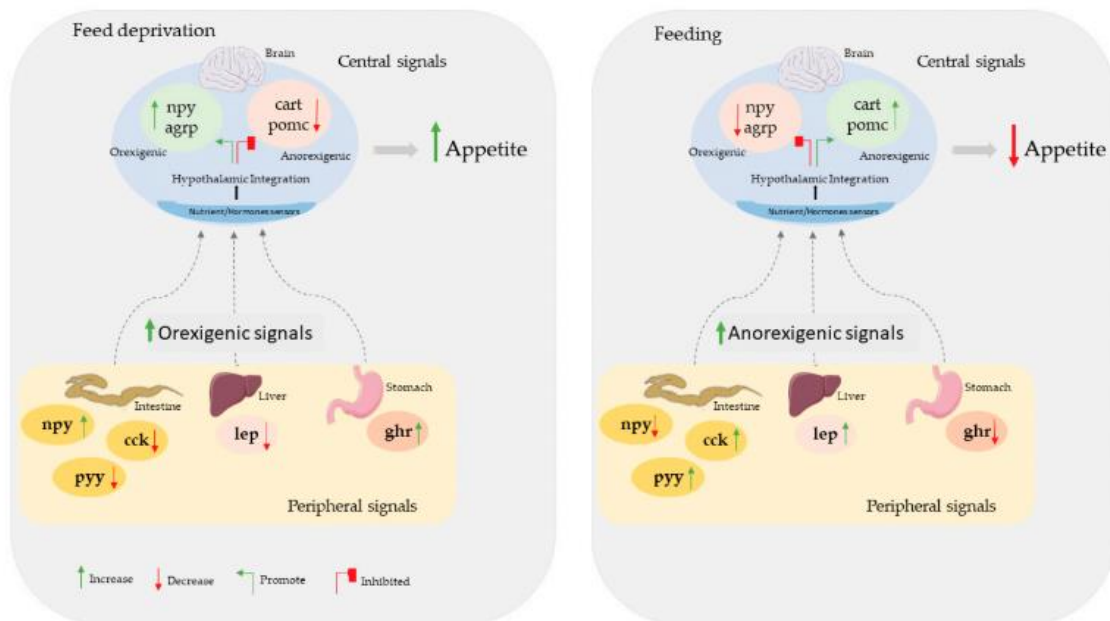
Figure 2: Endocrine regulation of appetite in fish before and after a meal. The diagram shows the interaction of peptides and hormones at the central (brain) and peripheral levels (intestine, liver, and stomach) involved in the regulation of feed intake in fish and how they can be affected by feed deprivation and feeding. agrp: Agouti-related peptide; cart: cocaine- and amphetamine-regulated transcript; cck: cholecystokinin; npy: neuropeptide Y; pomc: proopiomelanocortin; pyy: peptide YY; ghrl: ghrelin (Martins et al., 2022).

1.6 Activation of Ghrelin- membrane-bound O- acyltransferases (MBOAT4)

All organisms, including fish, have a family of integral transmembrane enzymes called *membrane-bound O- acyltransferases* (MBOAT) (Yang *et al.,* 2008). In all vertebrates, this superfamily is responsible for lipid and peptide modifications, which will promote protein acylation (Yang *et al*., 2008; Chang & Magee, 2009). The enzyme that is responsible for acylating ghrelin is called Ghrelin O-acyltransferase (GOAT), also known as MBOAT4. MBOAT4 is a member of the membrane-bound acetyltransferase family and is critical for modifying ghrelin into its active form by attaching octanoate (fatty acid) to the ghrelin peptide. This modification is important for regulating energy homeostasis and ghrelin activity. (Chang & Magee, 2009; Chen *et al.,* 2009; Müller *et al.,* 2015; Romero *et al.,* 2010; Shlimun & Unniappan, 2011). Levels of *mboat4* in stomach, pancreas and hypothalamus are altered by energy status and contribute to regulating food intake and energy expenditure. Studies show that levels of MBOAT4 increase during fasting and decrease when the organism has a positive energy balance (Li *et al.,* 2016). This may suggest that *MBOAT4* turns into a hunger signal in Atlantic salmon when feed is available (Kalananthan *et al.,* 2023)

1.7 Aims and objectives of the study

The primary objective of this study was to investigate the effects of temperature on appetite, feed intake, and growth in Atlantic salmon. Furthermore, the study sought to explore potential associations between gastrointestinal transit and the expression of ghrelin splice variants (*ghrl-I* and *ghrl-II*) as well as the activating enzyme (*mboat4*) in the stomach. Additionally, it aimed to document any possible correlations between feeding status, stomach filling, ghrelin expression in stomach tissue, and ghrelin plasma levels. The overall objective was to generate findings that could be utilized in developing appetite-controlled feeding models, grounded in a fundamental biological comprehension of appetite regulation, stomach filling, gut transit, and feed uptake.

1.7.1 Hypothesis

Examine the relationship between feed intake and growth for Atlantic salmon at different temperatures (low (8℃), control (12℃) and high (15℃))

- H0: Temperature will not have a significant effect on feed intake and growth of Atlantic salmon
- H1: Temperature will have a significant effect on feed intake and growth of Atlantic salmon. Feed intake and growth will be highest in the 15℃ group and lowest in the 8℃

Examine the temporal change in stomach-, midgut- and hindgut filling for Atlantic salmon reared at different water temperatures (low (8℃), control (12℃) and high (15℃))

- H0: There will be no differences in the temporal change of stomach-, midgut- or hindgut filling between fish at the different rearing temperatures during the 24 hours post feeding
- H1: There will be a difference in the temporal change of stomach-, midgut- or hindgut filling between fish at the different rearing temperatures during the 24 hours post feeding

Examine stomach filling for Atlantic salmon reared at different water temperatures (low (8℃), control (12℃) and high (15℃)) right after feeding as an indicator for feed intake.

- H0: There will be no differences in the stomach filling between fish at the different rearing temperatures during right after feeding.

- H1: There will be significant difference in the stomach filling between fish at the different rearing temperatures right after feeding.

Asses to what extent stomach fullness impacts the relative expression of *ghrl-I, ghrl-II* and *mboat4* in stomach tissue of Atlantic salmon at different rearing temperatures (low (8℃), control (12℃) and high (15℃)).
- H0: There will be no significant effect of the degree of stomach fullness on the relative expression of *ghrl-I, ghrl-II* or *mboat4* in stomach tissue of Atlantic salmon at different rearing temperatures
- H1: There will be a significant effect of the degree of stomach fullness on the relative expression of *ghrl-I, ghrl-II* or *mboat4* in stomach tissue of Atlantic salmon at different rearing temperatures

Examine the temporal change in mRNA expression of *ghrl-I, ghrl-II*, and *mboat4* in Atlantic salmon stomach tissue from 2 hours to 24 hours post-feeding at different rearing temperatures (low (8℃), control (12℃) and high (15℃)).
- H0: There will be no significant temporal change in the expression of *ghrl-I, ghrl-II* and *mboat4* in stomach tissue 2-24 h post-feeding between the temperature groups.
- H1: There will be a significant temporal change in the expression of *ghrl-I, ghrl-II* and *mboat4* in stomach tissue 2-24 h post-feeding between the temperature groups.

Examine the link between *ghrl-I* and *ghrl-II* mRNA expression in stomach and the expression of *mboat4*.
- H0: There will be no significant link between the *ghrl-I* or *ghrl-II* and the expression of *mboat4* in stomach tissue.
- H0: There will be a significant link between the *ghrl-I* or *ghrl-II* and the expression of *mboat4* in stomach tissue.

Examine the temporal change in Ghrl plasma levels in Atlantic salmon from 2 hours to 24 hours post-feeding at different rearing temperatures (low (8℃), control (12℃) and high (15℃))
- H0: There will be no significant temporal change in Ghrl plasma levels in Atlantic salmon from 2 hours to 24 hours post-feeding at different rearing temperatures.

- H1: There will be a significant temporal change in Ghrl plasma levels in Atlantic salmon from 2 hours to 24 hours post-feeding at different rearing temperatures. With a temporal gradual increase from 2 -24 hours.

Asses if there is a correlation between stomach fullness and Ghrl levels in plasma of Atlantic salmon reared at different temperatures (low (8°C), control (12°C) and high (15°C)).
- H0: Degree of stomach fullness will not have any significant impact on Ghrl plasma levels in Atlantic salmon at different rearing temperatures.
- H1: Degree of stomach fullness will have any significant impact on Ghrl plasma levels in Atlantic salmon at different rearing temperatures.

Identify if there is a correlation between mRNA expression of *ghrl-I* and *ghrl-II* in stomach tissue and Ghrl plasma levels.
- H0: The Ghrl levels in plasma is not affected by the mRNA expression of *ghrl-I* and *ghrl-II* in stomach tissue.
- H1: The Ghrl levels in plasma are correlated by the mRNA expression of *ghrl-I* and *ghrl-II* in stomach tissue.

2. Material and methods

2.1 Fish and experimental design

This master thesis was part of a larger experiment where the aim was to investigate some of the key signalling pathways in the gut-brain axis particularly targeting control of appetite and feed intake and how they are affected by temperature. This thesis focused on the gut transit, stomach filling and links to ghrelin, a potential orexigenic hormone. In the trial, groups of Atlantic salmon were kept at three different temperatures for eight weeks: 8 °C (low-temperature; LT), 12 °C (control; C) and 15 °C (high-temperature; HT). As part of the final sampling, fish were collected at regular intervals during 24 h to follow the gut transit of a meal and the response in gene expression and plasma levels of ghrelin. All animal handling and procedures described in this study were approved by the National Animal Research Authority in Norway (FOTS 28416).

On January 2022, 540 post-smolt Atlantic salmon of approximately 200 g were PIT tagged and randomly distributed into 9 tanks (600 L; n = 60/tank) at the Department of Biological Sciences at the University of Bergen. Tanks were constantly supplied with flow-through seawater at a temperature of 10 ± 0 °C, and a LD12:12 light regime (from 07:00 to 19:00). After an acclimation period of three weeks, fish weight and fork length were measured, and water temperature gradually changed to establish three experimental temperature groups at week 0 (W0) (Figure 3). The rearing temperatures were kept constant until the end of the trial after 8 weeks (W8). Water temperature, salinity, and oxygen saturation were monitored daily in all tanks and header tanks (OxyGuard system, Farun, Denmark) connected to a remotely controlled PC. Temperature during the experiment was (LT, 8.4 ± 0.00; C, 12.3 ± 0.00 °C; HT, 14.8 ± 0.01), oxygen (group LT, $93 \pm 0.05\%$; group CB $95 \pm 0.08\%$; group HT $93 \pm 0.12\%$), and salinity (25-29 ‰.) All tanks and fish were observed daily to detect irregularities and checked for mortality.

2.2 Feeding and collection of feed waste

Fish were fed once a day for two hours from 08:00 to 10:30 using automatic feeders, and feed intake was assessed by collecting and quantifying uneaten feed from each tank. Each temperature group had a 15-minute shift between the first feeding of each tank to ensure precise collection of the feed waste and standardised collection of biological samples during sampling. Tank numbers 1, 4 and 8 were fed from 08:00-10:00, tank numbers 2, 6, and 9 from 08:15-10:15 and tanks 3, 7 and 10 from 08:30-10:30 (Figure 3). Light was turned on 1 h prior to feeding to signalize feeding one hour later. From the start of the trial till the end, feed waste was collected from tanks 1, 4, 8 at 11:00, tanks 2, 6 and 9 at 11:15 and tanks 3, 7 and 10 at 11:30. The amount of feed administered for each respective tank was calculated on behalf of biomass estimations and regulated every day accordingly. Feed waste was collected and weighed every day, and the weight was used to analyse how much feed was eaten and how much surplus feed was present in each respective tank. The feed was placed on a feeding belt for the following day with an automatic start at 08:00.
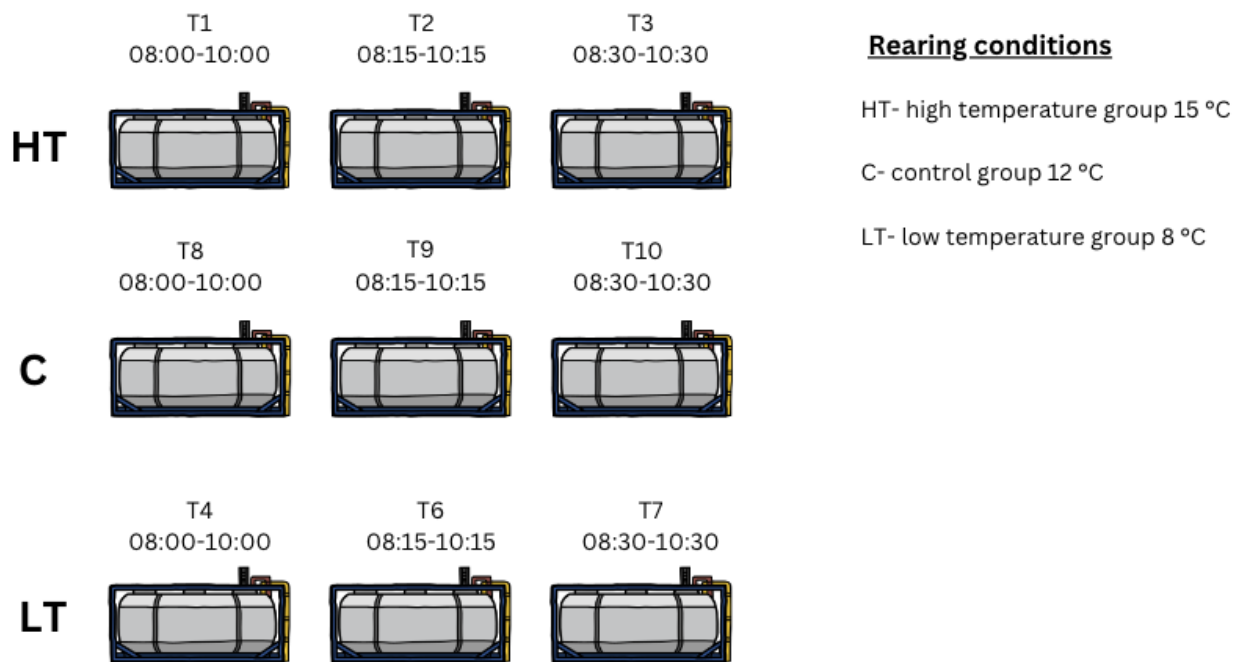
Figure 3: Experimental trial set-up. Fish were reared at three different temperatures with triplicate tanks: LT (Low temperature) 8°C, C (Control) 12°C, HT (High temperature) 15 °C. The fish were fed every day for 2 hours with a 15-minute shift between the triplicate tanks to ensure similar and standardized conditions during sampling and feed waste collection.

2.3 Sampling

Fish were collected at seven sampling points during the 24-hour sampling to follow the gut transit of a meal and the response in gene expression and plasma levels of ghrelin. Ten fish from each temperature group were collected 2 hours post-feeding, followed by sampling every 4th hour for 24 hours, resulting in sampling points at 11:00, 13:00, 17:00, 21:00, 01:00, 05:00 and 09:00 (CEST). On day 1 of sampling (29.03.22-30.03.22), fish reared at 15 °C from tanks 1, 2 and 3 were collected. At day 2 (30.03.22-31.03.22), fish reared at 8 °C from tanks 4, 6 and 7 were collected. Day 3 (31.0.3.22-01.04.22) fish reared at 12 °C from tanks 8, 9, and 10 were sampled. (Figure 4)
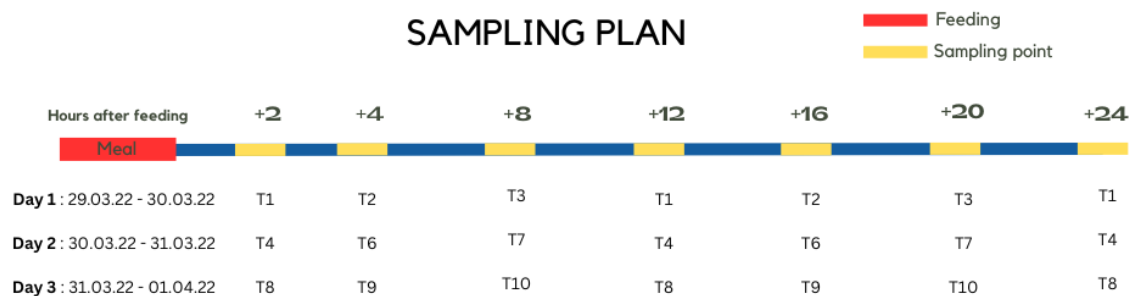
Figure 4: Sampling schedule during the 24-hour sampling. 10 Atlantic salmon from each tank were collected at set sampling points after feeding, where 8°C was reared in T4, T6, T7, 12°C in T8, T9, T10 and 15°C in T1, T2 and T3. Day 1 (29.03.22-30.03.22), day 2 (30.03.22-31.03.22) and day 3 (31.0.3.22-01.04.22).

Every fish was randomly collected and euthanized with an overdose of tricaine (*NaCO3-buffered tricaine methanesulfonate 200 mg/L, Finquel vet,* Argent, Redmond, USA). Before dissecting the fish, body weight (grams) and fork length (centimetres) were measured to the nearest 0.1 g/cm respectively (Ohaus Valor 2000 W and scale respectively) to measure the growth of fish. Fish were further dissected, gender assessed, and liver, gallbladder, heart, and gonads were weighed for the calculation of somatic indexes. Blood was manually extracted from the caudal vein using 2 ml heparinized sterile syringes and further centrifuged (5000 rpm, 3 min) to separate plasma from the blood cells. The plasma was collected in separate 0.6 ml PCR tubes (Axygen, Glendale, Arizona), kept on dry ice, and stored at -80 °C until analysis. The gastrointestinal (GI) tract was removed using surgical clamps at both ends to avoid loss or content transfer between the compartments and further separated into stomach (ST), midgut (MG) and hindgut (HG) by surgical clamps and dissection (Figure 1). The inner content (feed and digesta) from the different segments was carefully emptied into the pre-weighted, labelled

bags/vials (120x170x0.05mm, VWR International, Oslo, Norway / Eppendorf, Oslo, Norway). A distinct piece of the ST tissue for each individual was collected, rinsed in 1 x PBS (*phosphate buffered saline*) (VWR, Oslo, Norway) and stored in tubes with RNA later (Thermo Fischer, Oslo, Norway) at –80 °C until further analysis. Wet weight of gut content from ST, MG and HG was weighted and stored in at –20 °C for further analysis. From the head of each fish, the brain and pituitary were dissected and stored in 2 ml and 0,6 ml PCR tubes (Axygen) with RNA later (Thermo Fischer, Oslo, Norway). Further, the wet content from ST, MG and HG was tawed and dehydrated using a dehydrator (Excalibur 10, Excalibur, USA). All samples were exposed to 74°C, and the exposure time was standardized to 24 hours to ensure proper dehydration. Once dried, samples were individually weighted.

2.4 Calculations – biometrics, growth, and feed utilization

Relative growth rate (RGR) was calculated according to the following formula (Lugert *et al.,* 2016)

$$RGR\ (\%) = \frac{(w_t - w_i)}{w_i} * 100$$

Specific growth rate (SGR) was calculated according to the following formula (Lugert *et al.,* 2016)

$$SGR\ (\%) = \frac{(\log(w_t) - \log(w_i))}{t} \cdot 100$$

Where w_t is the final weight/length, w_i is the initial weight/length in grams of individually PIT-tagged fish, and *t* is time in days. This calculation was used on stock data from the 24-hour sampling with a t (time) from 49-51 days. RGR displays the absolute increase in relation to the initial weight/length and is reported as a percentage increase over time. SGR is a method to convert the analogy between absolute – and relative growth to the instantaneous growth rate. It provides a measure of increase in daily growth as a percentage, which is practical when comparing groups in short-term and nutrition experiments. (Lugert *et al.,* 2016)

Condition factor (K) is an indicator for the health, energy allocation and well-being of the fish and was calculated using the following equation (Chellappa *et al.,* 1995; Le Cren, 1951):

$$K = \frac{w}{l^3} * 100$$

Where *w* is the weight (g), and *l* is the length of the fish (cm) (Datta *et al.,* 2013). It is a method to assess if the fish is in good condition, where a value above 1.0 suggests that the fish is in good condition and robust (Datta *et al.,* 2013; Le Cren, 1951)

Gonadosomatic index (GSI) is an indicator for maturation status was calculated for both genders using the following equation (Martinez *et al.,* 2021):

$$GSI\ (\%) = \frac{gonad\ weight(g)}{total\ body\ weight\ (g)} * 100$$

Hepatosomatic index (HSI) is an indicator of energy status and was calculated using the following equation (Chellappa *et al.*, 1995).

$$HSI\ (\%) = \frac{liver\ weight(g)}{total\ body\ weight\ (g)} * 100$$

Feed intake was calculated from week 0 to week 8.

$$Feed\ intake\ = feed\ given\ (g) - feed\ waste\ (g)$$

Feed conversion rate (FCR) was calculated using the following equation (Bai *et al.,* 2021):

$$FCR = \frac{Total\ weight\ of\ feed\ consumed\ (g)}{Total\ body\ mass\ gained\ (g)}$$

Stomach fullness index (SFI) was calculated as a percentage to assess the degree of ST filling normalized to body weight, and further asses the temporal changes in stomach fullness after feeding. Midgut fullness index (MFI) and hindgut fullness index (HFI) are calculated using an analogue equation, where "*stomach dry content (g)*" in the numerator was substituted with midgut and hindgut content accordingly.

$$SFI\ (\%) = \frac{stomach\ dry\ weight\ (g)}{fish\ weight(g) - (stomach\ weight\ wet(g) + midgut\ wet\ weight\ (g) + hindgut\ wet\ weight\ (g))} * 100$$

Gastrointestinal distribution was calculated to investigate how much content was present in each segment (ST, MG, and HG) relative to the total dry content in the gastrointestinal tract. This was normalized by fish body weight by using stomach-, midgut- and hindgut fullness indexes. MG and HG distribution were calculated using an analogue equation, where "*SFI*" in the numerator was substituted with MFI and HFI accordingly.

$$GIT\ distribution = \frac{stomach\ fullness\ index}{stomach\ fullness\ index + midgut\ fullness\ index + hindgut\ fullnes\ index} * 100$$

2.5 Gene expression analysis: qPCR

Expression analysis for the selected genes was conducted as described below. Sample preparation (starting from RNA extraction) of ST tissue analysis was performed at the MDB laboratories at BIO, Bergen, Norway.

2.5.1 RNA isolation with TRI reagent

RNA was extracted from stomach samples with TRI Reagent (Sigma-Aldrich, MO, USA) following the manufacturer's protocol. 1 ml TRI-reagent was added to centrifuge tubes with one 0.6-0.7 g ceramic sphere (Qiagen, Hilden, Germany). 50-100 µg of ST tissue was cut into small pieces and added to tubes with TRI reagent. The samples were homogenized using the Precellys 24 homogenizer (Bertin, Bretonneu, France), where the program was run three times to achieve proper homogenization. Further, 200 µl chloroform was added and tubes were centrifuged for 15 min at 4°C at maximum speed. The supernatant was transferred to new tubes where 500 µl of isopropanol was added. Tubes were centrifuged for 10 min, at 4°C at maximum speed to separate the pellet from the supernatant. The pellet was washed in 1 ml 80 % cold EtOH and stored in the –80°C freezer. When RNA was extracted from all 210 samples, the pellet was thawed and centrifuged for 5 min at 4°C at 7500 g (9000 rpm). The EtOH was carefully removed, the pellet was reconstituted in 100-250 µl nuclease-free water, and the RNA was quantified using the NanoDrop ND-1000 spectrophotometer (ThermoFischer, Oslo, Norway). Further, the RNA was precipitated by adding 1/10 of 3M NaAc (Sodium Acetate), pH 5.2 and 2-2.5 vol of -20°C 100% EtOH. Samples were further controlled by using NanoDrop spectrophotometer.

2.5.2 DNase- treatment

Total RNA was treated with TURBO DNA-free kit (Ambion Applied Biosystem) to remove all traces of genomic DNA. This procedure was conducted following the manufacturer´s protocol for all 210 samples. Volume calculations were based on RNA concentration (section 2.5.1) to achieve a maximum of 10 µg of RNA. Nuclease-free H20 and sample were mixed with 5 µl (0.1 volume) 10X TURBO DNase Buffer and 1 µl TURBO DNase. The mixture was incubated at 37 °C for 30 minutes. 5 µl resuspended DNase Inactivation Reagent (0.1 volume) was added to the samples and mixed before incubating for 5 minutes at room temperature. All samples were centrifuged at 10 000 g for 1.5 min, and the supernatant (RNA) was moved to a new tube. The concentration of RNA was measured using NanoDrop before being stored at – 80°C.

2.5.3 cDNA synthesis

First-Strand cDNA Synthesis from total RNA was performed using SuperScript III Reverse Transcriptase (Invitrogen, California, USA) and Oligo(dT)$_{20}$ (50 µM) in a total reaction volume of 20 µl. RNA samples were diluted to a concentration of 1.2 µg cDNA with a total volume of 11 µl of cDNA and water.

For each sample,

1 µl	Oligo(dT)$_{20}$ (50 µM)
x µl	total RNA (1,5 µg)
1 µl	10 mM dNTP Mix
y µl	RNase free water
------	
13 µl	Total volume

The mixture was heated to 65°C for 5 minutes and incubated on ice for at least 1 minute in the PCR machine. Further

4 µl	5X First-Strand Buffer
1 µl	0.1 M DTT
1 µl	RNaseOUT™ Recombinant RNase Inhibitor
1 µl	SuperScript™ III RT (200 units/µl)
-----	
7 µl	Total volume

was added to the mixture before incubated at 50°C for 60 minutes, and further, the reaction was inactivated by heating at 70°C for 15 minutes. For 210 samples, 6 NRT control samples were made where 1 µl SuperScript™ III RT was replaced by 1 µl pure water. The cDNA and NRTs were stored at – 20°C till amplification in qPCR.

2.5.4 qPCR

All primers are listed in Table 1. The specific reference genes for Atlantic salmon were *beta-actin (actb)* and *ribosomal protein s20 (rps20).* Specific primers for *ghrl- I, ghrl-II* and *mboat4* were used to measure mRNA concentration levels of target genes.

Table 1: Overview of primer sequences. Primer sequences for reverse transcription quantitative PCR (RT-qPCR) expression analysis in Atlantic salmon.

Gene	GeneBank ID	Primer Sequence	Size (bp)	Efficiency (%)	R^2	Ref
actb	NM_01123525.1	**F**: CCAAAGCCAACAGGGAGAAG	91	101,2	0,997	Olsvik *et al.,* 2005
		R: AGGGACAACACTGCCTGGAT				
rps20	NM_001140843.1	**F**:GCAGACCTTATCCGTGGAGCTA	85	107,8	0,997	Olsvik *et al.,* 2005
		R:TGGTGATGCGCAGAGTCTTG				
ghrl-I	NM_001142709.1	**F**:CCAGAAACCACAGGTAAGACAGGGTA	128	89,2	0,992	Del Vecchio *et al.,* 2021
		R:GAGCCTTGATTGTATTGTGTTTGTCT				
ghrl-II	NM_001139585.1	**F**:TCCCAGAAACCACAGGGTAAA	121	95,4	0.989	Del Vecchio *et al.,* 2021
		R:GAGCCTTGATTGTATTGTGTTTGTCT				
mboat4	XM_045703012.1	**F**:GGGTTGGCAAACATTCTGGC	89	96,6	0,998	Kalananthan *et al.,* 2022
		R:ACACTGATAGGAGAAGCCTGG				

The primers were analysed for quantification cycle (Cq), primers efficiency (E) and melting peaks to detect potential nonspecific product and/or primer dimers. The efficiency for the primers was determined by using a 10-fold dilution standard curve (1.00E+07 to 1.00E+02 copies amplicon/ml). qPCR analyses were carried out using 10 µl of SYBR Green I Master Mix - iTaq (Roche Diagnostic, Basel, Switzerland), 0.6 µl of each forward and reverse primers (10 mM), 6.8 µl Ultra-Pure Water (Biochrom, Berlin, Germany) and 2 µl cDNA template (6 ng/µl) (Lai *et al.,* 2021).

For all reactions, duplicates were run into 96-well plates. Two positive controls were included, no-template control (NTC), no-reverse transcriptase (NRT) and one positive control. The following qPCR protocol was performed:

1) 95 °C for 30 s,
2) 95 °C for 5 s,
3) 60 °C for 25 s,
4) repeating step 2–3 for 39 more times.

Detecting nonspecific products and primer dimers was achieved by performing a melting curve analysis over a temperature range of 65 to 95 °C, with an increment of 0.5 °C for 2 seconds. The quantitative PCR (qPCR) was conducted using a CFX96 Real-Time System (Bio-Rad Laboratories, California, USA), in conjunction with CFX Manager Software version 3.1 (Bio-Rad Laboratories, California, USA). The absolute mRNA expression levels for each target gene were then calculated using the following equation.

$$\text{Copy number} = 10^{\frac{Cq - intercept}{slope}}$$

The copy number was normalized using each target gene's total ng of RNA. The geometric average of target gene expression was used to normalize the data sets to reference genes (*actb* and *rsp 20).* This method has been shown to prerequisite for accurate qPCR expression analysis and facilitating for studying small expression differences (Vandesompele *et al.,* 2002; Hellemans *et al.,* 2007).

2.6 Ghrelin- Enzyme Linked Immunosorbent Assay (ELISA)

An ELISA kit was used to quantify Ghrelin protein levels in plasma. Plasma was added into ghrelin pre-coated plates, which allowed the Ghrl present in the sample to bind to the antibodies coated on wells. The analysis was performed according to the producer's protocol (Cat.NO MBS1601713, MyBiosource, San Diego, USA). All reagents, standard solutions and samples were prepared and brought to room temperature. Before reading the plate, standard curves from solutions were prepared. Standard curves no.5 to no.1 had concentrations respectively, 240 ng/l, 120 ng/l, 60 ng/l, 30 ng/l and 15 ng/l. 50 µl standard were added to all standard wells before 40 µl samples were added to all sample wells. Further, 10 µl anti-Ghrl antibodies were added to all sample wells, followed by 50 µl streptavidin- HRP, which were added to all samples and standard wells, excluding the blank control well. The plate was covered with a plate sealer and mixed for 2 minutes at 600 rpm before being incubated at 37°C for 60 minutes. The plate was washed five times with 300 µl wash buffer for 30-60 seconds each. After being washed, 50 µl substrate solution A and a further 50 µl substrate solution B were added to each well. The plate was covered with a plate sealer and aluminium foil and incubated at 37°C for 10 minutes in the dark. The plate sealer was removed, and 50 µl Stop Solution was added to all wells, causing the solution to change colour from blue to yellow instantly. Further, the optical density of each well was determined immediately by using a microplate reader set to 450 nm (must be read within 10 minutes after adding the stop solution).

2.7 Statistical analysis

All statistical analyses and graphs were performed using GraphPad Prism 9.1.0. (GraphPad Software, La Jolla, CA, United States). Before statistical evaluations, all data were tested using ROUT outlier test. The morphometric data (length, weight, growth performance and feed consumption) was tested for normality and equal variance using the D'Agostino-Person test. The morphometric data were further analysed using a one-way ANOVA for production parameters such as SGR, RGR, HSI and K-factor, with temperature being the class variable, followed by a post-hoc test to identify differences between the temperature groups. Dunn's multiple comparisons test was performed on datasets that did not pass D' Agostino-Person test, whereas the Holm-Šídák's multiple comparisons test was performed on datasets that passed the normal distribution test. Due to unequal sample sizes between males and females in different temperature groups, GSI

was analysed for males and females separately by performing a one-way ANOVA with a Kruskal-Wallis test. Relative gene expressions were log-transformed to achieve normal distribution and further analysed for differential expression between different temperature groups with a two-way ANOVA (Bruce & Bruce, 2017). A two-way ANOVA test was performed for gene expression in ST, gut content in different gut sections and Ghrl plasma levels, followed by Tukey's multiple comparison tests. Additionally, Pearson correlation test was utilized to determine the correlation between mRNA gene expression and ST filling. The same analysis was used to determine correlation between ghrelin plasma levels and gene expression, gut filling, and gallbladder index. For all tests, $p < 0.05$ was considered significant (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). All data are presented as mean $\pm$ SEM; otherwise, it is stated.

3. Results

The results are described in four parts: 1) effects of temperature on growth performance; 2) feed intake and gut filling (dry); 3) *ghrl-I*, *ghrl-II* and *mboat4* gene expression in the stomach; 4) Ghrl levels in plasma.

3.1 Effects of temperature on growth performance

At the start of the experiment, the average weight and length were similar in all groups (APPENDIX B, Table 6). By the end of the trial, the average weight was significantly lower in the 8°C group (418.2 ± 15.2 cm) compared to the 12°C (512.3 ± 17.8 cm, p=0.0008) and 15°C (480.5 ± 20.4 cm, p=0.0392) (APPENDIX B, Table 6).

The 12°C group had the best overall growth performance, whereas the 8°C group performed weakest. The 8°C had a significantly lower SGR (1.23 ± 0.0) than both the 12°C (1.73 ± 0.0, p< 0.0001) and the 15°C group (1.63 ± 0.0, p< 0.0001) (APPENDIX B, table 7 & 8). Whereas the 12°C showed a significantly higher RGR (143.7 ± 2.3) than both the 15°C (125.5 ± 3.3, p = 0.0018) and 8°C (85.9 ± 1.5, p< 0.001) groups (APPENDIX B, Table 9 & 10).
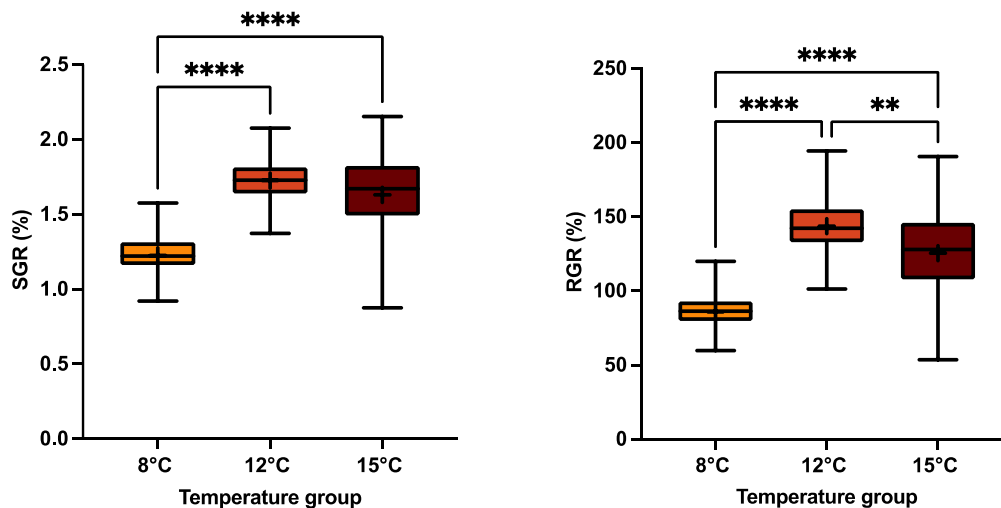
Figure 5: Specific Growth Rate (%) and Relative Growth Rate (%) in Atlantic salmon. The plot shows the distribution of SGR and RGR for each temperature groups (8°C, 12°C, 15°C). Each box plot represents the interquartile range (IQR) with the lower and upper quantiles (25th and 75th percentiles). The horizontal line indicates the median value, while the whiskers represent the minimum to maximum values of the data, the + sign indicates the mean value. A total of 210 fish were used for the experiment, with 70 individuals in each temperature group. Asterisks indicate statistically significant differences between groups ***(p<0.001) **(p<0.01) *(p<0.05). Non-significant results are not shown.

In addition to growth, the 12°C group had a higher mean K-factor (1.37 ± 0.0) than the 8°C (1.30 ± 0.0, p<0.0001) and 15°C (1.34 ± 0.0, p=0.0331) (Figure 6). The total range of K-values varied from 1.11 to 1.62, with a significant effect of temperature on K-factor on the different groups (p<0.0001) (APPENDIX B, Table 11 & 12).
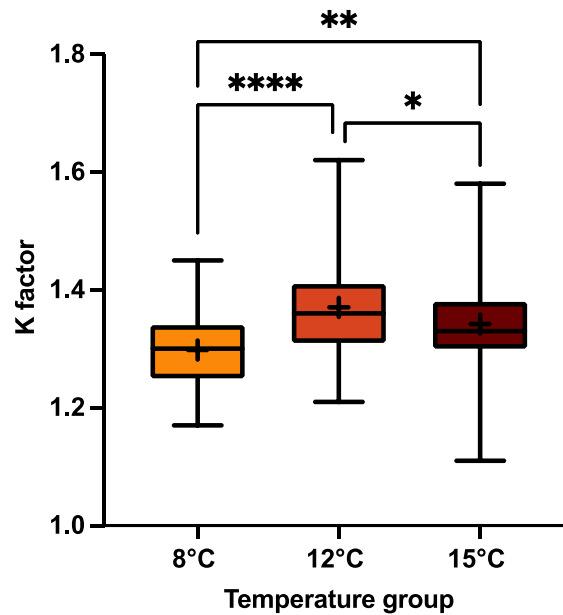
Figure 6: Condition factor (K-factor) for Atlantic salmon. The plot shows the distribution of K-factor values for each temperature groups (8°C, 12°C, 15°C). Each box plot represents the interquartile range (IQR) with the lower and upper quantiles (25th and 75th percentiles). The horizontal line indicates the median value, while the whiskers represent the minimum to maximum values of the data, the + sign indicates the mean value. A total of 210 fish were used for the experiment, with 70 individuals in each temperature group. Asterisks indicate statistically significant differences between groups ***(p<0.001) **(p<0.01) *(p<0.05). Non-significant results are not shown.

The hepatosomatic index (HSI) was significantly affected by temperature (p=0.0189) (Figure 7). The 15°C group had the lowest mean HSI (1.09 ± 0.0), significantly lower than both the 12°C (1.14 ± 0.0, p=0.036) and the 8°C group (1.14 ± 0.0, p=0.035) (APPENDIX B, Table 13 & 14).
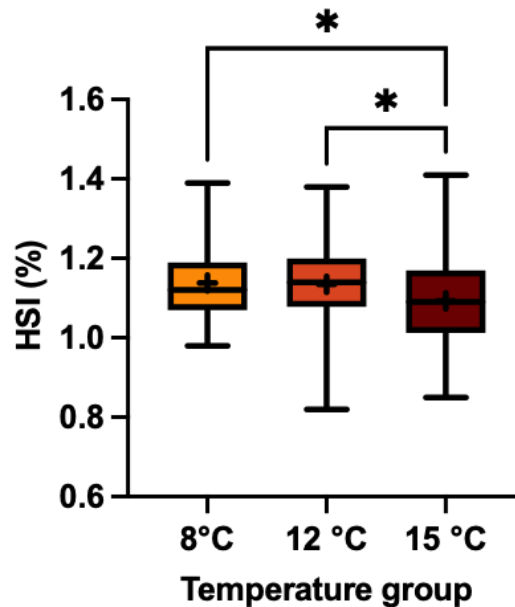
Figure 7: Hepatosomatic index (%) in Atlantic salmon at different temperatures. The plot illustrates the distribution of HSI values for each temperature groups (8°C, 12°C, 15°C). Each box plot represents the interquartile range (IQR) with the lower and upper quantiles (25th and 75th percentiles). The horizontal line indicates the median value, while the whiskers represent the minimum to maximum values of the data, the + sign indicates the mean value. A total of 210 fish were used for the experiment, with 70 individuals in each temperature group. Asterisks indicate statistically significant differences between groups ***(p<0.001) **(p<0.01) *(p<0.05). Non-significant results are not shown.

Analysis showed that temperature influenced gonadosomatic index (GSI) in both females (p = 0.0012) and males (p = 0.0066). The GSI was lower in females compared to males, with significantly higher values in the temperature group 15°C for both sexes. In the 15°C female group, the GSI was higher than the 8°C (p=0.0029) and 12°C (p=0.0072). In males, the 8°C group had a significantly lower GSI compared to both 12°C (p=0.0167) and 15°C (p=0.0169) (Figure 8) (APPENDIX B, Table 15-18).

Results from the Pearson correlation test showed that there were moderate negative correlations between GSI and SGR for males in temperature group 12°C (r= -0.6489, R^2=0.4210, p<0.0001) and 15°C (r= -0.6421, R^2=0.4122, p<0.0001) (APPENDIX B, Table 19).
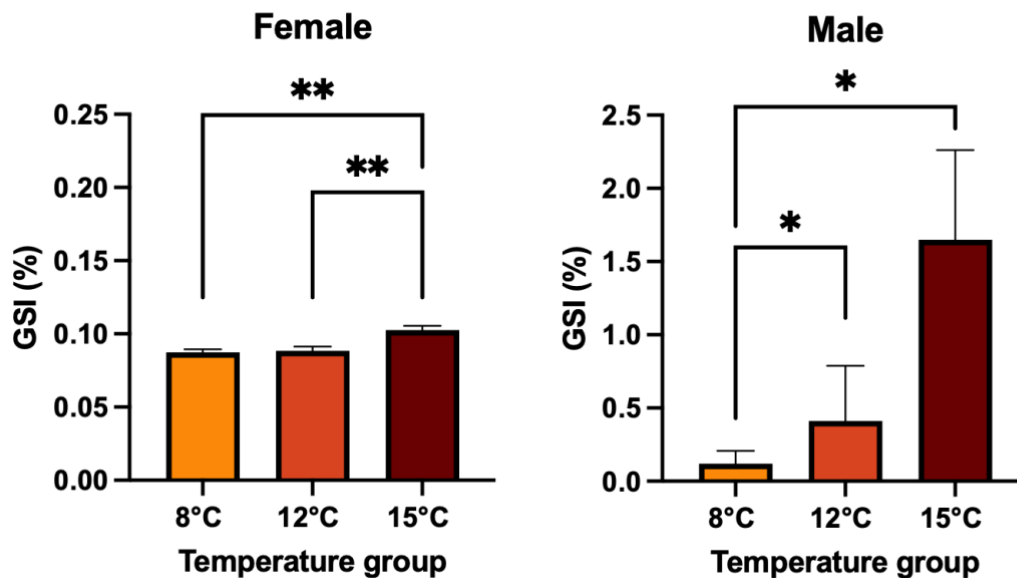
Figure 8: Gonadosomatic index (%) for Atlantic salmon. The figure illustrates the mean ± SEM gonadosomatic index (GSI) for Atlantic salmon as a percentage on the y-axis and the different rearing temperatures (8°C, 12°C, 15°C) on the x-axis. Female and male are analysed separately. A total of 210 fish were used for the experiment, with 70 individuals in each temperature group. Asterisks indicate statistically significant differences between groups ***(p<0.001) **(p<0.01) *(p<0.05). Non-significant results are not shown.

3.2 Feed intake, FCR and gut filling

3.2.1 Feed intake

Feed intake was highest in the 12°C group during the experimental period. Feed intake was lower in the 8°C group compared to both the 12°C ($p<0.0001$) and 15°C ($p=0.0013$) groups (Figure 9) (APPENDIX C, Table 29)
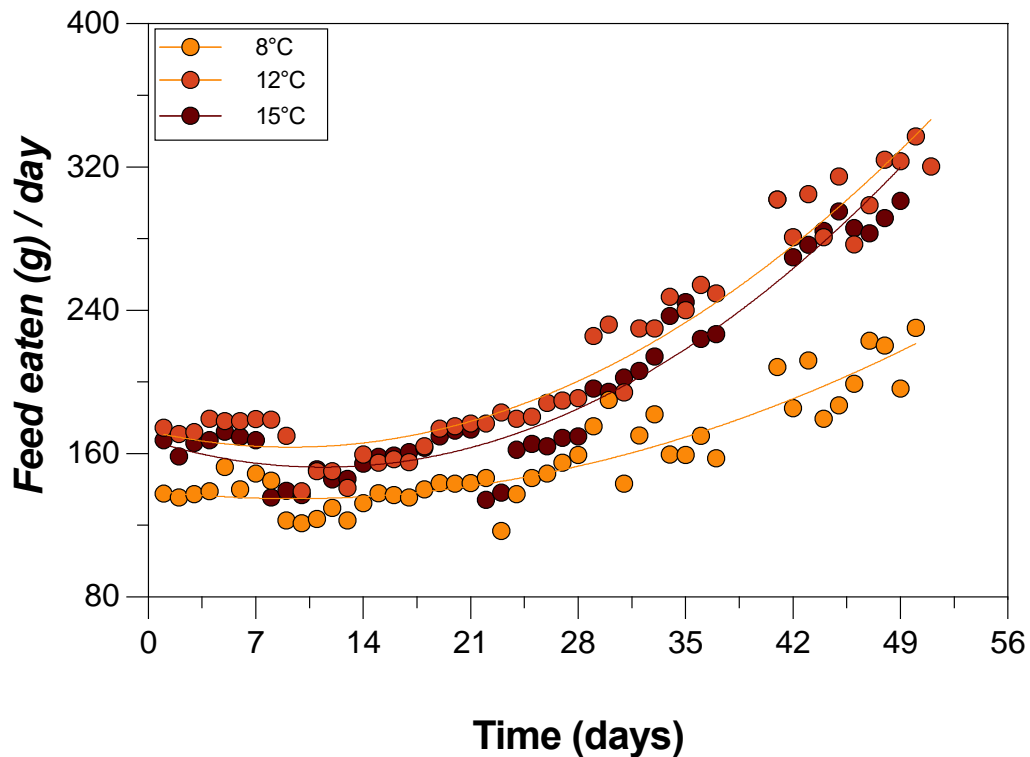
Figure 9: Relationship between daily feed intake (grams per day) and water temperature (°C) in Atlantic salmon. The graph shows individual values of daily feed intake as well as the best-fitted non-linear regression line for Atlantic salmon reared at three different temperatures (8°C, 12°C and 15°C). The numbers on the x-axis represents the days from the start till the end of an 8 week long trial.

3.2.2 Feed Conversion Rate (FCR)

The feed conversion rate (FCR) varied across the three temperature groups. Fish in the 8°C group exhibited a higher mean FCR compared to the 12°C and 15°C groups (Table 2). The 8°C group required more feed per unit of gained biomass, in contrast, the 12°C and 15°C groups showed the same FCR and more efficient utilization of feed.

Table 2: **Feed conversion rates (FCR) for Atlantic salmon.** Table represents the FCR for Atlantic salmon reared at three different temperature groups (8°C, 12°C and 15°C) during an eight-week trial. FCR was calculated for each respective tank, and the average FCR was calculated for the temperature groups.

Treatment	Tank	FCR	Average FCR
8°C	4	0.94	**0.95**
	6	0.99	
	7	0.92	
12°C	8	0.83	**0.78**
	9	0.75	
	10	0.75	
15°C	1	0.92	**0.79**
	2	0.76	
	3	0.70	

3.2.3 Temporal changes in GIT filling in Atlantic Salmon

ST, MG and HG content were analysed separately to investigate the temporal change of gut content. The dissected ST with the sampled content was photographed at the different sampling points sampling to visualise the temporal change in SFI from 2-24 hours post-feeding (Figure 10). The photos show the steady and gradual decrease in ST content from 2-24 h post-feeding in all temperature groups.
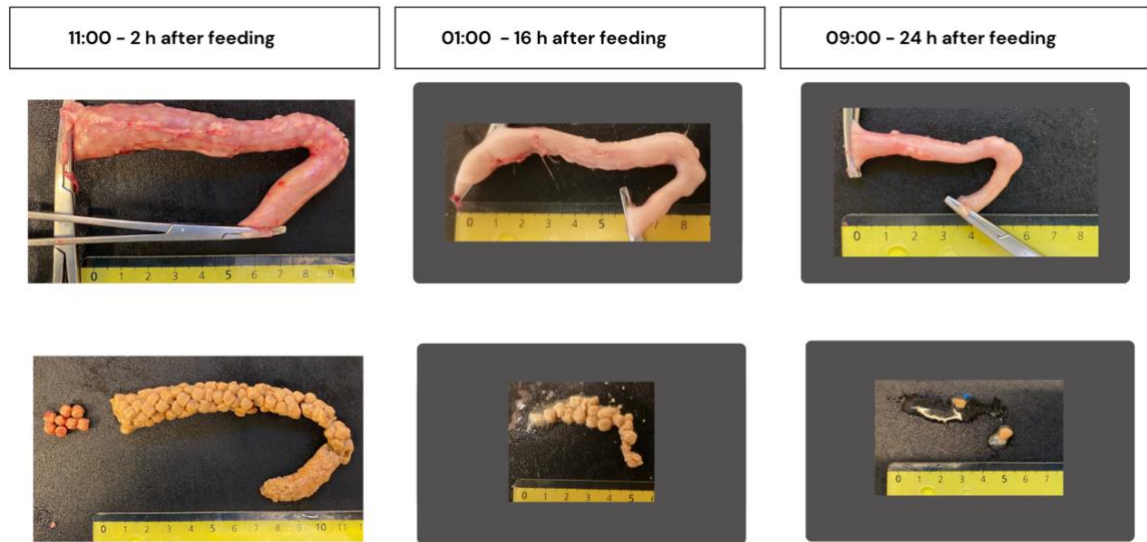
Figure 10: Stomach filling. Illustration of stomach fullness at three sampling points during the 24-hour sampling after the last meal. The illustrated pictures are from Atlantic salmon reared at 12°C.

3.2.2.1 Stomach

Stomach fullness index (SFI) was significantly affected by time (p = 0.0024) in all temperature groups (APPENDIX C, Table 21), where graphs showed a gradual decrease in ST content the following hours after feeding. At the first sampling point, 2 hours post feeding, all temperature groups show similar SFI, where the 12°C reveals the highest SFI (0.77 ± 0.1). There was no statistical difference in SFI between the temperature groups at this sampling point. The ST content evacuation exhibited a more rapid evacuation rate for fish reared at elevated temperatures (12°C and 15°C), while a somewhat slower rate was observed for the 8°C group. (Figure 11). Results from a two-way ANOVA showed a significant difference in ST content over time (p = 0.0024). These findings support that the amount of ST content decreases over time, however, temperature did not have a main effect on the SFI (p = 0.6629) (APPENDIX C, Table 21). Tukey's multiple comparison test revealed a significant difference in ST content across two sampling points (+8 and +16 h). At 8 hours after feeding, the 8°C group showed lower SFI compared to the 12°C group (p=0.0074) and the 15°C group (p=0.0019). At 16 hours after feeding, the 8°C group showed higher SFI % than both the 12°C (p=0.0249) and 15°C (p=0.0396) groups (Figure 11) (APPENDIX C, Table 22).
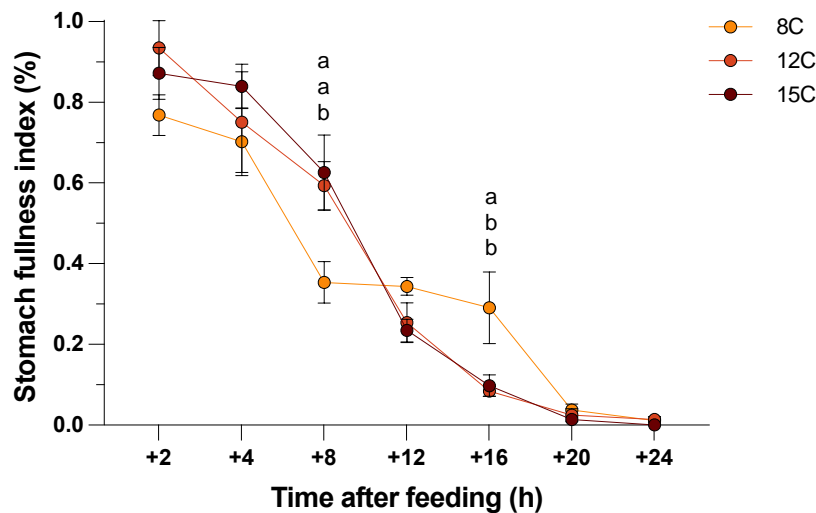
Figure 11: Temporal change in stomach fullness (%) for Atlantic salmon at different rearing temperatures (°C). Degree of stomach fullness for dry content in relation to water temperature and time after feeding in Atlantic salmon. Graphs show the mean± SEM for fish held at different water temperatures (8°C, 12°C, and 15°C) at different time intervals (2, 4, 8, 12, 16, 20, and 24 h) after feeding. The stomach fullness index is on the y-axis, and the time after feeding is on the x-axis. Letters indicate significant differences between groups (same letter means not significantly different, and different letters indicate significant differences in mean).

3.2.2.2 Midgut

Midgut fullness index (MFI) was significantly affected by temperature ($p<0.0001$) and time ($p<0.0001$) (APPENDIX C, Table 24). MFI % was generally lower in the 15°C group during the 24-hour sampling, with a significant difference from 12°C ($p=0.0081$) 2 hours post-feeding. A similar trend in MG filling was observed for all temperature groups, with a unimodal curve. The 12°C group had its highest HFI 8 hours post-feeding, while the 8°C group had the highest index 12 and 16 hours (Figure 12). Tukey multiple comparisons analysis revealed a sgnificant difference in MFI across multiple sampling points (+2, 8, 12, 16, 20, and 24) (APPENDIX C, Table 25), as illustrated in Figure 12.
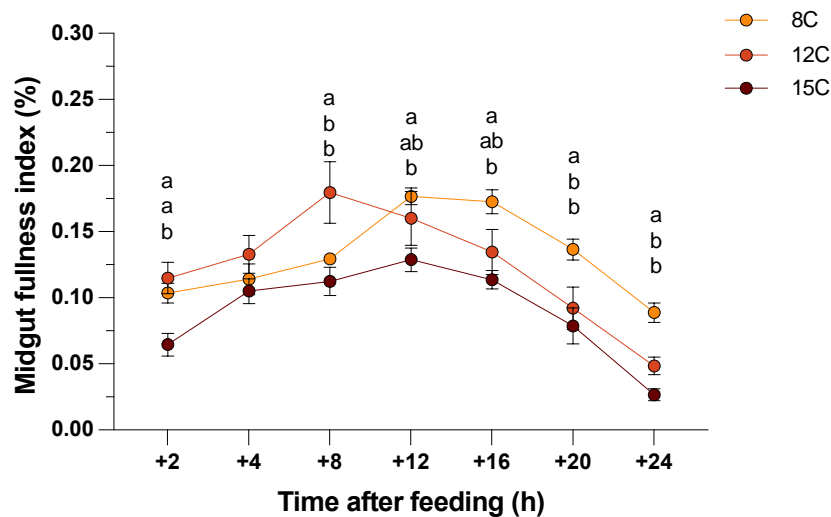
Figure 12: Temporal change in midgut fullness (%) for Atlantic salmon at different rearing temperatures (°C). Degree of midgut fullness for dry content in relation to water temperature and time after feeding in Atlantic salmon. Graphs show the mean MFI ($\pm$ SEM) for fish held at different water temperatures (8°C, 12°C, and 15°C) at different time intervals (2, 4, 8, 12, 16, 20 and 24 hours) after feeding. The midgut fullness index is represented on the y-axis, and the time after feeding is on the x-axis. Letters indicate significantly differences between groups (same letter means not significantly different, and different letters indicate significant differences in mean).

3.2.2.3 Hindgut

Hindgut fullness index (HFI) was significantly affected by temperature (p<0.0001) (APPENDIX C, Table 27). There was a significantly lower degree of filling in the 15°C group 2 hours post-feeding compared to both 8°C (p=0.0029) and 12°C (p<0.0001). Tukey multiple comparison tests revealed a significant difference in HFI across multiple sampling points (+2, 4, 8, 12, and 24). At 24 hours post feeding, the 15°C had a lower degree of filling compared to the 8°C group (p=0.0490) (Figure 13) (APPENDIX C, Table 28).
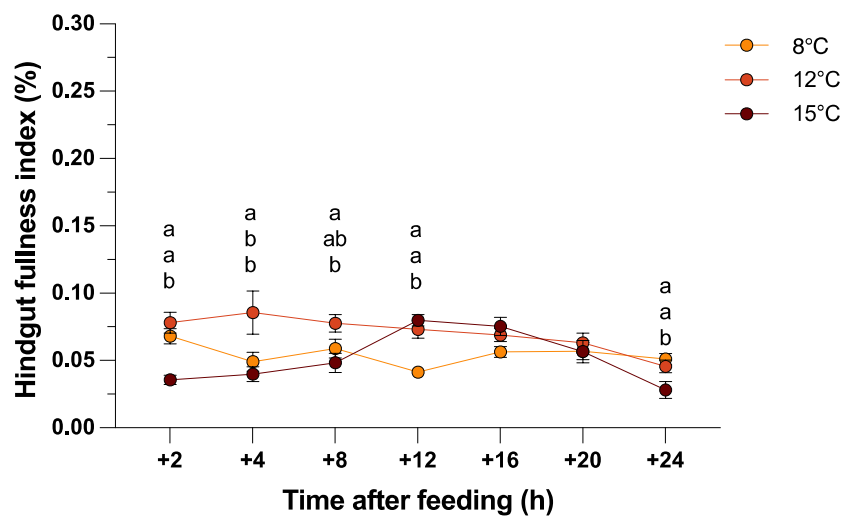
Figure 13: Temporal change in hindgut fullness index (%) for Atlantic salmon at different rearing temperatures (°C). Degree of hindgut filling for dry content in relation to water temperature and time after feeding in Atlantic salmon. Graphs show the mean (± SEM) for fish held at different water temperatures (8°C, 12°C, and 15°C) at different time intervals (2, 4, 8, 12, 16, 20 and 24 hours) after feeding. The hindgut fullness index is represented on the y-axis, and the time after feeding is on the x-axis. Letters indicate significant differences between groups (same letter means not significantly different, and different letters indicate significant differences in mean).

3.2.3 GIT transit

The data presented in Figure 14 demonstrates the temporal distribution of gut content in ST, MG, and HG for each temperature group. 2 hours after feeding, fish in the 15°C group had the highest degree of ST filling, with about 90 % of the total gut content in the ST. The 8°C and 12°C groups had about 80% of the total gut content in the ST at the same sampling point. The stomach fullness decreases gradually for all temperature groups over time. The 12°C and 15°C reached ≈ 50 % stomach fullness after 12 hours, however, the 8°C group reached ≈ 50 % ST fullness between 12 and 16 hours after feeding. 24 hours post-feeding, the 15°C had almost evacuated all content from ST. The gut content was distributed ≈ 51 % in MG and ≈ 47 % in HG, resulting in ≈ 2 % left in the ST. In contrast, the 8°C group had ≈ 59 % in MG and ≈ 35 % in the HG section (Figure 14).
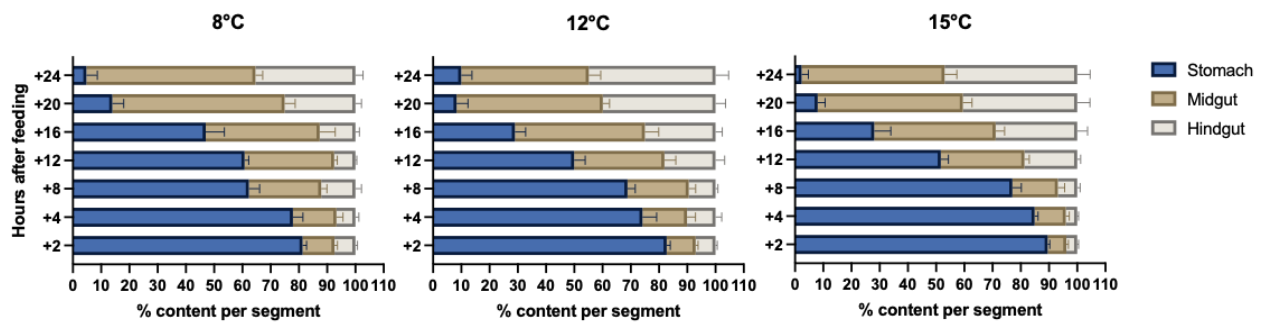
Figure 14: Temporal change of gut content in Atlantic salmon at different rearing temperatures.
The chart illustrates the distribution of dry content and gut transit for each temperature group. Bars show the percentage (%) distribution of dry content in the stomach, midgut and hindgut of Atlantic salmon reared at different temperature groups (8°C, 12°C, and 15°C), 2-24 hours after feeding. The percentage of each segment is calculated as the segments dry content, relative to total amount of dry content in the gastrointestinal tract. Time after feeding is represented on the y-axis and how much content is present in the different gut segments on the x-axis. Graphs show the mean percentage of content (± SEM) in stomach, midgut and hindgut.

3.2.4 Gall bladder index

All temperature groups exhibited a similar temporal trend of decreasing gall bladder index (GBI), 2-12 hours after the meal. Results of a two-way ANOVA show that time (p<0.0001) and temperature (p=0.0005) had a significant effect on GBI (APPENDIX C, Table 30). After 12 hours post-meal, GBI % gradually increases in all temperature groups and reached its peak at 24 hours post-feeding. At 20 hours post-feeding, the 12°C group showed a higher GBI compared to the 8°C group (p=0.0076). At the end of the trial, 24 hours after the meal, the 15°C group had a significantly higher GBI % than the 8°C (p<0.0001) and 12°C (p=0.0049) groups. Additionally, the 12°C group had a significantly higher index compared to the 8°C group (p=0.0003) (Figure 15) (APPENDIX C, Table 31).
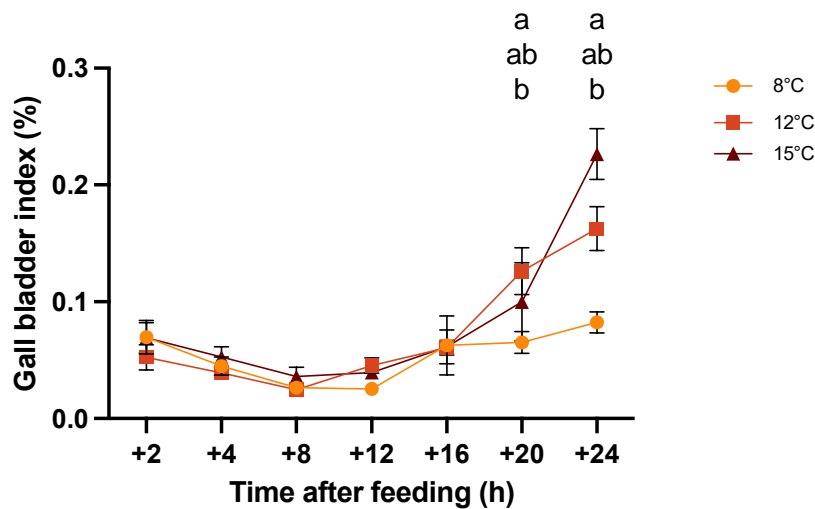
Figure 15: Temporal change in gallbladder index (%) for Atlantic salmon at different rearing temperatures. Data presented as mean ± SEM gall bladder index (GBI). A total of 210 fish were used for the experiment, with 70 individuals for every temperature group. Letters indicate significantly differences between groups (different letters denote significantly mean difference; same letter means not significantly different).

Pearson correlation test showed a moderate negative correlation between GBI and MFI in all temperature groups. The correlation was strongest in the 12°C group(r=-0.6598, R2=0.4354, p<0.0001), and somewhat weaker in the 8°C (r=-0.5495, R2=0.3020, p<0.0001), and 15°C groups (r=-0.5273, R2=0.2781, p<0.0001) (Figure 16). Additionally, the 12°C group revealed weak negative correlations between GBI and SFI, and HFI (APPENDIX C, Table 32).
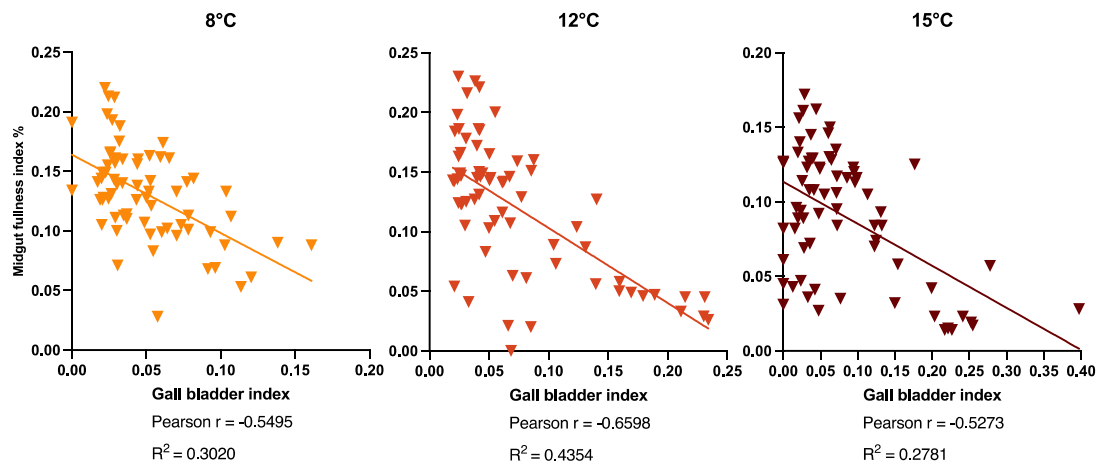
Figure 16: Correlation between gall bladder index and midgut content in Atlantic salmon at different rearing temperatures (8°C,12°C and 15°C). Gallbladder index is expressed as a percentage on the x-axis and midgut content in percentage in ratio to body weight on the y-axis. The line represents the best linear fit, calculated by simple linear regression. Pearson r is the Pearson correlation coefficient where -1 and +1 represent a perfect linear model, and 0 represents no linear relationships between the variables.

3.3 Expression of *ghrl-I*, *ghrl-II* and *mboat4* in the stomach

3.3.1 *ghrl-I*

Two-way ANOVA analysis indicated a significant effect of temperature on the mRNA expression of *ghrl-I* in ST tissue (p<0.05) (APPENDIX D, Table 32). No significant effect on gene expression was found for time (p>0.05). (Figure 17). Tukey's multiple comparisons test identified a significant difference in mRNA expression +16 h after feeding. Gene expression was significantly lower for the 15°C group compared to the 8°C and 12°C groups (p = 0.0129 and p = 0.0171, respectively). (Figure 17). The graph shows a gradual increase in gene expression for the 12°C group, with its highest point of expression 20 hours post-feeding. The 8°C group showed a gradual increase in mRNA expression of *ghrl-I* till it reached its peak at +16 and further decreased to its lowest point 24 hours post-feeding (APPENDIX D, Table 34).
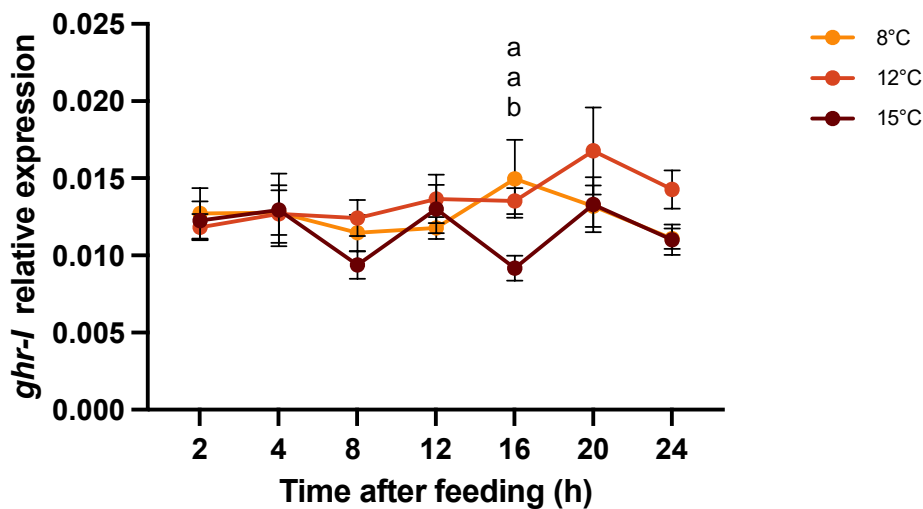
Figure 17: mRNA expression levels of *ghrl-I* in stomach tissue of Atlantic salmon at different rearing temperatures. Graph points represent mean ± SEM (n = 10/group) of normalized mRNA copy number to the total ng of RNA for target gene and the geometric mean copy number of actb and rps20. A total of 210 fish were used for the experiment, with 70 individuals for every temperature group (8°C,12°C and 15°C). A two-way ANOVA test shows significant interaction effects, letters indicate significant differences between groups (same letter means not significantly different, different letters significantly mean difference).

3.3.2 *ghrl-II*

A two-way ANOVA analysis indicated no significant effect of either time or temperature on the expression of mRNA for *ghrl-II* in ST tissue (p>0.05) (APPENDIX D, Table 35). The graph shows a gradual increase in *ghrl-II* gene expression for the 12°C group, with the lowest expression 2 hours post-meal and the highest expression 24 hours post-feeding. The 8°C group showed a gradual increase in mRNA expression of *ghrl-II* till it reaches its peak at +16 and further decreased to its lowest point 24 hours post-feeding (Figure 18), similar as *ghrl-I* expression (Figure 17) (APPENDIX D, Table 36).
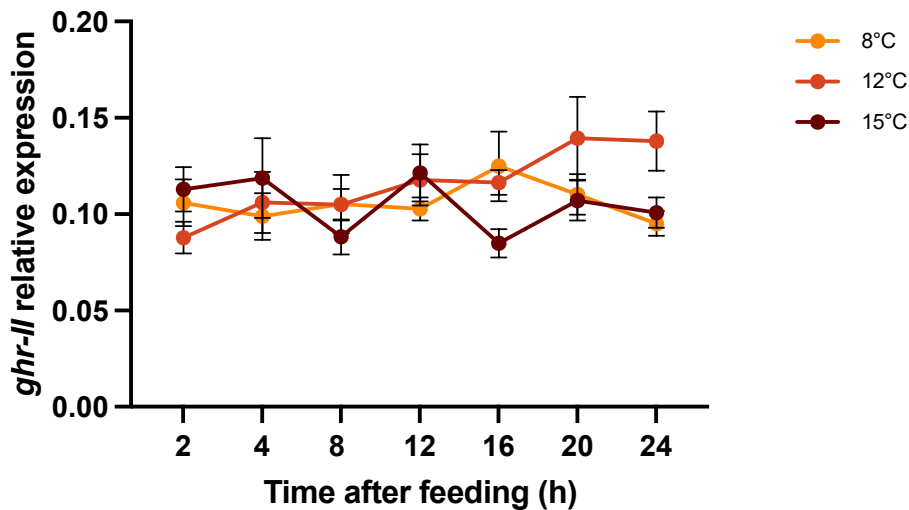
Figure 18: mRNA expression levels of *ghrl-II* in stomach of Atlantic salmon at different rearing temperatures. Graph points represent mean ± SEM (n = 10/group) of normalized mRNA copy number to the total ng of RNA for target gene and the geometric mean copy number of *actb* and *rps20*. A total of 210 fish were used for the experiment, with 70 individuals for every temperature group (8°C,12°C and 15°C). A two-way ANOVA test shows significant interaction effects, letters indicate significant differences between groups (same letter means not significantly different, different letters significantly mean difference).

3.3.3 *mboat4*

A two-way ANOVA analysis showed no significant effect of either time or temperature on mRNA expression for *mboat4* in ST tissue ($p > 0.05$) (APPENDIX D, Table 37) except for one sampling point. The graph shows similar expressions in all temperature groups +2 h post-feeding and +24 h post-feeding. Expression for the temperature group 15°C had a significantly higher expression than the 12°C group ($p = 0.0076$) at +12 hours post sampling (Figure 19) (APPENDIX D, Table 38).
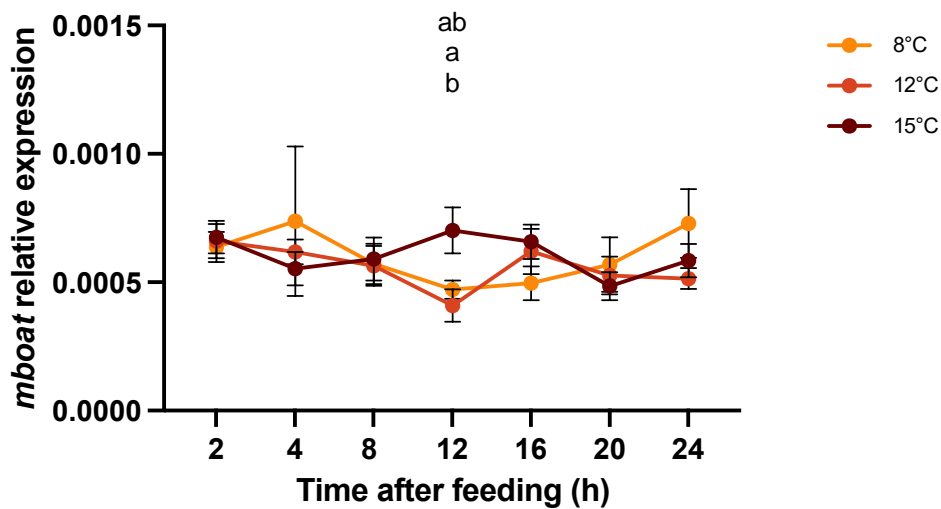
Figure 19: mRNA expression levels of *mboat4* in stomach of Atlantic salmon at different rearing temperatures. Graph points represent mean ± SEM (n = 10/group) of normalized mRNA copy number to the total ng of RNA for target gene and the geometric mean copy number of *actb* and *rps20*. A total of 210 fish were used for the experiment, with 70 individuals for every temperature group (8°C, 12°C and 15°C). A two-way ANOVA test shows significant interaction effects, letters indicate significant differences between groups (same letter means not significantly different, different letters significantly mean difference).

3.3.4 Correlation between stomach filling and mRNA expression

A Pearson correlation test was performed to determine correlation between SFI and *ghrl-I, ghrl-II*, or *mboat4* expression in different temperature groups. The results indicated that there was no significant correlation between SFI and mRNA expression of any of the target genes in any of the temperature groups, as presented in Figure 20. (APPENDIX D, Table 39).
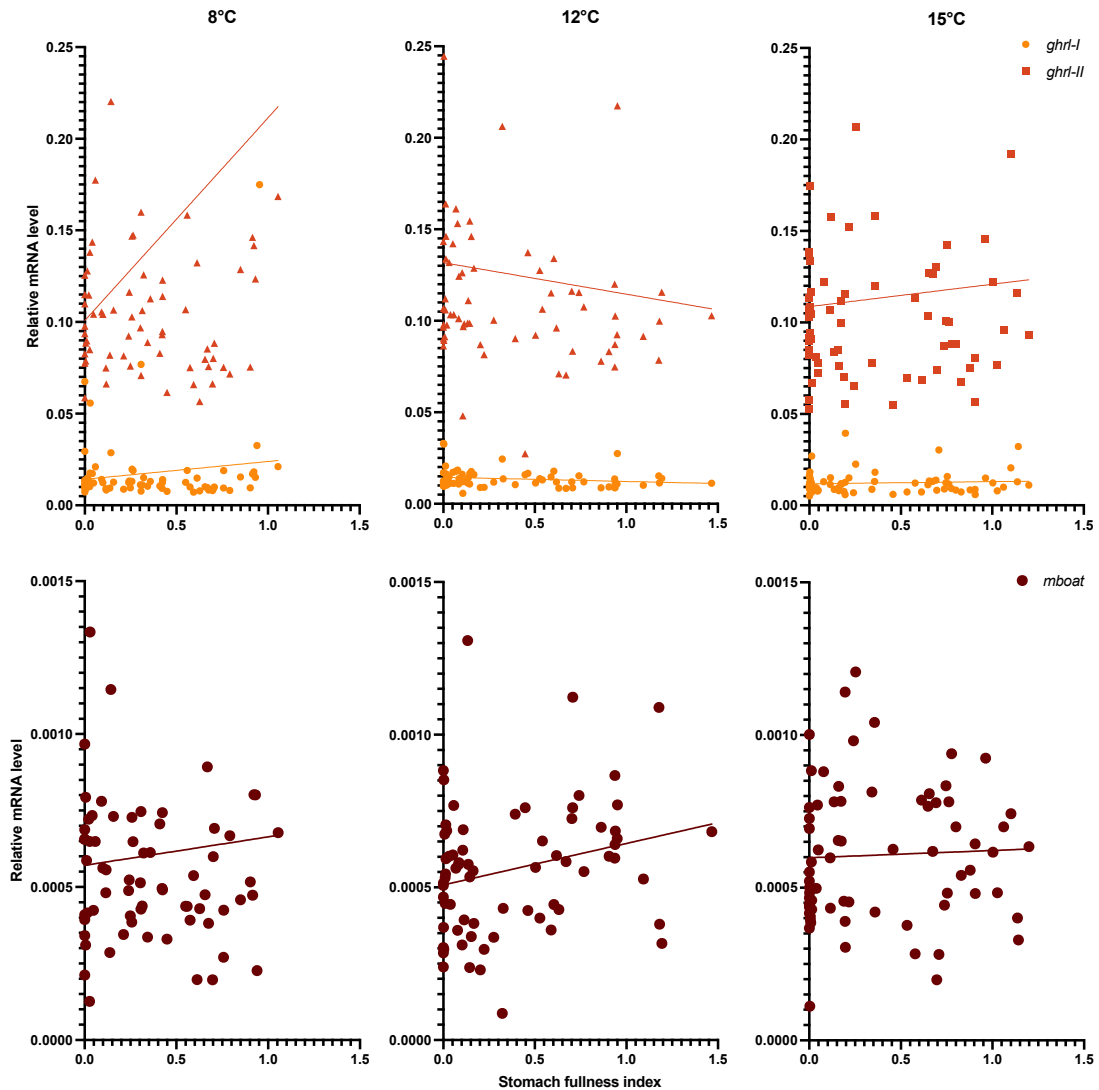
Figure 20: Correlation between degree of stomach fullness (%) and expression of mRNA for *ghrl-I, ghrl-II* and *mboat4* in Atlantic salmon. Stomach fullness percentage is expressed as weight of dry stomach content standardized by wet body weight. The relative mRNA level is the normalized mRNA copy number to the total ng of RNA for target gene and the geometric mean copy number of *actb* and *rps20*. The line in all six graphs represents the best linear fit, calculated by simple linear regression.

3.4 Ghrl levels in plasma

There were identified different temporal trends in Ghrl plasma levels for the different temperature groups (APPENDIX E, Table 40). 2 hours post-feeding, Ghrl plasma concentration was significantly higher for fish reared at 12°C than fish reared at 8°C (p=0.0025) and 15°C (p=0.0228). Ghrl levels increased rapidly from 2-4 hours post-feeding in the 15°C group, while there was a slower increase in the 8°C group (p=0.0369). In contrast, the graph shows that the 12°C group had a gradual decrease in expression. At 24 hours post-feeding, the 8°C group had its highest expression, in contrast to both the 12°C (p=0.0023) and 15°C (p=0.0063) groups (Figure 21). A two-way ANOVA showed significant differences between temperature groups at specific time points (p=0.0122). Tukey's multiple comparisons test identified a significant difference in plasma Ghrl concentration 2,4 and 24 h after feeding (APPENDIX E, Table 41).
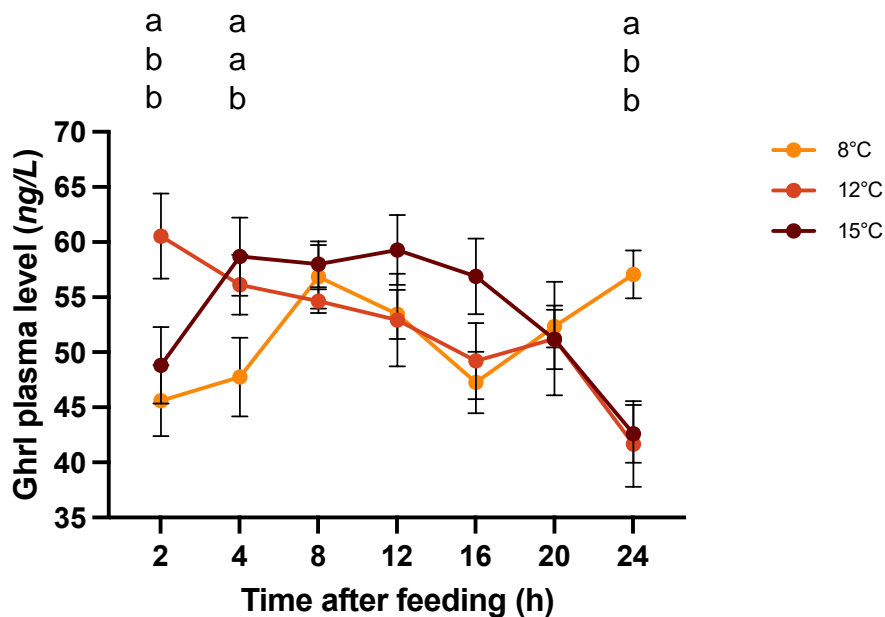
Figure 21: Plasma Ghrl level of Atlantic salmon reared at different temperatures, +2 h to +24 h after feeding. A total of 210 fish were used for the experiment, with 70 individuals for every temperature group (8°C,12°C and 15°C). Graph points represent mean ± SEM (n = 10/group). The graph is presented as a fitted non-linear regression curve. A two-way ANOVA followed by Tukey multiple comparison tests was used to analyse the effect of time and temperature. A two-way ANOVA test shows significant interaction effects, letters indicate significant differences between groups (the same letter indicates no significant different means and different letters indicate significant mean difference).

A Pearson correlation analysis of the relationship between Ghrl plasma levels and various parameters related to the digestive system and appetite regulation in Atlantic salmon at different temperatures was performed. The findings will be summarized in temperature groups.

3.4.1 Ghrl plasma levels in LT group (8°C)

There was no correlation of significance between Ghrl plasma levels and *ghrl-I, ghrl-II* or *mboat4* mRNA abundance. No significant correlations were observed between Ghrl plasma levels and SFI, MFI, HFI or GBI. (APPENDIX E, Table 42)

3.4.2 Ghrl plasma levels in C group (12°C)

There was a weak positive correlation between Ghrl plasma levels, and SFI (r=0.3452, R^2=0.1192, p=0.0042). Ghrl plasma levels showed no significant correlations with *ghrl-I, ghrl-II, mboat4*, MFI, HFI, or GBI. (APPENDIX E, Table 42)

3.4.3 Ghrl plasma levels in HT group (15°C)

A moderate significant correlation was observed between Ghrl plasma levels, MFI (r=0.5111, R^2=0.2612, p<0.0001). While a weak negative correlation was observed between Ghrl plasma levels and GBI (r=-0.4007, R^2=0.1605, p=0.0006. No significant correlations were observed between Ghrl plasma levels and *ghrl-I, ghrl-II, mboat4*, SFI or HFI. (APPENDIX E, Table 42)

4. Discussion

4.1 Discussion of materials and methods

4.1.1 Experimental design

This study considers three different rearing temperatures to represent low (8°C), control (12°C) and high-temperature (15°C) conditions and their effect on appetite, feed intake and growth in Atlantic salmon. Triplicate tanks within each temperature group were used to increase the statistical power of the experiment. The experimental design and temperature range should give a good representation of the span of temperature Atlantic salmon may experience in the Norwegian fjords. Before the 24-hour sampling, the fish had been exposed to the respective temperatures for 8 weeks to enable the fish to acclimatise to the new temperatures and feeding regime. In addition to temperature, photoperiod is one of the major regulators for growth in Atlantic salmon. Photoperiod in Atlantic salmon is a synchroniser for growth, smoltification and maturation, whereas photoperiod manipulation often is used to optimise the production of Atlantic salmon (Martinez *et al.,* 2021). Two commonly used photoperiods are LD24:00 and LD12:12. LD24:00, or constant light, is commonly used to mimic standard commercial production and optimise growth (Hansen *et al.,* 1992). However, a combination of high temperatures and constant light (LD24:00) can cause early sexual maturation, which is undesirable (Fjelldal *et al.,* 2011). In this study a light regime of LD12:12 was used, as it simulates natural winter conditions and was used throughout the experimental period (Björnsson *et al.,* 2000). This regime was chosen to facilitate for a natural photoperiod regime.

4.1.2 Controlled variables (dissolved oxygen, salinity, temperature, feed)

To achieve trustworthy justifications for the relationship between the independent variable (temperature) and dependent variable (feed intake), an experiment should be arranged in a way that other environmental factors are under constant control. This study strictly controlled and monitored dissolved oxygen, salinity, and temperature during the duration of the experiment. The temperature was the most important environmental parameter in this trial and was strictly monitored and controlled. The mean values $\pm$ SEM was calculated, resulting in (LT, 8.4 $\pm$ 0.00; C, 12.3 $\pm$ 0.00 ºC; HT, 14.8 $\pm$ 0.01) (APPENDIX A). The values were rounded (up/down) and referred to as the 8°C, 12°C and 15°C groups. In addition to automatic sensors monitoring temperature and salinity, one person physically monitored and logged the temperature for each tank and water supply tank every day. The oxygen in all experimental tanks was kept above 90% O_2 saturation by automatically bubbling pure O_2 into the header tanks. The sensors that monitored temperature and oxygen tended to become overgrown by biological waste (biofilm), which resulted in a lower oxygen saturation being reported. A daily routine was therefore implemented to clean the sensors to get a better and more realistic representation of the environment in the tanks.

The amount of feed administered to each tank was adjusted in accordance with predicted biomass for each tank, and fish were always overfed. This resulted in differences in feeding amounts between the tanks. Feed intake was assessed by collecting and quantifying uneaten feed from each tank. The study aimed to investigate gut transit, and ideally, the fish should have an equal relative feed intake to standardize conditions. However, this can only be achieved by force-feeding and would have affected the fish negatively (Bolasina *et al.,* 2007; Peters, 1982). The data in this study therefore represent voluntary feed intake when the fish is offered a meal in excess once a day.

4.1.3 Sampling for GIT transit calculations

Seven samplings per temperature group were conducted to assess the GIT compartmental filling and transit of digesta. The sampling and handling procedures used in this experiment followed standardized protocols from previous studies in the research group (Kalananthan *et al.*, 2023). Some modifications were made to the protocol as the experiment had a strict time schedule to standardize conditions for all triplicates within the temperature groups. During the 24-hour trial, the sampling teams worked shifts, one day and one night team. Day and night teams were the same for the whole sampling period, facilitating sampling accuracy. Before the first sampling, personnel from day and night teams trained together to ensure more precise and standardized samples though the whole period. This was a measure to ensure accuracy and to limit the individual variation between samples. Even though the group trained together beforehand, there may still be some individual variation in sample accuracy between the day and night teams.

During the samplings, different biological samples were collected from the fish. To minimize handling and stress for the remaining fish in the tanks, all fish (n=10) were collected at the same time and immediately euthanised. After euthanasia, fish were kept on ice until further processed. All plasma samples were carefully handled and placed on ice within a short period to avoid any degradation. To avoid RNA degradation, all tissue samples, in particular ST were processed fast and transferred immediately to RNA later. After sampling, ST tissue were stored at 4°C (fridge) overnight to allow the solution to thoroughly penetrate the tissue before being stored at -80°C until further analysis. In addition to collecting tissue, feed waste was collected from all tanks. A simple method was used to collect feed waste from each tank by separating water and waste using a simple mesh. In advance of the experiment, a simple test on pellet was conducted to quantify to what extent the pellet size expanded in water. This was to improve the calculation of feed intake, and further determine the FCR. It should be taken into consideration that on some occasions, there was undigested food present when the feed waste was weighed. Ideally, the undigested food should be separated from the uneaten pellets as it could add some extra weight to the "feed waste", however, the amount of undigested food was not taken into consideration in the calculations.

4.1.4 Statistical analysis

Prior to performing statistical analysis, ROUTs outlier test and normality test were performed to ensure that an ANOVA analysis could be performed on the datasets. A one-way ANOVA analysis was chosen as statistical analysis for data with one independent predictor variable/factor. A two-way ANOVA analysis was chosen as a statistical analysis of the data with two independent predictor variables. For datasets that did not pass the normality test (mRNA expression of *ghrl-I, ghrl-II* and *mboat4),* values were log-transformed. As the different response variables were examined, a *post-hoc* analysis of multiple comparison tests was considered ideal for all combinations of response- and predictor variables. The post-hoc analysis (Tukey's multiple comparisons test) was beneficial in this trial as it provided valuable information about the variation between temperature groups at specific sampling points. This allowed us to investigate the temporal changes of SFI, MFI and HFI, mRNA expression of target genes, Ghrl plasma levels and GBI. As in most other biological studies, a p-value of >0.05 was utilized as the significance level for the statistical analysis (Fay & Gerow, 2013).

One statistical analysis that should be discussed is the analysis of GSI. GSI was analysed for females and males separately. When running the ROUTs outlier test on males, there were detected several rather large outliers. In this analysis, the outliers characterised mature fish, or fish starting to mature. In temperature group 15℃, there were, in total, seven mature fish, which resulted in an elevated total mean GSI for the group. In the 12℃ the GSI was elevated because of one mature male. It was discussed whether to include or exclude the outliers and ultimately decided to include them. There were, in total 107 males, whereas 35 of the individuals were part of the 15℃ group. Seven out of the 35 individuals had GSI > 2 %, which represents 20 % of the population. In addition, a Pearson correlation test was performed to investigate the relationship between GSI and SGR for all temperature groups. The negative correlation between GSI and SGR in males for temperature groups 12℃ and 15℃ strengthened the decision to include the outliers.

The number of individuals per sampling point was n=10, which was a satisfying sampling size in this experiment. There was a tight time schedule during the 24-hour sampling, where a higher number of individuals could have led to logistical difficulties. Increasing the number of individuals may have enhanced the precision of the sample mean, yet it

could have led to a delay during the sampling process, which could have had an adverse effect on the results.

4.1.5 Gene expression analysis

In addition to analysing ST tissue for the three target genes *ghrl-I, ghrl-II* and *mboat4*, two reference genes, *actb* and *rps20,* were also analysed. To avoid large errors and to increase the accuracy of the results, it is recommended always to use at least two reference genes (Kozera & Rapacz, 2013), where *actb* and *rps20* are commonly used reference genes for Atlantic salmon (Olsvik *et al.,* 2005). The reference genes were used to normalise target genes and to calculate a relative mRNA expression. During analysis, the expression of *actb* was less stable than predicted, therefore, it was decided to rerun the qPCR plate for *actb* twice. The new analysis revealed similar results in terms of Cq and efficiency values, which could indicate that the unstableness was due to some technical mistakes. The geometric average was used to normalize the reference genes (*actb* and *rsp 20)*, as the method has been shown to add accuracy to gene expression analysis (Hellemans *et al.,* 2007; Vandesompele *et al.,* 2002). Relative quantification of mRNA is a valid method of determining gene abundance (Bustin, 2000).

4.2 Discussion of results

The aim of this study was to investigate temperature affects appetite, feed intake and growth in Atlantic salmon. The study aimed to describe if there is a link between gut transit and the mRNA expression in stomach between ghrelin (*ghrl-I* and *ghrl-II)* and *mboat4*. Additionally, the study aimed to document if there was a correlation between feeding status, stomach filling, ghrelin expression in stomach and ghrelin plasma levels.

4.2.1 Effects of Temperature on growth performance

Multiple points can be discussed from the experimental results. Firstly, it was hypothesised that temperature would have a significant impact on feed intake and growth. This hypothesis was proven to be true. Further, it was hypothesised that both feed intake and growth would be highest in the 15℃ group and lowest in the 8℃ group, which was partially true. The 8℃ had the lowest growth and feed intake during the experimental period. However, the 12℃ group had the best growth rates in terms of SGR, RGR and K-factor during the eight weeks trial. Water temperature is a key environmental factor that affects the growth of Atlantic salmon (Volkoff & Rønnestad, 2020). All ectotherms have a thermal tolerance range which determines the organism's performance, and it is generally accepted that an increase in temperature within the optimal range results in increased food intake and growth rates and declines when out of this range (Huey & Stevenson,1979; Miller & Stillman, 2012; Volkoff & Rønnestad, 2020). The results of this current study agree with previous reports that show increased growth rates at elevated temperatures (Handeland *et al.,* 2000; Handeland *et al.,* 2003). In this study, SGR and RGR were used to evaluate the increased growth per day and increased growth over time, respectively. SGR gives an indication of increased daily growth, which is practical when comparing groups in short-term and nutrition experiments (Lugert *et al*., 2016). A study by Handeland *et al*. (2008) suggested that the optimum temperature for growth in Atlantic salmon post-smolts (170-300 g) is roughly 14℃. The study used mean SGR ± SE as the indicator of growth, and based on their findings, one would expect a higher SGR in the 15℃-group compared to the 12℃ groups in this current study. However, there was no significant difference in growth (SGR) between the 12℃ and 15℃ group (Figure 5). All fish were reared with the same oxygen conditions, salinity, photoperiod, and feeding schedule. This indicates that the variation in growth between the 8℃ group and the other groups must be explained by either internal physiological factors or temperature.

Biochemical reaction rates increase when temperature increases in ectothermic fish, which increases the standard metabolic rate.(van de Pol *et al.,* 2017; Volkoff & Rønnestad, 2020) The results of this study imply that there could have been significant differences in metabolic rate due to differences in rearing temperatures, thereby caused differences in growth. There are physiological processes that could have impacted growth in this study. Mature males were observed in all temperature groups, with a significantly higher proportion of maturation in the 15°C group. 20 % of the males in the 15°C group had started to mature, and a correlation of negative order was observed between GSI and SGR for 12°C and 15°C groups. The negative correlation between GSI and SGR suggest that the 15°C has had higher energy allocation towards maturation, resulting in differences in growth between the groups. Development and energy allocation into the gonads involve major physiological changes, which also affect the appetite. During the early stages of maturation, the feed intake increases, however, it decreases later in the maturation process. Early maturation in farmed Atlantic salmon is a common challenge as it will result in decreased muscle (fillet) growth as energy is allocated to reproduction rather than growth. (Aksnes *et al.,* 1986) Energy can also be stored in the liver, and how much is depends on age, sex, nutritional status, and maturation status. While some surplus energy is stored in liver, some may also be stored as intramuscular fat or visceral fat. (Kryvi & Poppe, 2016) To investigate the energy storage in fish, researchers use HSI, which is a measure of the relative size of the liver relative to the total body weight of fish (Higgs *et al.,* 2009; Chellappa *et al.,* 1995). In this study, the lowest HSI was observed in the 15°C group, significantly lower than the two other temperature groups. These results may suggest that as temperature increases in this experiment, the energy was allocated towards gonadal development and growth, resulting in less energy stored in the liver. Metabolic processes in fish are affected by temperature (Prosser & Nelson, 1981), which may lead to changes in liver size and function. As the liver is an essential organ for energy storage and metabolism, HSI is commonly used to evaluate fish's nutritional condition and health status. (Chellappa *et al.,* 1995) Combining the HSI results with the GSI, the findings suggests that more energy has been allocated towards gonadal development rather than somatic growth in the elevated temperature group. Elevated temperatures have previously been documented to influence both maturation and energy storage in the liver (Adams & Thorpe, 1989; Chellappa *et al.,* 1995). The result of this current study supports the previous knowledge about how temperature impact maturation in male Atlantic salmon.

4.2.2 Feed intake and FCR

It was hypothesised that temperature would have a significant effect on feed intake, which was proven to be true. Further, it was hypothesised that both feed intake and growth would be highest in the 15°C group and lowest in the 8°C group, which was proven partially true. The feed intake during the 8-week trial was lowest in the 8°C group and highest 12°C group, only slightly higher than the 15°C group (Figure 9). This indicates that fish at higher rearing temperatures ate more. Voluntary food intake and growth usually increase with increased temperatures (Brett, 1971; Prosser & Nelson, 1981; Volkoff & Rønnestad, 2020). The 12°C and 15°C groups had an overall greater growth and feed intake during the 8-week trial. As the experiment relied on overfeeding the fish, the amount of feed given was increased when biomass increased. During the 8 weeks, there were certain days when little feed waste was collected in the waste collectors for the 12°C and 15°C groups. This suggested that the fish in these groups required more feed. The amount of feed was upregulated in all temperature groups to achieve sufficient overfeeding in all groups. In addition to investigating the feed intake during the 8-week experimental period, SFI from 2 hours post-feeding was used as an estimate of the feed intake during a single meal All groups showed similar SFI 2 hours post-feeding, indicating that they ate equal amounts of feed relative to their body weight (approx. 0,7-0,9 % of BW). However, the SFI was slightly higher in the 12°C and 15°C. The results of the current study partially agree with the results from Handeland *et al.* (2008), who found that there is a close link between feed intake and temperature. They found that fish reared at 14°C had the highest feed intake, whereas minimum feed intake in their study was at 18 and 6°C (Handeland *et al.,* 2008). This indicates that moderately high temperatures result in higher feeding activity. Based on this, one could predict that the feed intake would be higher in the 15°C compared to the 12°C group. Feed waste was a measure of feed given minus the feed waste. The FCR was calculated to obtain a measure of feed intake in relation to the biomass for the different groups. The FCR was lower for fish reared in the 12°C and 15°C groups and higher for the 8°C group. FCR is a measure of how efficiently the fish converts feed into weight gain. A low FCR value indicates that the feed is efficiently converted into weight gain, whereas a higher value indicates that more feed is required per unit of gained biomass. (Bai *et al.*, 2021) This indicator is an important biological indicator for economic performance. The mean FCR values in this current study are generally lower than common in commercial aquaculture, where it

typically yields an FCR of 1.1-1.4 (Aas *et al.*, 2022). The FCR in Norwegian aquaculture has improved due to better feeding strategies, new technologies and better feed composition. A study on Atlantic salmon reared at 4.3°C, 9.4°C and 14.3°C revealed that the optimum temperature for FCE for fish in seawater was 13°C (Handeland *et al.,* 2003). The FCE a measure of the efficiency of which feed is transformed into body mass, differentiating from FCR, which calculates the amount of feed required to yield a specific amount of body mass (Bai *et al.,* 2021; Handeland *et al.,* 2003). Their experiment followed the two strains of fish from freshwater through smoltification and further transfer to sea. FCR was reduced the following 2 to 7 weeks after transfer to sea (Handeland *et al.,* 2003). Another study by Handeland *et al* (2008) revealed similar FCR values (0.5-0.8) for fish reared at different temperatures 4-8 weeks after transfer to sea (Handeland *et al.,* 2008). A third study investigated the growth performance of fish fed three different diets. It investigated the FCR for fish during a period of 6 weeks in freshwater, the period through smoltification, followed by a 3-month growth period in salt water. Results revealed that the fish had generally lower FCR in the saltwater phase, with a mean FCR of 0.80-0.86 during this period (Espe *et al.,* 2020). The fish of this current study was followed 8 weeks in seawater after smoltification. The results revealed a higher FCR in the 8°C group, in contrast to 12°C and 15°C, which showed an equal mean FCR value. This suggests that the 8°C required more feed per unit of gained biomass compared to the other two groups. The FCR values in this trial are very low, indicating a very efficient utilization of the feed. The efficient utilization could be due to the fish being in an environment with minimal stressors, low density, and limited physical activity, resulting in more energy being allocated directly to growth. Combining feed intake during the 8-week experimental period, SFI and FCR, one can with certainty state that the 12°C and 15 °C had the overall best appetite, where it was generally lower in the 8°C group. However, the low FCR could also indicate insufficient control of the collection of surplus feed combined with some inclusion of faeces that would have contributed to lower FCR. Thus, to calculate precise FCR, improved systems for the collection of uneaten pellets and faeces should be implemented in the experimental set-up.

4.2.3 The effect of temperature on temporal changes in GIT compartmental filling

It was hypothesised that there would be differences in the stomach-, midgut- and hindgut filling between fish at the different rearing temperatures during the 24 hours post feeding. This hypothesis was partially true as both midgut and hindgut fullness were significantly affected by temperature. Stomach fullness index (SFI) 2 h after the meal was not influenced by temperature in this trial. This means they ate a similar relative amount of feed during the last meal. Nevertheless, there were temporal differences in SFI between the different temperature groups, suggesting differences in stomach evacuation. Stomach filling is an important short-term regulator for regulating meal size and the frequency of meals, it is considered critical in regulating gut transit rate (Camilleri, 2015; Rust, 2003; Sam *et al.,* 2012). The stomach serves as a reservoir for ingested feed, where it responds to both mechanical and chemical stimuli (Grove *et al.,* 1978; Krogdahl, 2001; Latorre *et al.,* 2016). Research suggests that appetite returns when the stomach is empty and that temperature affects the stomach evacuation rate (Gwyther & Grove, 1981; Jobling *et al.,* 1977). The results indicate that fish ate equal amounts in relation to body mass in all temperature groups. In addition, the gut transit differed between temperature groups. The 15°C group revealed a more rapid evacuation rate than both 8°C and 12°C. Temperature impacts the secretion of digestive juices, GIT motility, and the activity of digestive enzymes. Lower water temperatures may limit nutrient digestibility by slowing digestion, increasing gut transit time, and decreasing gastrointestinal evacuation rates. (Aas *et al.,* 2021; Handeland *et al.,* 2008; Mock *et al.,* 2022; Volkoff & Rønnestad, 2020) The results support that gut transit time increases with lower temperatures and increases with higher temperatures. The 15°C had the fastest gut transit, indicating more effective digestion. As a result of a higher gut transit- and digestion rate in this group, the fish had less content from the previous meal in the GIT at 24 hours. This would have resulted in a higher proportion of gut content in the ST 2 hours after feeding compared to the other two groups. Researchers have hypothesised that feed consumed in a following meal is equivalent to the amount of digesta (or chyme) transferred from the ST to the MG. This suggests that fish might eat more if they have evacuated a large portion of the ST content (Huebner & Langton, 1982), the results of the current study partially contradict this. The 12°C group had the highest feed intake during the eight-week trial and the highest ST filling during 24-hour sampling. In contrast, when investigating the gut transit, the 15°C group had the greatest proportion of feed in the stomach, the greatest ST evacuation rate and digestion. Understanding the mechanisms behind gut transit may be critical for

understanding the optimal utilization of feed in Atlantic salmon. The findings of this study suggest that digestion increases with temperature, resulting in a higher gut transit rate. Further the results on gut transit suggests that enabling the stomach to empty before the next meal affects the gut transit rate.

The GBI showed the same temporal trend in all temperature groups. The index was low right after feeding, followed by a gradual increase, reaching its highest point 24 hours after the last meal. The highest index at the end of the sampling period was observed in the 15°C group and the lowest in the 8°C group. The gallbladder is a small organ that stores and releases bile. Bile is produced in the liver and released into the proximal intestine in response to the anorexigenic GI peptide CCK (Raybould, 2007). CCK is an important factor for regulating short-time satiation and food intake, as it promotes and control digestion and has a key role in regulating the pace of gut transit (Rønnestad *et al.,* 2017). When CCK is secreted, it stimulates contractions of the gallbladder, which results in a discharge of bile (Ivy & Oldberg, 1928). Previous studies have identified that the gallbladder becomes progressively fuller when fish is starved (Talbot & Higgins, 1982), which is consistent with the results of this study. Temperature was identified to have a significant effect on the GBI with great differences 24 hours post-feeding where it was highest in the 15°C group and lowest in the 8°C. A negative correlation between MFI and GBI was observed for all temperature groups. Researchers have found that the secretion of bile is highly influenced by midgut content (Krogdahl, 2001; Mock *et al*, 2022; Rust; 2003), the result of this present study strongly support this. Considering that the 15°C group had a higher GBI than both 12°C and 8°C groups, and the gut transit was highest in the 15°C group, one could suggest that a higher gut transit rate is somewhat dependent on midgut fullness and evacuation. As CCK is the initiator for gall bladder contraction, it could have been interesting to investigate the relationship between CCK expression, MFI, GBI and concentration to understand more about how these factors are involved in the digestive process, gut transit, and appetite control.

4.2.4 Effect of feeding status on mRNA expression of key genes in stomach

It was hypothesised that stomach fullness would significantly affect the expression of *ghrl-I, ghrl-II* and *mboat4* in stomach tissue of Atlantic salmon, but this hypothesis was rejected. The results from this current study show that there is no correlation between SFI and relative mRNA expression of *ghrl-I* or *ghrl-II* in ST tissue in either temperature groups. The temporal change in SFI indicates that changes in feeding status from 2 hours to 24 hours post-feeding have not significantly affected the expression of either of *ghrl-I* or *ghrl-II* in ST tissue. Ghrelin has been studied for years in humans due to its involvement in metabolic energy balance, appetite, and food intake regulation, as it could help us understand obesity and eating disorders (Vikeså, 2020). There are few studies of how ghrelin affects appetite in Atlantic salmon, but it should be of great interest for fish farmers as feed intake optimisation is key for salmon farming to utilise its protentional growth. In humans, the ST is the primary site of ghrelin synthesis (Kojima *et al,* 1999), which is the basis for investigating gene expression in ST tissue in this study. In mammals, ghrelin has been shown to influence gastrointestinal motility and gastric acid secretion, which facilitates more effective digestion of food (Cummings, 2006). There is still limited knowledge about ghrelin's role on appetite and digestion in salmon, but Moen and colleagues found an up-regulation of *ghrl* in salmon larvae at first feeding, which suggests that ghrelin is an appetite signal for salmon (Moen *et al.,* 2010). Cummings suggests that one can identify an increase in *ghrl* before meals when fish is adapted to fixed feeding schedules (Cummings, 2006). Vikeså found that muscle *ghrl* activity is more closely linked to mealtimes, feed intake and appetite than temperature (Vikeså, 2017). Even though the statistical analysis showed no correlation between SFI and mRNA expression of *ghrl* in ST tissue, a gradual increase in *ghrl-I* and *ghrl-II* was identified from 2-24 hours post-feeding in the temperature group 12°C. These findings partially agree with Vikeså's predictions of an upregulation of *ghrl* when Atlantic salmon is adapted to set mealtimes. In the current study fish was adapted to a consistent feeding regime with one meal every 24 hour; however, one would expect to see the same upregulation close to mealtime for all temperature groups as they have been adapted to the same feeding regimes. Previous and current findings provide conflicting results, the contradicting results make it ambiguous and challenging to state how feeding status and gene expression of *ghrl-I* and *ghrl-II* in Atlantic salmon are correlated.

It was further hypothesised that there would be a significant temporal change in the expression of *ghrl-I, ghrl-II* and *mboat4* in stomach tissue 2-24 h after a meal, which was rejected. The results showed no significant difference in mRNA expression of either *ghrl-I* or *ghrl-II,* 24 hours after feeding. A moderate, not significant upregulation trend was observed in the 12°C group in mRNA expression of both *ghrl-I* and *ghrl-II.* Several studies have been conducted on how fasting affects *ghrl* mRNA expression in stomach, however, results about *ghrl* role in Salmoniformes are not consistent (Del Vecchio *et al.,* 2021). Previous studies have investigated the relationship between *ghrl-I* and *ghrl-II* in stomach in response to starvation. Murashita *et al.* (2009) found that 6 days of fasting resulted in increased mRNA expression of *ghrl-I.* In contrast, Hevrøy *et al.* (2011) observed a significantly lower level of *ghrl-I* mRNA in fish starved for 14 days. A recent study by Mangersnes (2020) found no correlation between 3 days of starvation and *ghrl-I* in stomach tissue of Atlantic salmon. However, Mangersnes (2020) observed a significant upregulation of *ghrl-II* in stomach tissue and suggested that *ghrl-II* has a major appetite regulatory impact on short-term regulation. Del Vecchio *et al.* (2021) showed that short-term fasting of Atlantic salmon did not influence *ghrl* mRNA expression in stomach. The results of this present study agree with Del Vecchio *et al.* (2021), as no significant differences were detected in either *ghrl-I* or *ghrl-II* during the 24 hours post-feeding. In contrast to most other studies, the fish in this experiment was not starved. The fish were fed according to their usual schedule, with 24 hours between meals. 24 hours is not considered a period of starvation but rather a temporary deprivation of food. Del Vecchio *et al.* (2021) suggested that 4 days of starvation is too short of initiating GI responses in Atlantic salmon, which could be supported by the findings of this current study, as no starvation gives the same results as 4 days of starvation.

In addition to investigating the mRNA expression of ghrelin, the present experiment investigates the expression of its activating enzyme *mboat4.* It was hypothesised that there would be a link between the expression of the two ghrelin splice variant and the expression of *mboat4,* which was rejected. There was no correlation between the expression of either *ghrl-I* or *ghrl-II* and *mboat4* in this study. MBOAT4 is the enzyme responsible for acylating ghrelin into its active form and enables ghrelin to bind to the GHSR in the ARC (Kojima *et al*., 1999; Yang *et al*., 2008). Kalananthan *et al.* (2023) investigated the effect of 4 and 6 weeks of fasting on *mboat4* expression in stomach, however, the expression was unaffected (Kalananthan *et al.,*2023) There are few studies

investigating the link between *ghrl* and *mboat4* in teleosts. In zebrafish, *mboat4* has been reported to increase in response to fasting, suggesting it to be an orexigenic peptide important for appetite regulation (Hatef *et al.*, 2015). In contrast, Kalananthan *et al.* (2023) found no significant effect of *mboat4* expression in response to long-term fasting in Atlantic salmon. They suggest that *mboat4* turns into a hunger signal when feed is available after a long fasting period (Kalananthan *et al*., 2023). There was no significant correlation between SFI and *mboat4* expression in any of the temperature groups in this study. A weak positive correlation between MFI and the mRNA expression of *mboat4* in temperature group 12°C. MBOAT4 was discovered in recent years, and there are limited studies and knowledge on how the enzyme is regulated in both higher and lower vertebrates. The results of the present study support Kalananthan's findings that *mboat4* might turn into a hunger signal after a long period without feed, considering that the expression was not affected by either temperature or time.

4.2.5 Effect of temperature on expression of key genes in stomach

It was hypothesised that temperature would have a significant effect on the relative expression of *ghrl-I, ghrl-II* and *mboat4* in stomach tissue. This was proven partially true. The results showed a significant effect of temperature on *ghrl-I* mRNA expression in stomach tissue between fish reared at 8°C, 12°C and 15°C. There was no significant effect of temperature on *ghrl-II* or *mboat4* mRNA expression in stomach tissue in any of the temperature groups. An important temporal trend was observed in the 12°C group, where there was a gradual increase of *ghrl-I* and *ghrl-II*. In mammals, ghrelin is the only peripheral peptide hormone with an orexigenic role (Rønnestad *et al.,* 2017). Considering the importance temperature has on metabolic processes (Brett, 1979), food intake and growth, one could predict that the genes regulating the production of orexigenic would be affected as well. Few studies have explored how temperature affects the regulation of ghrelin activity in teleost and specifically Atlantic salmon. Studies have been conducted on other species, such as goldfish (*Carassius auratus*), Burbot (*Lota lota*), Chinese perch (*Siniperca chuatsi*) and Arctic char (*Salvelinus alpinus)*. A study on goldfish showed a lower mRNA level of *ghrl* in the intestine for fish reared at 35°C compared to 15°C (Nadermann *et al.,* 2019). A study on Burbot showed that temperature did not significantly affect ghrelin expression (Nieminen *et al.,* 2003). In contrast, a study on Chinese perch revealed elevated mRNA levels of preproghrelin (inactive ghrelin) stomach tissue of fish reared at 26°C and 32°C compared to 8°C and 18°C (Song *et al.,* 2017). Frøiland *et al.* (2010) found seasonal differences in ghrelin expression, with reduced levels during warmer months and elevated levels in cooler months. A study by Mangersnes in 2020 investigated the relationship between *ghrl-I* and *ghrl-II* levels in Atlantic salmon and temperature. The "*study did not see any significant difference of ghrl-I and ghrl-II mRNA levels between fish reared at 12.5°C and 15°C*"(Mangersnes, 2020). The findings of the current study are in contrast with both Frøiland's and Mangersnes findings, as a significant effect of temperature on *ghrl-I* was observed. However, no effect of temperature was found on *ghrl-II* mRNA levels. The observed differential response to temperature suggests that there may be functional differences between the two *ghrl* splice variants. It implies that *ghrl-I* is more responsive to temperature during short periods of feed deprivation. Contrasting findings suggest a need for additional research on how temperature influences mRNA expression of *ghrl-I* and *ghrl-II* in Atlantic salmon.

4.2.6 Ghrl levels in plasma

It was hypothesised that there would be temporal changes in Ghrl plasma levels, which was proven true. All temperature groups had temporal changes in Ghlr concentrations 2-24 hours post-feeding. Further, it was hypothesised that the concentration of Ghrl in plasma would be at its lowest +2 h post-feeding and gradually increase to its peak + 24 h post-feeding. This was partially proven to be true as elevated plasma Ghrl levels 24 hours post-feeding for the 8°C group were identified. However, the 12°C and 15°C groups exhibited reduced levels. Cumming (2006) found that the circulating levels of Ghrl in humans and rodents decreased with feeding and increased before meals. Jönsson *et al.* (2007) reported no variation in plasma Ghrl levels postprandially, indicating that feeding does not affect the endocrine release of ghrelin in rainbow trout. A study by Pankhurst *et al.* (2008) showed that plasma Ghrl levels were elevated 24 h after feeding in rainbow trout, which suggests that ghrelin is a possible orexigenic in fish. Their study suggests that the elevation in Ghrl occurred in relation to mealtime, as the fish had been adapted (three months) to one meal per day (Pankhurst *et al.,* 2008). Jönsson *et al.* (2007) and Pankhurs *et al.* (2008) findings contradict each other, as Jönsson *et al.* (2007) results do not support that circulating Ghrl triggers meal initiation or that it is associated with short-term hunger in rainbow trout. A study on Japanese quail (*Coturnix japonica*) found increased Ghlr plasma levels 24 hours after a meal, suggesting it to be a short-time regulator of energy homeostasis (Shousha *et al.,* 2005). Vikeså's results on salmon reared at 12°C showed that Ghrl plasma levels peaked before each adapted mealtime (Vikeså, 2015). However, the results of this study partially differ from Pankhurst, Cummings and Vikeså´s results. In this current study, the fish was adapted to one meal a day at the exact same time every day. Fish reared at 8°C showed elevated Ghrl plasma levels 24 hours post-feeding, before the expected meal. The 12°C and 15°C groups showed the lowest Ghrl plasma levels 24 hours post-feeding. One could expect that all groups would show similar levels as all fish have been adapted to the same feeding regimes and mealtimes. These results suggest that temperature, in fact, does have a remarkable impact on Ghrl plasma levels in Atlantic salmon. Previous studies (Hevrøy *et al.,* 2012; Kullgren *et al.,* 2013) have found contrasting results when investigating the relationship between Ghrl plasma levels and temperature in Atlantic salmon. Kullgren *et al.* (1) described no difference in Ghrl plasma levels between fish reared at 8°C, 12°C and 18°C, while Hevrøy *et al.* (2) describe lower levels at 19°C compared to 14°C. The current study follows a similar experimental design as Kullgren *et al.* (2013), using nine 600L tanks with

triplicates for each temperature group. Fish in both studies (1 and 2) were fed twice a day, in contrast to this study, where fish were adapted to one meal a day. In contrast to this current study, Kullgren *et al.* (2013) and Hevrøy *et al.* (2012) investigated Ghrl plasma levels at one sampling point and not how Ghrl changes over time. It is not stated specific sampling points in relation to feeding regimes in Kullgren *et al.* (2013), however, in Hevrøy *et al.* (2012) study, fish were sampled 4 hours after feeding. In this current study, there were observed significant differences in Ghrl levels between the temperature groups at 2-, 4- and 24 hours post-feeding. However, after 4 till 20 hours post-feeding, the concentration showed no statistically significant difference between the temperature groups. The data from this research illustrates the relevance of time after feeding on Ghrl plasma levels, as rapid temporal changes in concentration were observed.

Further, it was hypothesised that stomach filling would affect the Ghrl plasma levels, however, the results show no correlation between SFI and Ghrl plasma levels. One could assume that there would be a correlation as the ST is the major site for ghrelin production, and thereby also the secretion of Ghrl in the bloodstream. As time after feeding passes, the SFI and stomach expansion decrease. As stomach filling and stretching are considered short-term regulators for feed intake (Blundell *et al.,* 2015; Camilleri, 2015; Krogdahl, 2001), one could predict that there would be a correlation between stomach filling and the Ghlr plasma levels and vice versa. A study on rodents revealed that stomach filling did not change plasma ghrelin levels, suggesting that the secretion of ghrelin is not affected by stomach expansion in rodents (Shiiya *et al.,* 2002; Tschöp *et al.,* 2000). The result in this current study is consistent with these findings, suggesting that stomach filling does not impact either the mRNA expression of ghrelin or the levels of Ghrl in the bloodstream.

An important aim of the study was to explore the link between mRNA and plasma levels of Ghrl. It was hypothesised that mRNA expression of *ghrl* in stomach tissue and Ghrl plasma levels was correlated. This was rejected as no correlation of significance was identified between the mRNA expression of the target genes and Ghrl plasma levels. A central dogma in molecular biology is the transfer of information from mRNA to proteins. Genes get transcribed, mRNA gets processed and translated in sequences into chains of amino acids that fold to become functional proteins. One would assume there to be a tight link between the mRNA expression and abundance of the proteins it codes for, however,

only weak correlations have been discovered. (Maier *et al.,*2009) Ghrelin is a protein that is produced by EEC cells in the stomach, researcher suggest that it is further secreted into the bloodstream to act on the hypothalamus (Kojima *et al.,* 1999). Based on biological knowledge, it was predicted that there would be a tight correlation between the two factors. To my knowledge, there are few studies that investigate the correlation between ghrelin mRNA expression in stomach and Ghrl plasma levels. A study from 2007, investigated the relationship between ghrelin mRNA expression in the proventriculus and plasma levels in chicken (*Gallus gallus domesticus*). The gene expression and plasma levels increased in response to fasting, however, no change in food intake as a response to increased circulating ghrelin was observed. (Kaiya *et al.,* 2007) Kaiya and colleagues found that changes in ghrelin expression were relatively slow in bullfrogs compared to mammals and birds, however, a close link between mRNA expression and plasma levels of ghrelin has been observed when fasted (Kaiya *et al.,* 2006). The absence of correlation between mRNA expression of *ghrl-I* and *ghrl-II* and the concentration of circulating Ghrl in this study was unanticipated. It is a critical observation that contradicts previous knowledge. These findings must be taken into consideration in further studies on appetite in Atlantic salmon.

5. Conclusion

In summary, the present study shows that temperature had a significant effect on appetite, feed intake and growth performance in Atlantic salmon. Fish in the 12°C and 15°C group had an overall greater growth, resulting in a higher feed intake compared to the 8°C. Gut transit was higher at higher temperatures, however, the temporal change in gastrointestinal transit did not influence gene expression of either *ghrl-I, ghrl-II* or *mboat4* in stomach tissue. Temperature did influence the mRNA expression of *ghrl-I,* whereas *ghrl-II* and *mboat4* were unaffected. The temporal change in Ghrl plasma concentrations differed significantly between the different temperature groups, however, no correlation was observed between gene expression of either splice variants of ghrelin (*ghrl-I* and *ghrl-II*) and Ghlr plasma levels. As no correlation was observed between stomach fullness, mRNA expression of ghrelin and Ghrl plasma levels, this indicate that the ghrelin does not have the orexigenic function in Atlantic salmon as previously predicted.

6. References

Aas, T.S., Sixten, H. J., Hillestad, M., Sveier, H., Ytrestøyl, T., Hatlen, B., & Åsgård, T. (2017). Measurement of gastrointestinal passage rate in Atlantic salmon (*Salmo salar*) fed dry or soaked feed. *Aquaculture Reports*, *8*, 49–57. https://doi.org/10.1016/J.AQREP.2017.10.001

Aas, T. S., Ytrestøyl, T., & Åsgård, T. (2019). Utilization of feed resources in the production of Atlantic salmon (*Salmo salar*) in Norway: An update for 2016. *Aquaculture Reports*, *15,* 100216. https://doi.org/10.1016/j.aqrep.2019.100216

Aas, T. S., Ytrestøyl, T., & Åsgård, T. E. (2022). *Utnyttelse av fôrressurser i norsk oppdrett av laks og regnbueørret i 2020.* (Rapport 2/2022). Nofima rapportserie. Accessed from: https://nofima.brage.unit.no/nofima-xmlui/bitstream/handle/11250/2977260/Korrigert%20Rapport%202%202022%20Ressurs%202020.pdf?sequence=6&isAllowed=y

Adams, C.E., & Thorpe, J. E. (1989). Photoperiod and temperature effects on early development and reproductive investment in Atlantic salmon (*Salmo salar* L.). *Aquaculture*, 79(1–4), 403–409. https://doi.org/10.1016/0044-8486(89)90483-3

Aksnes, A., Gjerde, B., & Roald, S. O. (1986). Biological, chemical and organoleptic changes during maturation of farmed Atlantic salmon, *Salmo salar*. *Aquaculture*, *53*(1), 7–20. https://doi.org/10.1016/0044-8486(86)90295-4

Ariyasu, H., Takaya, K., Tagami, T., Ogawa, Y., Hosoda, K., Akamizu, T., Suda, M., Koh, T., Natsui, K., Toyooka, S., Shirakami, G., Usui, T., Shimatsu, A., Doi, K., Hosoda, H., Kojima, M., Kangawa, K., & Nakao, K. (2001). Stomach is a major source of circulating ghrelin, and feeding state determines plasma Ghrelin-like immunoreactivity levels in humans. *The Journal of Clinical Endocrinology & Metabolism*, *86*(10), 4753–4758. https://doi.org/10.1210/JCEM.86.10.7885

Bauer, P.V., Hamr, S.C., & Duca, F.A. (2015). Regulation of energy balance by a gut–brain axis and involvement of the gut microbiota. *Cellular and Molecular Life Sciences, 73*(4), 737–755. https://doi.org/10.1007/S00018-015-2083-Z

Bai, S.C., Hardy, R.W. & Hamidoghli, A. (2021). Chapter 10 - Diet analysis and evaluation. In: Hardy, R.W. & Kaushik, S.J., (ed.). Fish Nutrition. 4th ed, pp.1-882.

Calo, J., Blanco, A.M., Comesaña, S., Conde-Sieira, M., Morais, S., & Soengas, J. L. (2021). First evidence for the presence of amino acid sensing mechanisms in the fish gastrointestinal tract. *Scientific Reports, 11*(1), 1–14. https://doi.org/10.1038/s41598-021-84303-9

Blundell, J. E., Finlayson, G., Gibbons, C., Caudwell, P., & Hopkins, M. (2015). The biology of appetite control: Do resting metabolic rate and fat-free mass drive energy intake? *Physiology & Behavior*, *152*, 473–478. https://doi.org/10.1016/J.PHYSBEH.2015.05.031

Bolasina, S.N., Tagawa, M., & Yamashita, Y. (2007). Changes on cortisol level and digestive enzyme activity in juveniles of Japanese flounder, *Paralichthys olivaceus*, exposed to different salinity regimes. *Aquaculture*, *266*(1–4), 255–261. https://doi.org/10.1016/J.AQUACULTURE.2007.01.046

Brett, J. R., & Higgs, D. A. (1970). Effect of temperature on the rate of gastric digestion in fingerling sockeye salmon, *Oncorhynchus nerka*. *Journal of Fish Research Board of Canada, 27,* 1767-1779. https://doi.org/10.1139/f70-197

Brett, J.R. (1971). Energetic responses of salmon to temperature. A study of some thermal relation in the physiology and freshwater ecology of Sockeye Salmon *(Oncorhynchus nerka)*. *American zoologist, 11*(1), 99-113. https://doi.org/10.1093/icb/11.1.99

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

Bruce, P., & Bruce, A. (2017) Practical Statistics for Data Scientists. O'Reilly Media. Chapter 3 "*Exploring One-Way ANOVA*"

Björnsson, B.T., Hemre, G.I., Bjornevik, M., & Hansen, T. (2000). Photoperiod regulation of plasma growth hormone levels during induced smoltification of underyearling Atlantic Salmon. *General and Comparative Endocrinology*, *119*(1), 17–25. https://doi.org/10.1006/gcen.2000.7439

Bustin, S.A. (2000). Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *Journal of Molecular Endocrinology*, *25*(2), 169–193. https://doi.org/10.1677/jme.0.0250169

Camilleri, M. (2015). Peripheral mechanisms in appetite regulation. *Gastroenterology*, *148*(6), 1219–1233. https://doi.org/10.1053/J.GASTRO.2014.09.016

Chang, S.C., & Magee, A. (2009). Acyltransferases for secreted signalling proteins. *Molecular Membrane Biology, 26*(1-2), 104-113. https://doi.org/10.1080/09687680802706432

Chellappa, S., Huntingford, A., Strang, H.C., & Thomson, R.Y. (1995). Condition factor and hepatosomatic index as estimates of energy status in male three-spined

stickleback. *Journal of fish biology, 47*(5), 775–787. https://doi.org/10.1111/j.1095-8649.1995.tb06002.x

Chen, C.Y., Asakawa, A., Fujimiya, M., Lee, S.D., & Inui, A. (2009). Ghrelin gene products and the regulation of food intake and gut motility. *Pharmacological Reviews, 61*(4), 430–481. https://doi.org/10.1124/pr.109.001958

Cummings, D. E. (2006). Ghrelin and the short- and long-term regulation of appetite and body weight. *Physiology & Behavior*, *89*(1), 71–84. https://doi.org/10.1016/j.physbeh.2006.05.022

Cummings, D. E., Purnell, J. Q., Frayo, R. S., Schmidova, K., Wisse, B. E. & Weigle, D. S. (2001). A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans. *Diabetes, 50*(8), 1714-1719. doi:10.2337/diabetes.50.8.1714

Date, Y., M. Kojima, H. Hosoda, A. Sawaguchi, M. S. Mondal, T. Suganuma, S. Matsukura, K. Kangawa & M. Nakazato (2000). Ghrelin, a novel growth hormone-releasing acylated peptide, is synthesized in a distinct endocrine cell type in the gastrointestinal tracts of rats and humans. *Endocrinology, 141*(11), 4255-4261. https://doi.org/10.1210/endo.141.11.7757

Date, Y., & Kangawa, K. (2012). Ghrelin as a starvation signal. *Obesity Research & Clinical Practice, 6*(4), 263-269. https://doi.org/10.1016/j.orcp.2012.08.195

Datta, S.N., Kaur, V.I., Dhawan, A. & Jassal, G. (2013*)* Estimation of length-weight relationship and condition factor of spotted snakehead Channa punctata (Bloch) under different feeding regimes *Springerplus*, 2, 436. https://doi.org/10.1186/2193-1801-2-436

Del Vecchio, G., Lai, F., Gomes, A.S., Verri, T., Kalananthan, T., Barca, A., Handeland, S., & Rønnestad, I. (2021). Effects of short-term fasting on mRNA expression of Ghrelin and the peptide transporters PepT1 and 2 in Atlantic Salmon *(Salmo salar)*. *Frontiers in Physiology*, *12,* 666670. https://doi.org/10.3389/fphys.2021.666670

Delhanty, P.J.D., Neggers, S.J., & Van der Lely, A.J. (2012). Mechanisms in endocrinology: Ghrelin: The differences between acyl- and des-acyl ghrelin. *European Journal of Endocrinology, 167*(5), 601–608. https://doi.org/10.1530/EJE-12-0456

Espe, M., Vikeså, V., Helgøy Thomsen, T., Adam, A. C., Saito, T., & Skjærven, K. H. (2020). Atlantic salmon fed a nutrient package of surplus methionine, vitamin B12, folic acid and vitamin B6 improved growth and reduced the relative liver size, but when in excess growth reduced. *Aquaculture Nutrition*, *26*(2), 477–489. https://doi.org/10.1111/ANU.13010

FAO. (2016). The State of World Fisheries and Aquaculture 2016. Contributing to food security and nutrition for all. Rome. Accessed from: https://www.fao.org/3/i5555e/i5555e.pdf

FAO. (2020). The State of World Fisheries and Aquaculture 2020. Sustainability in action. Rome. https://doi.org/10.4060/ca9229en

Fay, D. S., & Gerow, K. (2013). A biologist's guide to statistical thinking and analysis. *WormBook: The Online Review of C. Elegans Biology*, 1–54. https://doi.org/10.1895/WORMBOOK.1.159.1

Fjelldal, P.G., Hansen, T., & Huang, T. (2011). Continuous light and elevated temperature can trigger maturation both during and immediately after smoltification in male Atlantic salmon (*Salmo salar*). *Aquaculture*, *321*(1–2), 93–100. https://doi.org/10.1016/j.aquaculture.2011.08.017

Frøiland, E., Murashita, K., Jørgensen, E. H., & Kurokawa, T. (2010). Leptin and ghrelin in anadromous Arctic charr: Cloning and change in expressions during a seasonal feeding cycle. *General and Comparative Endocrinology*, *165*(1), 136–143. https://doi.org/10.1016/J.YGCEN.2009.06.010

Føre, M., Frank, K., Norton, T., Svendsen, E., Alfredsen, J. A., Dempster, T., Eguiraun, H., Watson, W., Stahl, A., Sunde, L. M., Schellewald, C., Skøien, K. R., Alver, M. O., & Berckmans, D. (2018). Precision fish farming: A new framework to improve production in aquaculture. *Biosystems Engineering*, *173*, 176-193. https://doi.org/10.1016/j.biosystemseng.2017.10.014

Gomes, A.S., Zimmermann, F., Hevrøy, E.M., Søyland, M.A.L., Hansen, T.J., Nilsen, T.O., & Rønnestad, I. (2023) Statistical modelling of voluntary feed intake in individual Atlantic salmon (*Salmo salar*). *Frontiers in Marine Science,* 10, 1127519. https://doi.org/10.3389/fmars.2023.1127519

Grove, D.J., Loizides, L.G., & Nott, J. (1978). Satiation amount, frequency of feeding and gastric-emptying rate in Salmo gairdneri. *Journal of Fish Biology*, *12*(5), 507-516. https://doi.org/10.1111/j.1095-8649.1978.tb04195.x

Gwyther, D., & Grove, D.J. (1981). Gastric emptying in *Limanda limanda* (L.) and the return of appetite. *Journal of Fish Biology*, *18*(3), 245–259. https://doi.org/10.1111/J.1095-8649.1981.TB03767.X

Handeland, S. O., Berge, Å., Björnsson, B. T., Lie, O., & Stefansson, S. O. (2000). Seawater adaptation by out-of-season Atlantic salmon (*Salmo salar* L.) smolts at

different temperatures. *Aquaculture*, *181*(3–4), 377–396. https://doi.org/10.1016/S0044-8486(99)00241-0

Handeland, S. O., Björnsson, B. T., Arnesen, A. M., & Stefansson, S. O. (2003). Seawater adaptation and growth of post-smolt Atlantic salmon (*Salmo salar*) of wild and farmed strains. *Aquaculture*, *220*(1–4), 367–384. https://doi.org/10.1016/S0044-8486(02)00508-2

Handeland, S. O., Imsland, A. K., & Stefansson, S. O. (2008). The effect of temperature and fish size on growth, feed intake, food conversion efficiency and stomach evacuation rate of Atlantic salmon post-smolts. *Aquaculture*, *283*(1-4), 36-42. doi:10.1016/j.aquaculture.2008.06.042.

Hansen, T., Stefansson, S., & Taranger, G. L. (1992). Growth and sexual maturation in Atlantic salmon, *Salmon, salar* L., reared in sea cages at two different light regimes. *Aquaculture Research*, *23*(3), 275–280. https://doi.org/10.1111/j.1365-2109.1992.tb00770.x

Hatef, A., Yufa, R., & Unniappan, S. (2015). Ghrelin O-Acyl Transferase in Zebrafish Is an Evolutionarily Conserved Peptide Upregulated During Calorie Restriction. *Zebrafish*, *12*(5), 327. https://doi.org/10.1089/ZEB.2014.1062

Hellemans, J., Mortier, G., de Paepe, A., Speleman, F., & Vandesompele, J. (2008). qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome Biology*, *8*(2), 1–14. https://doi.org/10.1186/gb-2007-8-2-r19

Hevrøy, E. M., Azpeleta, C., Shimizu, M., Lanzén, A., Kaiya, H., Espe, M., & Olsvik, P. A. (2011). Effects of short-term starvation on ghrelin, GH-IGF system, and IGF-binding proteins in Atlantic salmon. *Fish Physiology and Biochemistry*, *37*(1), 217–232. https://doi.org/10.1007/s10695-010-9434-3

Hevrøy, E.M., Waagbø, R., Torstensen, B.E., Takle, H., Stubhaug, I., Jørgensen, S.M., Torgersen, T., Tvenning, L., Susort, S., Breck, O. & Hansen, T., (2012). Ghrelin is involved in voluntary anorexia in Atlantic salmon raised at elevated sea temperatures. *General and Comparative Endocrinology*, *175*(1), 118-134. https://doi.org/10.1016/j.ygcen.2011.10.007

Higgs, D.A., Sutton, J.N., Kim, H., Oakes, J.D., Smith, J., Biagi, C., Rowshandelim M., & Devlin, R.H. (2009). Influence of dietary concentrations of protein, lipid and carbohydrate on growth, protein and energy utilization, body composition, and plasma titres of growth hormone and insulin-like growth factor-1 in non-transgenic and growth

hormone transgenic coho salmon, *Oncorhynchus kisutch* (Walbaum). *Aquaculture* *286*(1-2), 127–137. https://doi.org/10.1016/j.aquaculture.2008.08.036

Holmgren, S., & Olsson, C. (2009). Chapter 10 The neuronal and endocrine regulation of gut function. *Fish Physiology*, *28*(C), 467–512. https://doi.org/10.1016/S1546-5098(09)28010-1

Huebner, J. D., & Langton, R. W. (1982). Rate of gastric evacuation for winter flounder, Pseudopleuronectes americanus. *Canadian Journal of Fisheries and Aquatic Science*, *39* (2), 356–360. https://doi.org/10.1139/f82-049

Huey, R. B., & Stevenson, R. D. (1979). Integrating thermal physiology and ecology of ectotherms: A discussion of approaches. *Integrative and Comparative Biology*, *19*(1), 357–366. https://doi.org/10.1093/ICB/19.1.357

Ivy, A. C., & Oldberg, E. (1928). A hormone mechanism for gall-bladder contraction and evacuation. *American Journal of Physiology-Legacy Content*, *86*(3), 599-613.

Jönsson, E. (2013). The role of ghrelin in energy balance regulation in fish. *General and Comparative Endocrinology*, *187*, 79–85. https://doi.org/10.1016/J.YGCEN.2013.03.013

Jönsson, E., Forsman, A., Einarsdottir, I. E., Kaiya, H., Ruohonen, K., & Björnsson, B. T. (2007). Plasma ghrelin levels in rainbow trout in response to fasting, feeding and food composition, and effects of ghrelin on voluntary food intake. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 147*(4), 1116–1124. https://doi.org/10.1016/J.CBPA.2007.03.024

Kaiya, H., Sakata, I., Yamamoto, K., Koda, A., Sakai, T., Kangawa, K., & Kikuyama, S. (2006). Identification of immunoreactive plasma and stomach ghrelin, and expression of stomach ghrelin mRNA in the bullfrog, Rana catesbeiana. *General and Comparative Endocrinology*, *148*(2), 236–244. https://doi.org/10.1016/J.YGCEN.2006.03.008

Kaiya, H., Saito, E. S., Tachibana, T., Furuse, M., & Kangawa, K. (2007). Changes in ghrelin levels of plasma and proventriculus and ghrelin mRNA of proventriculus in fasted and refed layer chicks. *Domestic Animal Endocrinology*, *32*(4), 247–259. https://doi.org/10.1016/j.domaniend.2006.03.006

Kalananthan, T., Folkedal, O., Gomes, A.S., Lai, F., Handeland, S., Tolås, I., Gelebart, V., & Rønnestad, I. (2023) Impact of long-term fasting on the stomach-hypothalamus appetite regulating genes in Atlantic salmon postsmolts. *Aquaculture 563*(2), 738917. https://doi.org/10.1016/j.aquaculture.2022.738917

Klockars, A., Levine, A. S., & Olszewski, P. K. (2019). Hypothalamic integration of the endocrine signaling related to food intake. *Topics in Behavioral Neurosciences*, *43*, 239–269. DOI: 10.1007/7854_2018_54

Kojima, M., Hosoda, H., Date, Y., Nakazato, M., Matsuo, H., & Kangawa, K. (1999). Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature*, *402*, 656–660. https://doi.org/10.1038/45230

Kojima, M., & Kangawa, K. (2005). Ghrelin: Structure and function. *Physiological Reviews, 85*(2), 495–522 https://doi.org/10.1152/physrev.00012.2004

Kojima, M., Ida, T., & Sato, T. (2007). Structure of mammalian and nonmammalian Ghrelins. *Vitamins and Hormones*, *77*, 31–46. https://doi.org/10.1016/S0083-6729(06)77003-0

Krogdahl, Å. (2001). Fordøyelsessystemet hos kaldtvannsfisk – oppbygging og funksjon. In: Waagbø, R., Espe, M., Hamre, K., & Lie, Ø. (Eds.) *Fiskeernæring* (pp.12-34). Kystnæringen Forlag & Bokklubb AS

Kryvi, H. & Poppe, T. (2016). Fiskeanatomi, Bergen, Fagbokforlaget. (In Norwegian)

Kullgren, A., Jutfelt, F., Fontanillas, R., Sundell, K., Samuelsson, L., Wiklander, K., Kling, P., Koppe, W., Larsson, D.G., Björnsson, B.T. & Jonsson, E. (2013). The impact of temperature on the metabolome and endocrine metabolic signals in Atlantic salmon (*Salmo salar*). *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 164*(1), 44-53. https://doi.org/10.1016/j.cbpa.2012.10.005

Kozera, B., & Rapacz, M. (2013). Reference genes in real-time PCR. *Journal of Applied Genetics, 54*(4), 391-406 https://doi.org/10.1007/s13353-013-0173-x

Lai, F., Royan, M. R., Gomes, A. S., Espe, M., Aksnes, A., Norberg, B., Gelebart, V., & Rønnestad, I. (2021). The stress response in Atlantic salmon (*Salmo salar* L.): identification and functional characterization of the corticotropin-releasing factor (crf) paralogs. *General and Comparative Endocrinology, 313*, 113894. https://doi.org/10.1016/j.ygcen.2021.113894

Latorre, R., Sternini, C., de Giorgio, R., & Greenwood-Van Meerveld, B. (2016). Enteroendocrine Cells: a review of their role in brain-gut communication. *Neurogastroenterology and motility*, *28*(5), 620-630.

Le Cren, E.D. (1951). The length-weight relationships and seasonal cycle in gonad weight and condition in the perch (*Perca fluviatilis*). *Journal of Animal Ecology, 20*(2), 201-219. https://doi.org/10.2307/1540

Li, Z., Mulholland, M., & Zhang, W. (2016). Ghrelin O-acyltransferase (GOAT) and energy metabolism. *Science China. Life Sciences, 59*(3), 281–291. https://doi.org/10.1007/S11427-015-4973-6

Little, T. J., Horowitz, M., & Feinle-Bisset, C. (2005). Role of cholecystokinin in appetite control and body weight regulation. *Obesity Reviews*, *6*(4), 297–306. https://doi.org/10.1111/J.1467-789X.2005.00212.X

Lugert, V., Thaller, G., Tetens, J., Schulz, C., & Krieter, J. (2016). A review on fish growth calculation: multiple functions in fish production and their specific application. *Reviews in Aquaculture*, *8*(1), 30–42. https://doi.org/10.1111/RAQ.12071

Ma, D., Wang, Z., Merrikh, C. N., Lang, K. S., Lu, P., Li, X., Merrikh, H., Rao, Z., & Xu, W. (2018). Crystal structure of a membrane-bound O-acyltransferase. *Nature*, *562*, 286–290. https://doi.org/10.1038/s41586-018-0568-2

Maier, T., Güell, M., & Serrano, L. (2009). Correlation of mRNA and protein in complex biological samples. *FEBS Letters*, *583*(24), 3966–3973. https://doi.org/10.1016/J.FEBSLET.2009.10.036

Mangersnes, M.H. (2020) *The effect of feeding and stomach filling on the gastric Ghrelin system in Atlantic salmon Salmo salar.* Master thesis, University of Bergen, Bergen, Norway

Martinez, E.P., Balseiro, P., Pedrosa, C., Haugen, T.S., Fleming, M. S., & Handeland, S.O. (2021). The effect of photoperiod manipulation on Atlantic salmon growth, smoltification and sexual maturation: A case study of a commercial RAS. *Aquaculture Research*, *52*(6), 2593–2608. https://doi.org/10.1111/ARE.15107

Martinez, E.P., Balseiro, P., Fleming, M.S., Stefansson, S.O., Norberg, B., Imsland, A. K.D., & Handeland, S.O. (2023). Interaction of temperature and photoperiod on male postsmolt maturation of Atlantic salmon (*Salmo salar* L.). *Aquaculture*, *568*, 739325. https://doi.org/10.1016/J.AQUACULTURE.2023.739325

Martins, N., Castro, C., Oliva-Teles, A. & Peres, H. (2022). The interplay between central and peripheral systems in feed intake regulation in European seabass (*Dicentrarchus labrax*) juveniles. *Animals 2022*, *12*(23), 3287. https://doi.org/10.3390/ani12233287

Mayer, E.A. (2011). Gut feelings: the emerging biology of gut–brain communication. *Nature Reviews Neuroscience*, *12*, 453-466. https://doi.org/10.1038/nrn3071

Mendieta-Zerón, H., López, M., & Diéguez, C. (2008). Gastrointestinal peptides controlling body weight homeostasis. *General and Comparative Endocrinology*, *155*(3), 481–495. https://doi.org/10.1016/J.YGCEN.2007.11.009

Miegel, R.P., Pain, S. J., van Wettere, W. H. E. J., Howarth, G. S., & Stone, D. A. J. (2010). Effect of water temperature on gut transit time, digestive enzyme activity and nutrient digestibility in yellowtail kingfish (*Seriola lalandi*). *Aquaculture*, *308*(3–4), 145–151. https://doi.org/10.1016/J.AQUACULTURE.2010.07.036

Miller, N. A., & Stillman, J. H. (2012) Physiological Optima and Critical Limits. *Nature Education Knowledge 3*(10):1

Mock, T. S., Alkhabbaz, Z. H. R. A. A., Rocker, M. M., Lewis, M. J., Cumming, E. E., Smullen, R. P., Turchini, G. M., & Francis, D. S. (2022). Gut transit rate in Atlantic salmon (Salmo salar) exposed to optimal and suboptimally high water temperatures. *Aquaculture Research*, *53*(13), 4858–4868. https://doi.org/10.1111/ARE.15979https://doi.org/10.1111/ARE.15979

Moe Føre, H., Thorvaldsen, T., Osmundsen, T. C., Asche, F., Tveterås, R., Fagertun, J. T., & Bjelland, H. V. (2022). Technological innovations promoting sustainable salmon (*Salmo salar*) aquaculture in Norway. *Aquaculture Reports*, *24*, 101115. https://doi.org/10.1016/j.aqrep.2022.101115

Moen, A.G.G., Murashita, K., & Finn, R. N. (2010). Ontogeny of energy homeostatic pathways via neuroendocrine signaling in Atlantic salmon. *Developmental Neurobiology*, *70*(9), 649–658. https://doi.org/10.1002/DNEU.20803

Müller, T. D., Nogueiras, R., Andermann, M. L., Andrews, Z. B., Anker, S. D., Argente, J., Batterham, R. L., Benoit, S. C., Bowers, C. Y., Broglio, F., Casanueva, F. F., D'Alessio, D., Depoortere, I., Geliebter, A., Ghigo, E., Cole, P. A., Cowley, M., Cummings, D. E., Dagher, A., Tschöp, M. H. (2015). Ghrelin. *Molecular Metabolism*, *4*(6), 437–460. https://doi.org/10.1016/J.MOLMET.2015.03.005

Murashita, K., Kurokawa, T., Nilsen, T.O., & Rønnestad, I. (2009). Ghrelin, cholecystokinin, and peptide YY in Atlantic salmon (*Salmo salar*): molecular cloning and tissue expression. *General and comparative Endocrinology*, *160*(3), 223-235 https://doi.org/10.1016/j.ygcen.2008.11.024

Nadermann, N., Seward, R. K., & Volkoff, H. (2019). Effects of potential climate change -induced environmental modifications on food intake and the expression of appetite regulators in goldfish. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *235*, 138–147. https://doi.org/10.1016/J.CBPA.2019.06.001

Navarro-Guillén, C., Gilannejad, N., Pérez-Hilario, D., Martínez-Rodríguez, G., & Yúfera, M. (2023). Gut transit of daily consecutive meals in greater amberjack juveniles reared at different temperatures. *Aquaculture*, *567*, 739244. https://doi.org/10.1016/j.aquaculture.2023.739244

Neubauer, P., & Andersen, K. H. (2019). Thermal performance of fish is explained by an interplay between physiology, behaviour and ecology. *Conservation Physiology*, *7*(1). https://doi.org/10.1093/CONPHYS/COZ025

Nieminen, P., Mustonen, A. M., & Hyvärinen, H. (2003). Fasting reduces plasma leptin- and Ghrelin-immunoreactive peptide concentrations of the Burbot (*Lota lota*) at 2°C but not at 10°C. *Zoological Science*, *20*(9), 1109–1115. https://doi.org/10.2108/ZSJ.20.1109

Nonogaki, K. (2007). Ghrelin and Feedback Systems. *Vitamins and Hormones*, *77*, 149–170. https://doi.org/10.1016/S0083-6729(06)77007-8

Olsvik, P.A., Lie, K.K., Jordal, A.E.O., Nilsen, T.O. & Hordvik, I., (2005). Evaluation of potential reference genes in real-time RT-PCR studies of Atlantic salmon. *BMC Molecular Biology*, *6*(21), 1–9. https://doi.org/10.1186/1471-2199-6-21

Pankhurst, N., King, H., & Ludke, S. (2008). Relationship between stress, feeding and plasma ghrelin levels in rainbow trout, *Oncorhynchus mykiss*. *Marine and Freshwater Behaviour and Physiology*, *41*(1), 53–64. https://doi.org/10.1080/10236240701661156

Peters, G. (1982). The effect of stress on the stomach of the European eel, *Anguilla anguilla* L. *Journal of Fish Biology*, *21*(5), 497–512. https://doi.org/10.1111/J.1095-8649.1982.TB02855.X

Prosser, C.L., & Nelson, D. O. (1981). The Role of Nervous Systems in Temperature Adaptation of Poikilotherms. *Annual Review of Physiology*, *43*, 281–300. https://doi.org/10.1146/annurev.ph.43.030181.001433

Raybould, H. E. (2007). Mechanisms of CCK signaling from gut to brain. *Current Opinion in Pharmacology*, *7*(6), 570–574. https://doi.org/10.1016/J.COPH.2007.09.006

Raybould, H. E. (2010). Gut chemosensing: interactions between gut endocrine cells and visceral afferents. *Autonomic Neuroscience*, *153*(1-2), 41-46. https://doi.org/10.1016/j.autneu.2009.07.007

Romero, A., Kirchner, H., Heppner, K., Pfluger, P. T., Tschöp, M. H., & Nogueiras, R. (2010). GOAT: the master switch for the ghrelin system? *European Journal of Endocrinology*, *163*(1), 1–8. https://doi.org/10.1530/EJE-10-0099

Rust, M.B. (2003). Nutritional Physiology. In J.E. Halver & R.W. Hardy (Eds.), *Fish Nutrition* (3rd ed., pp. 367-452). Academic Press. https://doi.org/10.1016/B978-012319652-1/50008-2

Rønnestad, I., Gomes, A.S., Murashita, K., Angotzi, R., Jönsson, E., & Volkoff, H. (2017). Appetite-controlling endocrine systems in Teleosts. *Frontiers in Endocinology, 8*(73). https://doi.org/10.3389/fendo.2017.00073

Sam, A. H., Troke, R. C., Tan, T. M., & Bewick, G. A. (2012). The role of the gut/brain axis in modulating food intake. *Neuropharmacology*, *63*(1), 46–56. https://doi.org/10.1016/j.neuropharm.2011.10.008

Shahbandeh, M. (2020) Global production of Atlantic salmon 2000-2021. Available at: https://www.statista.com/statistics/1179062/global-atlantic-salmon-production/ (Accessed 1 September 2022)

Shindou, H., Eto, M., Morimoto, R., & Shimizu, T. (2009). Identification of membrane O-acyltransferase family motifs. *Biochemical and Biophysical Research Communications, 383*(3), 320–325. https://doi.org/10.1016/J.BBRC.2009.04.013

Shlimun, A., & Unniappan, S. (2011). Ghrelin O-acyl transferase: Bridging ghrelin and energy homeostasis. *International Journal of Peptides*, *2011,* 217957. https://doi.org/10.1155/2011/217957

Shiiya, T., Nakazato, M., Mizuta, M., Date, Y., Mondal, M.S., Tanaka, M., Nozoe, S-I., Hosoda, H., Kangawa, K. & Matsukura, S., (2002). Plasma ghrelin levels in lean and obese humans and the effect of glucose on ghrelin secretion. *The Journal of Clinical Endocrinology & Metabolism, 87*(1), 240-244. https://doi.org/10.1210/jcem.87.1.8129

Shousha, S., Nakahara, K., Kojima, M., Miyazato, M., Hosoda, H., Kangawa, K., & Murakami, N. (2005). Different effects of peripheral and central ghrelin on regulation of food intake in the Japanese quail. *General and Comparative Endocrinology*, *141*(2), 178–183. https://doi.org/10.1016/J.YGCEN.2004.12.021

Song, Y., Zhao, C., Liang, X. fang, He, S., Tian, C., Cheng, X., Yuan, X., Lv, L., Guo, W., Xue, M., & Tao, Y.X. (2017). Effects of fasting, temperature, and photoperiod on preproghrelin mRNA expression in Chinese perch. *Fish Physiology and Biochemistry, 43*(3), 803–812. https://doi.org/10.1007/s10695-016-0335-y

Statistisk sentralbyrå (SSB). (2012). *Beregningene av næringene fiske, fangst og akvakultur i nasjo- nalregnskapet..* (Notater 28/2012). Economic Report. Available at: https://www.ssb.no/a/publikasjoner/pdf/notat_201228/notat_201228.pdf

Stefansson, S. O., Björnsson, B. T., Hansen, T., Haux, C., Taranger, G. L., & Saunders, R. L. (1991). Growth, parr–smolt transformation, and changes in growth hormone of Atlantic salmon (*Salmo salar*) reared under different photoperiods. *Canadian Journal of Fisheries and Aquatic Sciences*, *48*(11), 2100–2108.

Stengel, A., & Taché, Y. (2009). Regulation of food intake: the gastric X/A-like endocrine cell in the spotlight. *Current Gastroenterology Reports, 11*(6), 448-454. doi:10.1007/s11894-009-0069-4

Sugino, T., Hasegawa, Y., Kikkawa, Y., Yamaura, J., Yamagishi, M., Kurose, Y., Kojima, M., Kangawa, K., & Terashima, Y. (2002). A transient ghrelin surge occurs just before feeding in a scheduled meal-fed sheep. *Biochemical and Biophysical Research Communications*, *295*(2), 255–260. https://doi.org/10.1016/S0006-291X(02)00654-X

Talbot, C., & Higgins, P. J. (1982). Observations on the gall bladder of juvenile Atlantic salmon *Salmo salar* L., in relation to feeding. *Journal of Fish Biology, 21*(6), 663-669. https://doi.org/10.1111/j.1095-8649.1982.tb02870.x

Taranger, G. L., Karlsen, Ø., Bannister, R. J., Glover, K. A., Husa, V., Karlsbakk, E., Kvamme, B. O., Boxaspen, K. K., Bjørn, P. A., Finstad, B., Madhun, A. S., Morton, H. C., & Svåsand, T. (2014). Risk assessment of the environmental impact of Norwegian Atlantic salmon farming. *ICES Journal of Marine Science*, *72*(3), 997–1021. https://doi.org/10.1093/icesjms/fsu132

Timper, K., & Brüning, J. C. (2017). Hypothalamic circuits regulating appetite and energy homeostasis: pathways to obesity. *Disease Models & Mechanisms*, *10*(6), 679–689. https://doi.org/10.1242/dmm.026609

Tschöp, M., Smiley, D. L., & Heiman, M. L. (2000). Ghrelin induces adiposity in rodents. *Nature*, *407*, 908–913. https://doi.org/10.1038/35038090

van de Pol, I., Flik, G., & Gorissen, M. (2017). Comparative physiology of energy metabolism: Fishing for endocrine signals in the early vertebrate pool. *Frontiers in Endocrinology*, *8,* 36. https://doi.org/10.3389/fendo.2017.00036

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., & Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology*, *3*(7), 1–12. https://doi.org/10.1186/gb-2002-3-7-research0034

Vikeså, V. (2017). Regulation of appetite and growth of Atlantic salmon (*Salmo salar* L.) and effect of water oxygen, temperature and dietary energy. *Aquaculture Research*, *48*(8), 4086–4101. https://doi.org/10.1111/are.13229

Vikeså, V., Nankervis, L., & Hevrøy, E. M. (2017). Appetite, metabolism and growth regulation in Atlantic salmon (*Salmo salar* L.) exposed to hypoxia at elevated seawater temperature. *Aquaculture Research*, *48*(8), 4086–4101. https://doi.org/10.1111/are.13229

Volkoff, H. (2016). The Neuroendocrine regulation of food intake in fish: A review of current knowledge. *Frontiers in Neuroscience*, *10*, 540. https://doi.org/10.3389/FNINS.2016.00540

Volkoff, H. (2019). Fish as models for understanding the vertebrate endocrine regulation of feeding and weight. *Molecular and Cellular Endocrinology*, *497*, 110437. https://doi.org/10.1016/J.MCE.2019.04.017

Volkoff, H., & Rønnestad, I. (2020). Effects of temperature on feeding and digestive processes in fish. *Temperature 7*(4), 307–320. https://doi.org/10.1080/23328940.2020.1765950

Volkoff, H., Canosa, L. F., Unniappan, S., Cerdá-Reverter, J. M., Bernier, N. J., Kelly, S. P., & Peter, R. E. (2005). Neuropeptides and the control of food intake in fish. *General and Comparative Endocrinology, 142*(1–2), 3–19. https://doi.org/10.1016/j.ygcen.2004.11.001

Volkoff, H., Xu, M., MacDonald, E., & Hoskins, L. (2009). Aspects of the hormonal regulation of appetite in fish with emphasis on goldfish, Atlantic cod and winter flounder: Notes on actions and responses to nutritional, environmental and reproductive changes. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *153*(1), 8–12. https://doi.org/10.1016/J.CBPA.2008.12.001

White, P. (2013). Environmental consequences of poor feed quality and feed management. In M.R. Hasan and M.B. New, eds. On-farm feeding and feed management in aquaculture. *FAO Fisheries and Aquaculture Technical Pape*r No. 583. Rome, FAO. pp. 553–564.

Yang, J., Brown, M. S., Liang, G., Grishin, N.V., & Goldstein, J. L. (2008). Identification of the acyltransferase that octanoylates ghrelin, an appetite-stimulating peptide hormone. *Cell*, *132*(3), 387–396. https://doi.org/10.1016/J.CELL.2008.01.017

Ytrestøyl, T., Aas, T. S., & Åsgård, T. (2015). Utilisation of feed resources in production of Atlantic salmon (Salmo salar) in Norway: An update for 2012. *Aquaculture Reports*, *448*, 365–374. https://doi.org/10.1016/J.AQUACULTURE.2015.06.023

7. APPENDIX

APPENDIX A – Water parameters

Table 3: Descriptive statistics of water temperature for the low temperature group (8°C) during the experimental trial.

8°C	Tank 4	Tank 6	Tank 7	Combined
Number of values	3572	3572	3572	3572
Minimum	6,6	8,1	7,8	7,8
Maximum	10	10,7	8,8	9,667
Range	3,4	2,6	0,9998	1,867
Mean	8,399	8,64	8,16	8,4
Std. Deviation	0,3449	0,1972	0,1649	0,1968
Std. Error of Mean	0,005771	0,0033	0,002759	0,003292

Table 4: Descriptive statistics of water temperature for the control group (12°C) during the experimental trial.

12°C	Tank 8	Tank 9	Tank 10	Combined
Number of values	3572	3601	3601	3601
Minimum	10	10,6	10,5	10,4
Maximum	13,5	13,2	13,5	13
Range	3,5	2,6	3	2,6
Mean	12,05	12,47	12,46	12,33
Std. Deviation	0,3054	0,2817	0,2833	0,2742
Std. Error of Mean	0,005109	0,004694	0,004722	0,004569

Table 5: Descriptive statistics of water temperature for the high temperature group (15°C) during the experimental trial.

15°C	Tank 1	Tank 2	Tank 3	Combined
Number of values	3574	3574	3572	3574
Minimum	11,4	11,4	11,4	11,53
Maximum	15,9	16,3	16,1	16,1
Range	4,5	4,9	4,7	4,566
Mean	14,7	14,99	14,83	14,84
Std. Deviation	0,4432	0,4719	0,5037	0,4603
Std. Error of Mean	0,007413	0,007893	0,008428	0,0077

Table 6: **Growth data**. Growth and physiological parameters at initial (W0) and final (W8) weeks for Atlantic salmon reared at three different temperatures: 8°C, 12°C, and 15°C. Each parameter includes mean ± SEM. The last column provides the p-value associated with each parameter, indicating the statistical significance of the observed differences between the temperature groups.

	8°C	12°C	15°C	p-value
Weight (g) W0	228 ± 8.41	212.3 ± 7.83	213.2 ± 9.2	0.3330
Length (g) W0	25.9 ± 0.32	25.27 ± 0.31	25.29 ± 0.36	0.3140
Weight(g) W8	418.2 ± 15.19	512.3 ± 17.76	480.5 ± 20.40	0.001
Length(cm) W8	31.61 ± 0.36	33.22 ± 0.42	32.62 ± 0.47	0.0253
SGR (%)	1.225 ± 0.02	1.723 ± 0.24	1.630 ± 0.03	< 0.0001
RGR (%)	85.91 ± 1.53	143.7 ± 2.91	125.5 ± 3.25	< 0.0001
GSI (%) F	0.089 ± 0.00	0.089 ± 0.00	0.101 ± 0.00	0.0029
GSI (%) M	0.119 ± 0.09	0.409 ± 0.38	1.649 ± 0.61	0.0066
HIS (%)	1.15 ± 0.02	1.14 ± 0.01	1.12 ± 0.02	0.0349
K factor	1.287 ± 0.01	1.362 ± 0.01	1.329 ± 0.01	<0.0001

Table 7: **Specific growth rate (SGR).** Descriptive statistic of Specific Growth Rate (%) for Atlantic salmon reared at three different temperatures: 8°C, 12°C, and 15°C during the eight-week trial.

SGR (%)	8°C	12°C	15°C
Number of values	69	70	70
Minimum	0,9195	0,8432	0,876
Maximum	1,576	2,359	2,154
Range	0,6563	1,516	1,278
Mean	1,225	1,723	1,63
Std. Deviation	0,1374	0,197	0,25
Std. Error of Mean	0,01654	0,02355	0,02988

Table 8: Spesific gowth rate (SGR). Results of a non-parametric one-way ANOVA, Dunn's multiple comparisons test, to identify differences between Specific Growth Rate (%) for Atlantic salmon reared at three different temperatures: 8°C, 12°C, and 15°C.

Dunn's multiple comparisons test	Mean rank diff,	Significant?	Summary	Adjusted P Value	
8°C vs. 12°C	-102,9	Yes	****	<0,0001	A-B
8°C vs. 15°C	-83,42	Yes	****	<0,0001	A-C
12°C vs. 15°C	19,49	No	ns	0,1279	B-C
Test details	Mean rank 1	Mean rank 2	Mean rank diff,	n1	n2
8°C vs. 12°C	42,59	145,5	-102,9	69	70
8°C vs. 15°C	42,59	126	-83,42	69	70
12°C vs. 15°C	145,5	126	19,49	68	70

Table 9: Relative growth rate (RGR). Descriptive statistic of Relative Growth Rate (%) for Atlantic salmon reared at three different temperatures: 8°C, 12°C, and 15°C during an eight week trial.

RGR (%)	8°C	12°C	15°C
Number of values	69	68	70
Minimum	59,83	101,4	53,61
Maximum	119,9	194,3	190,6
Range	60,05	92,96	137
Mean	85,91	143,7	125,5
Std. Deviation	12,71	18,66	27,21
Std. Error of Mean	1,53	2,263	3,252

Table 10: Relative growth rate (RGR). Results of a non-parametric one-way ANOVA, Dunn's multiple comparisons test, to identify differences between Relative Growth Rate (%) for Atlantic salmon reared at three different temperatures: 8°C, 12°C, and 15°C.

Dunn's multiple comparisons test	Mean rank diff,	Significant?	Summary	Adjusted P Value	
8°C vs. 12°C	-109,4	Yes	****	<0,0001	A-B
8°C vs. 15°C	-75,71	Yes	****	<0,0001	A-C
12°C vs. 15°C	33,71	Yes	**	0,0018	B-C
Test details	Mean rank 1	Mean rank 2	Mean rank diff,	n1	n2
8°C vs. 12°C	42,99	152,4	-109,4	69	70
8°C vs. 15°C	42,99	118,7	-75,71	69	70
12°C vs. 15°C	152,4	118,7	33,71	68	70

Table 11: Condition factor (K-factor). Descriptive statistic of condition factor (K-factor) for Atlantic salmon reared at three different temperatures: 8°C, 12°C, and 15°C during an eight week trail.

K-factor	8°C	12°C	15°C
Number of values	69	69	68
Minimum	1.170	1.210	1.110
Maximum	1,450	1,620	1,580
Range	0,2800	0,4100	0,4700
Mean	1,298	1,370	1,342
Std. Deviation	0,06468	0,07749	0,08731
Std. Error of Mean	0,007786	0,009328	0,01059

Table 12: Condition factor (K-factor). Results of a post-hoc test after a one-way ANOVA, Holm-Šídák's multiple comparisons test, to identify differences between Condition factor (K-factor) for Atlantic salmon reared at three different temperatures: 8°C, 12°C, and 15°C.

Holm-Šídák's multiple comparisons test	Mean Diff,	Below threshold?	Summary	Adjusted P Value		
8°C vs. 12°C	-0,07232	Yes	***	<0,0001	A-B	
8°C vs. 15°C	-0,04409	Yes	**	0,0019	A-C	
12°C vs. 15°C	0,02823	Yes	*	0,0331	B-C	
Test details	Mean 1	Mean 2	Mean Diff,	SE of diff,	n1	n2
8°C vs. 12°C	1,287	1,362	-0,07543	0,01887	70	70
8°C vs. 15°C	1,287	1,329	-0,04286	0,01887	70	70
12°C vs. 15°C	1,362	1,329	0,03257	0,01887	70	70

Table 13: Hepatosomatic index (HSI). Descriptive statistic of Hepatosomatic index (%) for Atlantic salmon reared at three different temperatures: 8°C, 12°C, and 15°C during the eight- week trial.

HSI (%)	8 °C	12 °C	15 °C
Number of values	68	70	68
Minimum	0,98	0,82	0,85
Maximum	1,39	1,38	1,41
Range	0,41	0,56	0,56
Mean	1,138	1,135	1,093
Std. Deviation	0,09075	0,1012	0,114
Std. Error of Mean	0,011	0,0121	0,01383

Table 14: Hepatosomatic index (HSI). Results of a post-hoc test after a one wayANOVA, Holm-Šídák's multiple comparisons test, to identify differences between Hepatosomatic index (%) for Atlantic salmon reared at three different temperatures: 8°C, 12°C, and 15°C.

Holm-Šídák's multiple comparisons test	Mean Diff,	Below threshold?	Summary	Adjusted P Value		
8°C vs. 12 °C	0,003223	No	ns	0,8536	A-B	
8°C vs. 15 °C	0,04471	Yes	*	0,0346	A-C	
12 °C vs. 15 °C	0,04148	Yes	*	0,0363	B-C	
Test details	Mean 1	Mean 2	Mean Diff,	SE of diff,	n1	n2
8°C vs. 12 °C	1,138	1,135	0,003223	0,01744	68	70
8°C vs. 15 °C	1,138	1,093	0,04471	0,01757	68	68
12 °C vs. 15 °C	1,135	1,093	0,04148	0,01744	70	68

Table 15: Gonadosomatic index (GSI) – female. Descriptive statistic of Gonadosomatic index (%) for female Atlantic salmon reared at three different temperatures: 8°C, 12°C, and 15°C during an eight-week trial.

GSI (%) female	8°C	12°C	15°C
Number of values	37	31	35
Minimum	0,06	0,06	0,07
Maximum	0,11	0,13	0,13
Range	0,05	0,07	0,06
Mean	0,08750	0,08839	0,1024
Std. Deviation	0,01228	0,01695	0,01759
Std. Error of Mean	0,002046	0,003044	0,003017

Table 16: Gonadosomatic index (GSI) – female. Results of a non-parametric one-way ANOVA, Dunn's multiple comparisons test, to identify differences between Gonadosomatic index (%) for female Atlantic salmon reared at three different temperatures: 8°C, 12°C, and 15°C.

Dunn's multiple comparisons test	Mean rank diff,	Significant?	Summary	Adjusted P Value	
8°C vs. 12°C	-1,023	No	ns	>0,9999	A-B
8°C vs. 15°C	-22,71	Yes	**	0,0029	A-C
12°C vs. 15°C	-21,69	Yes	*	0,0072	B-C
Test details	Mean rank 1	Mean rank 2	Mean rank diff,	n1	n2
8°C vs. 12°C	43,04	44,06	-1,023	36	31
8°C vs. 15°C	43,04	65,75	-22,71	36	34
12°C vs. 15°C	44,06	65,75	-21,69	31	34

Table 17: Gonadosomatic index (GSI) – males. Descriptive statistics of Gonadosomatic index (%) for males Atlantic salmon reared at three different temperatures: 8°C, 12°C, and 15°C during an eight-week trail.

GSI (%) male	8°C	12°C	15°C
Number of values	33	39	35
Minimum	0,02	0,02	0,02
Maximum	2,97	14,72	12,39
Range	2,95	14,7	12,37
Mean	0,1191	0,409	1,649
Std. Deviation	0,5121	2,352	3,621
Std. Error of Mean	0,08915	0,3766	0,612

Table 18: Gonadosomatic index (GSI) – males. Results of a non-parametric one-way ANOVA, Dunn's multiple comparisons test, to identify differences between Gonadosomatic index (%) for male Atlantic salmon reared at three different temperatures: 8°C, 12°C, and 15°C.

Dunn's multiple comparisons test	Mean rank diff,	Significant?	Summary	Adjusted P Value	
8°C vs. 12°C	-19,29	Yes	*	0,0167	A-B
8°C vs. 15°C	-19,77	Yes	*	0,0169	A-C
12°C vs. 15°C	-0,4766	No	ns	>0,9999	B-C
Test details	Mean rank 1	Mean rank 2	Mean rank diff,	n1	n2
8°C vs. 12°C	40,5	59,79	-19,29	33	39
8°C vs. 15°C	40,5	60,27	-19,77	33	35
12°C vs. 15°C	59,79	60,27	-0,4766	39	35

Table 19: Summary of Pearson correlation test between GSI (%) and SGR (%) for females and males in the different temperature groups (8°C, 12°C and 15°C).

GSI (%) vs. SGR (%)	8°C		12°C		15°C	
	Female	Male	Female	Male	Female	Male
r	-0,05452	-0,156	0,1186	-0,6489	-0,2102	-0,6421
R squared	0,002973	0,02433	0,01407	0,421	0,04418	0,4122
P value (two tailed)	0,7521	0,394	0,5251	<0,0001	0,2328	<0,0001

Table 20: Stomach fullness index (SFI). Descriptive statistics of Stomach fullness index (%) for female Atlantic salmon reared at three different temperatures: 8°C, 12°C, and 15°C during a 24-hour sampling.

Stomach fullness - grouped	8°C	12°C	15°C
Number of sampling points	7	7	7
Minimum (g)	0,0114	0,0135	0,0008
Maximum (g)	0,7687	0,9346	0,8721
Range	0,7573	0,9211	0,8713
Mean (g)	0,3583	0,3793	0,3834
Std. Deviation	0,2929	0,3773	0,3858
Std. Error of Mean	0,1107	0,1426	0,1458

Table 21: **Stomach fullness index (SFI).** Output from a 2-way ANOVA of stomach fullness index (%) for Atlantic salmon reared at three different temperatures: 8°C, 12°C, and 15°C, during a 24-hour sampling period.

Stomach fullness (dry) – 2wayANOVA					
Two-way ANOVA	Ordinary				
Alpha	0,05				
Source of Variation	% of total variation	P value	P value summary	Significant?	
Interaction	3,486	0,0024	**	Yes	
Time	75,9	<0,0001	****	Yes	
Temperature	0,08947	0,6629	ns	No	
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	0,9933	12	0,08277	F (12, 189) = 2,675	P=0,0024
Time	21,63	6	3,605	F (6, 189) = 116,5	P<0,0001
Temperature	0,02549	2	0,01275	F (2, 189) = 0,4120	P=0,6629
Residual	5,848	189	0,03094		
Data summary					
Number of columns (Temperature)	3				
Number of rows (Time)	7				
Number of values	210				

Table 22: Stomach fullness index (SFI). Output from Tukey's multiple comparison test on of stomach fullness index for differences in mean between three temperature groups (8°C, 12°C and 15°C) at various time intervals (2-24 hours). A statistically significant difference was considered p-value < 0.005 (∗p < 0.05; ∗∗p < 0.01; ∗∗∗p < 0.001).

Tukey's multiple comparisons test	Mean Diff,	95,00% CI of diff,	Below threshold?	Summary	Adjusted P Value
+2 h					
8°C vs. 12°C	-0,1659	-0,3517 to 0,01993	No	ns	0,0907
8°C vs. 15°C	-0,1034	-0,2892 to 0,08243	No	ns	0,3889
12°C vs. 15°C	0,0625	-0,1233 to 0,2483	No	ns	0,7068
+4 h					
8°C vs. 12°C	-0,0484	-0,2342 to 0,1374	No	ns	0,8119
8°C vs. 15°C	-0,1373	-0,3231 to 0,04853	No	ns	0,1911
12°C vs. 15°C	-0,0889	-0,2747 to 0,09693	No	ns	0,4967
+8 h					
8°C vs. 12°C	-0,2396	-0,4254 to -0,05377	Yes	**	0,0074
8°C vs. 15°C	-0,2724	-0,4582 to -0,08657	Yes	**	0,0019
12°C vs. 15°C	-0,0328	-0,2186 to 0,1530	No	ns	0,9087
+12 h					
8°C vs. 12°C	0,0893	-0,09653 to 0,2751	No	ns	0,4936
8°C vs. 15°C	0,1092	-0,07663 to 0,2950	No	ns	0,3491
12°C vs. 15°C	0,0199	-0,1659 to 0,2057	No	ns	0,9653
+16 h					
8°C vs. 12°C	0,2069	0,02107 to 0,3927	Yes	*	0,0249
8°C vs. 15°C	0,1931	0,007272 to 0,3789	Yes	*	0,0396
12°C vs. 15°C	-0,0138	-0,1996 to 0,1720	No	ns	0,9832
+20 h					
8°C vs. 12°C	0,0125	-0,1733 to 0,1983	No	ns	0,9862
8°C vs. 15°C	0,0241	-0,1617 to 0,2099	No	ns	0,9496
12°C vs. 15°C	0,0116	-0,1742 to 0,1974	No	ns	0,9881
+24 h					
8°C vs. 12°C	-0,0021	-0,1879 to 0,1837	No	ns	0,9996
8°C vs. 15°C	0,0106	-0,1752 to 0,1964	No	ns	0,99
12°C vs. 15°C	0,0127	-0,1731 to 0,1985	No	ns	0,9857

Table 23: **Midgut fullness index (MFI).** Descriptive statistics of midgut fullness index (%) for female Atlantic salmon reared at three different temperatures: 8°C, 12°C, and 15°C during a 24-hour sampling.

Midgut fullness - grouped	8°C	12°C	15°C
Number of sampling point	7	7	7
Minimum (g)	0,0888	0,0484	0,0266
Maximum (g)	0,1767	0,1796	0,1288
Range	0,0879	0,1312	0,1022
Mean (g)	0,1316	0,1232	0,08996
Std. Deviation	0,03335	0,04358	0,03557
Std. Error of Mean	0,01261	0,01647	0,01344

Table 24: **Midgut fullness index (MFI).** Output from a 2-way ANOVA of midgut fullness index (%) for Atlantic salmon reared at three different temperatures: 8°C, 12°C, and 15°C, during a 24-hour sampling period.

Midgut fullness (dry) - 2wayANOVA					
Two-way ANOVA	Ordinary				
Alpha	0,05				
Source of Variation	% of total variation	P value	P value summary	Significant?	
Interaction	7,198	0,0042	**	Yes	
Time	36,37	<0,0001	****	Yes	
Temperaure	11,55	<0,0001	****	Yes	
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	0,04239	12	0,003532	F (12, 189) = 2,526	P=0,0042
Time	0,2142	6	0,0357	F (6, 189) = 25,53	P<0,0001
Temperaure	0,06799	2	0,034	F (2, 189) = 24,31	P<0,0001
Residual	0,2643	189	0,001398		
Data summary					
Number of columns (Temperaure)	3				
Number of rows (Time)	7				
Number of values	210				

Table 25: Midgut fullness index (MFI). Output from Tukey's multiple comparisons test on of midgut fullness index for differences in mean between three temperature groups (8°C, 12°C and 15°C) at various time intervals (2-24 hours). A statistically significant difference was considered p-value < 0.005 (∗p < 0.05; ∗∗p < 0.01; ∗∗∗p < 0.001).

Tukey's multiple comparisons test	Mean Diff,	95,00% CI of diff,	Below threshold?	Summary	Adjusted P Value
+2 h					
8°C vs. 12°C	-0,0115	-0,05101 to 0,02801	No	ns	0,771
8°C vs. 15°C	0,039	-0,0005069 to 0,07851	No	ns	0,0539
12°C vs. 15°C	0,0505	0,01099 to 0,09001	Yes	**	0,0081
+4 h					
8°C vs. 12°C	-0,0188	-0,05831 to 0,02071	No	ns	0,5004
8°C vs. 15°C	0,0089	-0,03061 to 0,04841	No	ns	0,8556
12°C vs. 15°C	0,0277	-0,01181 to 0,06721	No	ns	0,2249
+8 h					
8°C vs. 12°C	-0,0502	-0,08971 to -0,01069	Yes	**	0,0085
8°C vs. 15°C	0,017	-0,02251 to 0,05651	No	ns	0,5674
12°C vs. 15°C	0,0672	0,02769 to 0,1067	Yes	***	0,0002
+12 h					
8°C vs. 12°C	0,0167	-0,02281 to 0,05621	No	ns	0,5787
8°C vs. 15°C	0,0479	0,008393 to 0,08741	Yes	*	0,0129
12°C vs. 15°C	0,0312	-0,008307 to 0,07071	No	ns	0,1516
+16 h					
8°C vs. 12°C	0,038	-0,001507 to 0,07751	No	ns	0,0622
8°C vs. 15°C	0,059	0,01949 to 0,09851	Yes	**	0,0015
12°C vs. 15°C	0,021	-0,01851 to 0,06051	No	ns	0,4221
+20 h					
8°C vs. 12°C	0,0445	0,004993 to 0,08401	Yes	*	0,0229
8°C vs. 15°C	0,0578	0,01829 to 0,09731	Yes	**	0,0019
12°C vs. 15°C	0,0133	-0,02621 to 0,05281	No	ns	0,7064
+24 h					
8°C vs. 12°C	0,0404	0,0008931 to 0,07991	Yes	*	0,0438
8°C vs. 15°C	0,0622	0,02269 to 0,1017	Yes	***	0,0008
12°C vs. 15°C	0,0218	-0,01771 to 0,06131	No	ns	0,395

Table 26: **Hindgut fullness index (HFI).** Descriptive statistics of hindgut fullness index (%) for female Atlantic salmon reared at three different temperatures: 8°C, 12°C, and 15°C during a 24-hour sampling.

Hindgut fullness - grouped	8°C	12°C	15°C
Number of sampling points	7	7	7
Minimum (g)	0,0415	0,0456	0,0281
Maximum (g)	0,068	0,0856	0,0799
Range	0,0265	0,04	0,0518
Mean (g)	0,05456	0,07033	0,05197
Std. Deviation	0,008385	0,01307	0,01978
Std. Error of Mean	0,003169	0,004939	0,007475

Table 27: Hindgut fullness index (HFI). Output from a 2-way ANOVA of midgut fullness index (%) for Atlantic salmon reared at three different temperatures: 8°C, 12°C, and 15°C, during a 24-hour sampling period.

Hindgut fullness (dry) - 2wayANOVA					
Two-way ANOVA	Ordinary				
Alpha	0,05				
Source of Variation	% of total variation	P value	P value summary	Significant?	
Interaction	18,28	<0,0001	****	Yes	
Time	8,822	0,0003	***	Yes	
Temperature	9,876	<0,0001	****	Yes	
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	0,02558	12	0,002132	F (12, 189) = 4,568	P<0,0001
Time	0,01235	6	0,002058	F (6, 189) = 4,409	P=0,0003
Temperature	0,01382	2	0,006911	F (2, 189) = 14,81	P<0,0001
Residual	0,08821	189	0,0004667		
Data summary					
Number of columns (Temperature)	3				
Number of rows (Time)	7				
Number of values	210				

Table 28: Hindgut fullness index (HFI). Output from a Tukey's multiple comparisons test on of hindgut fullness index for differences in mean between three temperature groups (8°C, 12°C and 15°C) at various time intervals (2-24 hours). A statistically significant difference was considered p-value < 0.005 (∗p < 0.05; ∗∗p < 0.01; ∗∗∗p < 0.001).

Tukey's multiple comparisons test	Mean Diff,	95,00% CI of diff,	Below threshold?	Summary	Adjusted P Value
+2 h					
8°C vs. 12°C	-0,0102	-0,03302 to 0,01262	No	ns	0,5428
8°C vs. 15°C	0,0323	0,009477 to 0,05512	Yes	**	0,0029
12°C vs. 15°C	0,0425	0,01968 to 0,06532	Yes	****	<0,0001
+4 h					
8°C vs. 12°C	-0,0365	-0,05932 to -0,01368	Yes	***	0,0006
8°C vs. 15°C	0,0093	-0,01352 to 0,03212	No	ns	0,6014
12°C vs. 15°C	0,0458	0,02298 to 0,06862	Yes	****	<0,0001
+8 h					
8°C vs. 12°C	-0,0188	-0,04162 to 0,004023	No	ns	0,1288
8°C vs. 15°C	0,0106	-0,01222 to 0,03342	No	ns	0,517
12°C vs. 15°C	0,0294	0,006577 to 0,05222	Yes	**	0,0075
+12 h					
8°C vs. 12°C	-0,0317	-0,05452 to -0,008877	Yes	**	0,0035
8°C vs. 15°C	-0,0384	-0,06122 to -0,01558	Yes	***	0,0003
12°C vs. 15°C	-0,0067	-0,02952 to 0,01612	No	ns	0,7676
+16 h					
8°C vs. 12°C	-0,0126	-0,03542 to 0,01022	No	ns	0,3946
8°C vs. 15°C	-0,019	-0,04182 to 0,003823	No	ns	0,1234
12°C vs. 15°C	-0,0064	-0,02922 to 0,01642	No	ns	0,7855
+20 h					
8°C vs. 12°C	-0,006	-0,02882 to 0,01682	No	ns	0,8088
8°C vs. 15°C	0,0004	-0,02242 to 0,02322	No	ns	0,9991
12°C vs. 15°C	0,0064	-0,01642 to 0,02922	No	ns	0,7855
+24 h					
8°C vs. 12°C	0,0054	-0,01742 to 0,02822	No	ns	0,842
8°C vs. 15°C	0,0229	7,657e-005 to 0,04572	Yes	*	0,049
12°C vs. 15°C	0,0175	-0,005323 to 0,04032	No	ns	0,1686

Table 29: Feed intake. Results of a non-parametric one-way ANOVA Dunn's multiple comparisons test, to identify differences between feed intake for Atlantic salmon reared at three different temperatures: 8°C, 12°C, and 15°C.

Dunn's multiple comparisons test	Mean rank diff,	Significant?	Summary	Adjusted P Value	
15°C vs. 8°C	29,85	Yes	**	0,0013	A-B
15°C vs. 12°C	-16,52	No	ns	0,15	A-C
8°C vs. 12°C	-46,37	Yes	****	<0,0001	B-C
Test details	Mean rank 1	Mean rank 2	Mean rank diff,	n1	n2
15°C vs. 8°C	75,33	45,48	29,85	46	47
15°C vs. 12°C	75,33	91,84	-16,52	46	48
8°C vs. 12°C	45,48	91,84	-46,37	47	48

Table 30: Gallbladder index (GBI). Output from a 2-way ANOVA of GBI in Atlantic salmon reared at three different temperatures: 8°C, 12°C, and 15°C, during a 24-hour sampling period.

2way ANOVA - gall bladder index					
Two-way ANOVA	Ordinary				
Alpha	0,05				
Source of Variation	% of total variation	P value	P value summary	Significant?	
Interaction	11,2	<0,0001	****	Yes	
Time	41,06	<0,0001	****	Yes	
Temperature	3,688	0,0005	***	Yes	
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	0,09674	12	0,008062	F (12, 189) = 4,006	P<0,0001
Time	0,3546	6	0,05909	F (6, 189) = 29,36	P<0,0001
Temperature	0,03185	2	0,01592	F (2, 189) = 7,912	P=0,0005
Residual	0,3804	189	0,002013		
Data summary					
Number of columns (Column Factor)	3				

Number of rows (Row Factor)	7				
Number of values	210				

Table 31: Gallbladder index (GBI). Output from Tukey's multiple comparisons test on GBI levels in Atlantic salmon at three different temperature groups (8°C, 12°C and 15°C) at various time intervals (2-24 hours). A statistically significant difference was considered p-value < 0.005 (∗p < 0.05; ∗∗p < 0.01; ∗∗∗p < 0.001).

Tukey's multiple comparisons test	Mean Diff,	95,00% CI of diff,	Below threshold?	Summary	Adjusted P Value
+2					
8°C vs. 12°C	0,01706	-0,03033 to 0,06446	No	ns	0,6721
8°C vs. 15°C	0,000458	-0,04694 to 0,04785	No	ns	0,9997
12°C vs. 15°C	-0,0166	-0,06400 to 0,03079	No	ns	0,6864
+4					
8°C vs. 12°C	0,005801	-0,04159 to 0,05320	No	ns	0,955
8°C vs. 15°C	-0,007721	-0,05512 to 0,03967	No	ns	0,9216
12°C vs. 15°C	-0,01352	-0,06092 to 0,03387	No	ns	0,7789
+8					
8°C vs. 12°C	0,001703	-0,04569 to 0,04910	No	ns	0,996
8°C vs. 15°C	-0,009654	-0,05705 to 0,03774	No	ns	0,8803
12°C vs. 15°C	-0,01136	-0,05875 to 0,03604	No	ns	0,8383
+12					
8°C vs. 12°C	-0,02005	-0,06744 to 0,02735	No	ns	0,5783
8°C vs. 15°C	-0,01401	-0,06140 to 0,03339	No	ns	0,7649
12°C vs. 15°C	0,006039	-0,04136 to 0,05343	No	ns	0,9513
+16					
8°C vs. 12°C	0,001976	-0,04542 to 0,04937	No	ns	0,9947
8°C vs. 15°C	0,00144	-0,04596 to 0,04884	No	ns	0,9972
12°C vs. 15°C	-0,000536	-0,04793 to 0,04686	No	ns	0,9996
+20					
8°C vs. 12°C	-0,06101	-0,1084 to -0,01361	Yes	**	0,0076
8°C vs. 15°C	-0,03466	-0,08205 to 0,01274	No	ns	0,1977

12°C vs. 15°C	0,02635	-0,02104 to 0,07375	No	ns	0,3894
+24					
8°C vs. 12°C	-0,08023	-0,1276 to -0,03283	Yes	***	0,0003
8°C vs. 15°C	-0,144	-0,1914 to -0,09663	Yes	****	<0,0001
12°C vs. 15°C	-0,0638	-0,1112 to -0,01640	Yes	**	0,0049

Table 32: Table of correlation. Output of a Pearson correlation test between GBI and SFI, MFI and HFI in Atlantic salmon reared at three different temperatures (8°C, 12°C and 15°C).

	Pearson correlation test	*GBI vs. SFI*	*GBI vs. MFI*	*GBI vs. HFI*
	R	-0.1623	-0.5495	-0.02989
	R squared	0.02634	0.30200	0.000089
8 C	P value (two tailed)	0.1827	<0.0001 ***	0.8074
	R	-0.4664	-0.6598	-0.3515
	R squared	0.2175	0.4354	0.1236
12 C	P value (two tailed)	<0.0001****	<0.0001****	0.0028 **
	R	-0.3568	-0.5273	-0.2446
	R squared	0.1273	0.2781	0.05985
15C	P value (two tailed)	0.0024	<0.0001****	0.0412 *

APPENDIX D – Analysis of gene expression

Table 33: *ghrl-I* **expression in stomach.** Output from a 2-way ANOVA of log-transformed mRNA expression of *ghrl-I* in stomach tissue for Atlantic salmon reared at three different temperatures: 8°C, 12°C, and 15°C, during a 24-hour sampling period.

ghrl-I - 2wayANOVA					
Two-way ANOVA	Ordinary				
Alpha	0,05				
Source of Variation	% of total variation	P value	P value summary	Significant?	
Interaction	5,519	0,5018	ns	No	
Time	3,479	0,3119	ns	No	
Temperature	4,496	0,011	*	Yes	
ANOVA table	SS (Type III)	DF	MS	F (DFn, DFd)	P value
Interaction	0,2335	12	0,01946	F (12, 178) = 0,9467	P=0,5018
Time	0,1472	6	0,02453	F (6, 178) = 1,193	P=0,3119
Temperature	0,1902	2	0,09511	F (2, 178) = 4,627	P=0,0110
Residual	3,659	178	0,02056		
Data summary					
Number of columns (Temperature)	3				
Number of rows (Time)	7				
Number of values	199				

Table 34: *ghrl-I* expression in stomach. Output from Tukey's multiple comparisons test on log-transformed *ghrl-I* mRNA expression in stomach, for differences in mean between three temperature groups (8°C, 12°C and 15°C) at various time intervals (2-24 hours). A statistically significant difference was considered p-value < 0.005 (∗p < 0.05; ∗∗p < 0.01; ∗∗∗p < 0.001).

Tukey's multiple comparisons test	Predicted (LS) mean diff,	95,00% CI of diff,	Below threshold?	Summary	Adjusted P Value
+ 2 h					
8°C vs. 12°C	0,01049	-0,1411 to 0,1620	No	ns	0,9854

8°C vs. 15°C	0,0007896	-0,1549 to 0,1565	No	ns	>0,9999
12°C vs. 15°C	-0,009697	-0,1654 to 0,1460	No	ns	0,9881
+ 4 h					
8°C vs. 12°C	0,01584	-0,1399 to 0,1715	No	ns	0,9686
8°C vs. 15°C	0,01938	-0,1363 to 0,1751	No	ns	0,9534
12°C vs. 15°C	0,003542	-0,1480 to 0,1551	No	ns	0,9983
+ 8 h					
8°C vs. 12°C	-0,03623	-0,1960 to 0,1235	No	ns	0,8537
8°C vs. 15°C	0,08628	-0,07346 to 0,2460	No	ns	0,4103
12°C vs. 15°C	0,1225	-0,03723 to 0,2823	No	ns	0,1684
+ 12 h					
8°C vs. 12°C	-0,05291	-0,2086 to 0,1028	No	ns	0,7016
8°C vs. 15°C	-0,02117	-0,1727 to 0,1304	No	ns	0,9417
12°C vs. 15°C	0,03173	-0,1240 to 0,1874	No	ns	0,88
+ 16 h					
8°C vs. 12°C	0,01138	-0,1443 to 0,1671	No	ns	0,9837
8°C vs. 15°C	0,1888	0,03315 to 0,3445	Yes	*	0,0129
12°C vs. 15°C	0,1775	0,02592 to 0,3290	Yes	*	0,0171
+ 20 h					
8°C vs. 12°C	-0,07476	-0,2305 to 0,08094	No	ns	0,4939
8°C vs. 15°C	0,008569	-0,1471 to 0,1643	No	ns	0,9907
12°C vs. 15°C	0,08333	-0,06821 to 0,2349	No	ns	0,3973
+ 24 h					
8°C vs. 12°C	-0,1029	-0,2675 to 0,06178	No	ns	0,3045
8°C vs. 15°C	0,01291	-0,1478 to 0,1736	No	ns	0,9803
12°C vs. 15°C	0,1158	-0,03991 to 0,2715	No	ns	0,187

Table 35: *ghrl-II* **expression in stomach.** Output from a 2-way ANOVA of log-transformed mRNA expression of *ghrl-II* in stomach tissue for Atlantic salmon reared at three different temperatures: 8°C, 12°C, and 15°C, during a 24-hour sampling period.

ghrl-II - 2wayANOVA					
Two-way ANOVA	Ordinary				
Alpha	0,05				
Source of Variation	% of total variation	P value	P value summary	Significant?	
Interaction	9,148	0,1	ns	No	
Time	2,977	0,4073	ns	No	
Temperature	1,502	0,2131	ns	No	
ANOVA table	SS (Type III)	DF	MS	F (DFn, DFd)	P value
Interaction	0,3902	12	0,03251	F (12, 179) = 1,583	P=0,1000
Time	0,1269	6	0,02116	F (6, 179) = 1,030	P=0,4073
Temperature	0,06407	2	0,03203	F (2, 179) = 1,560	P=0,2131
Residual	3,677	179	0,02054		
Data summary					
Number of columns (Temperature)	3				
Number of rows (Time)	7				
Number of values	200				

Table 36: *ghrl-II* **expression in stomach.** Output from Tukey's multiple comparisons test on log-transformed *ghrl-II* mRNA expression in stomach, for differences in mean between three temperature groups (8°C, 12°C and 15°C) at various time intervals (2-24 hours). A statistically significant difference was considered p-value < 0.005 (∗p < 0.05; ∗∗p < 0.01; ∗∗∗p < 0.001).

Tukey's multiple comparisons test	Predicted (LS) mean diff,	95,00% CI of diff,	Below threshold?	Summary	Adjusted P Value
+ 2 h					
8°C vs. 12°C	0,08351	-0,06797 to 0,2350	No	ns	0,3954
8°C vs. 15°C	-0,03692	-0,1925 to 0,1187	No	ns	0,8411
12°C vs. 15°C	-0,1204	-0,2761 to 0,03520	No	ns	0,1631
+ 4 h					
8°C vs. 12°C	-0,01865	-0,1832 to 0,1459	No	ns	0,9612
8°C vs. 15°C	-0,05639	-0,2171 to 0,1043	No	ns	0,6852
12°C vs. 15°C	-0,03774	-0,1934 to 0,1179	No	ns	0,8346
+ 8 h					
8°C vs. 12°C	-0,02063	-0,1763 to 0,1350	No	ns	0,9473
8°C vs. 15°C	0,06183	-0,09380 to 0,2175	No	ns	0,6164
12°C vs. 15°C	0,08247	-0,07721 to 0,2421	No	ns	0,4426
+ 12 h					
8°C vs. 12°C	-0,04935	-0,2050 to 0,1063	No	ns	0,7344
8°C vs. 15°C	-0,0494	-0,2009 to 0,1021	No	ns	0,7215
12°C vs. 15°C	-0,00004775	-0,1557 to 0,1556	No	ns	>0,9999
+ 16 h					
8°C vs. 12°C	0,0004993	-0,1510 to 0,1520	No	ns	>0,9999
8°C vs. 15°C	0,1465	-0,005002 to 0,2980	No	ns	0,0605
12°C vs. 15°C	0,146	-0,005501 to 0,2975	No	ns	0,0616
+ 20 h					
8°C vs. 12°C	-0,07753	-0,2332 to 0,07810	No	ns	0,4683
8°C vs. 15°C	0,01269	-0,1470 to 0,1724	No	ns	0,9807
12°C vs. 15°C	0,09022	-0,06541 to 0,2458	No	ns	0,3588
+ 24 h					
8°C vs. 12°C	-0,1502	-0,3058 to 0,005458	No	ns	0,0612
8°C vs. 15°C	-0,02099	-0,1766 to 0,1346	No	ns	0,9455
12°C vs. 15°C	0,1292	-0,02230 to 0,2807	No	ns	0,1114

Table 37: *mboat4* **expression in stomach.** Output from a 2-way ANOVA of log-transformed mRNA expression of *mboat4* in stomach tissue for Atlantic salmon reared at three different temperatures: 8°C, 12°C, and 15°C, during a 24-hour sampling period.

mboat4 - 2wayANOVA					
Two-way ANOVA	Ordinary				
Alpha	0,05				
Source of Variation	% of total variation	P value	P value summary	Significant?	
Interaction	6,209	0,3666	ns	No	
Time	4,26	0,1794	ns	No	
Temperature	0,7104	0,473	ns	No	
ANOVA table	SS (Type III)	DF	MS	F (DFn, DFd)	P value
Interaction	0,4964	12	0,04136	F (12, 188) = 1,095	P=0,3666
Time	0,3406	6	0,05676	F (6, 188) = 1,502	P=0,1794
Temperature	0,0568	2	0,0284	F (2, 188) = 0,7517	P=0,4730
Residual	7,102	188	0,03778		
Data summary					
Number of columns (Temperature)	3				
Number of rows (Time)	7				
Number of values	209				

Table 38: *mboat4* **expression in stomach.** Output from Tukey's multiple comparisons test on log-transformed *mboat4* mRNA expression in stomach, for differences in mean between three temperature groups (8°C, 12°C and 15°C) at various time intervals (2-24 hours). A statistically significant difference was considered p-value < 0.005 (∗p < 0.05; ∗∗p < 0.01; ∗∗∗p < 0.001).

Tukey's multiple comparisons test	Predicted (LS) mean diff,	95,00% CI of diff,	Below threshold?	Summary	Adjusted P Value
+ 2 h					
8°C vs. 12°C	-0,01656	-0,2219 to 0,1888	No	ns	0,9802
8°C vs. 15°C	-0,02355	-0,2289 to 0,1818	No	ns	0,9604
12°C vs. 15°C	-0,006987	-0,2123 to 0,1984	No	ns	0,9964
+ 4 h					
8°C vs. 12°C	-0,0569	-0,2622 to 0,1485	No	ns	0,79
8°C vs. 15°C	0,005577	-0,1998 to 0,2109	No	ns	0,9977
12°C vs. 15°C	0,06247	-0,1429 to 0,2678	No	ns	0,7527
+ 8 h					
8°C vs. 12°C	0,006115	-0,1992 to 0,2115	No	ns	0,9973
8°C vs. 15°C	-0,001371	-0,2067 to 0,2040	No	ns	0,9999
12°C vs. 15°C	-0,007487	-0,2128 to 0,1979	No	ns	0,9959
+ 12 h					
8°C vs. 12°C	0,113	-0,09794 to 0,3240	No	ns	0,4164
8°C vs. 15°C	-0,1512	-0,3622 to 0,05975	No	ns	0,2104
12°C vs. 15°C	-0,2643	-0,4696 to -0,05891	Yes	**	0,0076
+ 16 h					
8°C vs. 12°C	-0,1044	-0,3097 to 0,1010	No	ns	0,4542
8°C vs. 15°C	-0,1425	-0,3478 to 0,06286	No	ns	0,2317
12°C vs. 15°C	-0,03811	-0,2435 to 0,1672	No	ns	0,8995
+ 20 h					
8°C vs. 12°C	0,003624	-0,2017 to 0,2090	No	ns	0,999
8°C vs. 15°C	0,03736	-0,1680 to 0,2427	No	ns	0,9033
12°C vs. 15°C	0,03373	-0,1716 to 0,2391	No	ns	0,9204
+ 24 h					
8°C vs. 12°C	0,1065	-0,09881 to 0,3119	No	ns	0,4395
8°C vs. 15°C	0,06121	-0,1441 to 0,2666	No	ns	0,7614
12°C vs. 15°C	-0,04534	-0,2507 to 0,1600	No	ns	0,8609

Table 39: Table of correlation. Output of a Pearson correlation test of mRNA expression of *ghrl-I, ghrl-II* and *mboa4* and SFI in Atlantic salmon reared at three different temperatures (8°C, 12°C and 15°C).

Pearson correlation test		SFI vs. g*hrl-I*	SFI vs. *ghrl-II*	SFI vs. *mboat4*
	r	0,2059	0,1948	0,07049
8°C	R squared	0,04239	0,03794	0,004969
	P value(two tailed)	0,1027	0,1061	0,5649
	r	-0,1808	-0,09452	0,2496
12°C	R squared	0,03268	0,008933	0,06231
	P value(two tailed)	0,1432	0,4398	0,0372
	r	0,07657	0,08528	0,04125
15°C	R squared	0,005863	0,007273	0,001702
	P value(two tailed)	0,5287	0,4827	0,7346

APPENDIX E – Analysis of Ghrl plasma levels

Table 40: **Ghrelin plasma levels.** Output from a 2-way ANOVA of Ghrl plasma levels in Atlantic salmon reared at three different temperatures: 8°C, 12°C, and 15°C, during a 24-hour sampling period.

2way ANOVA- Ghlr plasma levels					
Two-way ANOVA	Ordinary				
Alpha	0,05				
Source of Variation	% of total variation	P value	P value summary	Significant?	
Interaction	16,92	0,0002	***	Yes	
Time	7,072	0,0122	*	Yes	
Temperature	0,6819	0,4448	ns	No	
ANOVA table	SS (Type III)	DF	MS	F (DFn, DFd)	P value
Interaction	3916	12	326,3	F (12, 181) = 3,365	P=0,0002
Time	1637	6	272,8	F (6, 181) = 2,813	P=0,0122
Temperature	157,8	2	78,92	F (2, 181) = 0,8138	P=0,4448
Residual	17553	181	96,98		
Data summary					
Number of columns (Temperature)	3				
Number of rows (Time)	7				
Number of values	202				

Table 41: Ghrl plasma levels. Output from Tukey's multiple comparisons test on Ghrl plasma levels in Atlantic salmon at three different temperature groups (8°C, 12°C and 15°C) at various time intervals (2-24 hours). A statistically significant difference was considered p-value < 0.005 (∗p < 0.05; ∗∗p < 0.01; ∗∗∗p < 0.001).

Tukey's multiple comparisons test	Predicted (LS) mean diff,	95,00% CI of diff,	Below threshold?	Summary	Adjusted P Value
+2					
8°C vs. 12°C	-14,93	-25,34 to -4,524	Yes	**	0,0025
8°C vs. 15°C	-3,199	-13,61 to 7,208	No	ns	0,7482
12°C vs. 15°C	11,73	1,325 to 22,14	Yes	*	0,0228
+4					
8°C vs. 12°C	-8,389	-18,80 to 2,018	No	ns	0,1402
8°C vs. 15°C	-10,94	-21,35 to -0,5316	Yes	*	0,0369
12°C vs. 15°C	-2,55	-12,96 to 7,857	No	ns	0,8315
+8					
8°C vs. 12°C	2,225	-8,468 to 12,92	No	ns	0,8754
8°C vs. 15°C	-1,126	-11,53 to 9,281	No	ns	0,9646
12°C vs. 15°C	-3,351	-14,04 to 7,342	No	ns	0,7397
+12					
8°C vs. 12°C	0,51	-9,897 to 10,92	No	ns	0,9926
8°C vs. 15°C	-5,856	-16,55 to 4,836	No	ns	0,4002
12°C vs. 15°C	-6,366	-17,06 to 4,326	No	ns	0,3394
+16					
8°C vs. 12°C	-1,958	-12,37 to 8,449	No	ns	0,8969
8°C vs. 15°C	-9,639	-20,33 to 1,053	No	ns	0,0866
12°C vs. 15°C	-7,681	-18,37 to 3,011	No	ns	0,2089
+20					
8°C vs. 12°C	1,086	-9,607 to 11,78	No	ns	0,9688
8°C vs. 15°C	1,169	-9,524 to 11,86	No	ns	0,9639
12°C vs. 15°C	0,08333	-10,89 to 11,05	No	ns	0,9998
+24					
8°C vs. 12°C	15,41	4,716 to 26,10	Yes	**	0,0023
8°C vs. 15°C	14,48	3,444 to 25,52	Yes	**	0,0063
12°C vs. 15°C	-0,9263	-12,23 to 10,38	No	ns	0,9796

Table 42: Table of correlation results. Output of a Pearson correlation test between Ghlr plasma levels and several other factors in Atlantic salmon reared at three different temperatures (8°C, 12°C and 15°C).

	Pearson correlation test	Ghrl plasma vs ghrl-I	Ghrl plasma vs. ghrl-II	Ghrl plasma vs. mboat4	Ghrl plasma vs. SFI	Ghrl plasma vs. MFI	Ghrl plasma vs. HFI	Ghrl plasma Vs. GBI
8°C	R	0.1784	0.1501	0.2440	-0.2742	-0.06939	-0.0659	-0.08041
	R squared	0.03183	0.02252	0.05953	0.07518	0.004815	0.00435	0.006466
	P value (two tailed)	0.1395	0.2150	0.0433 *	0.0216 *	0.5681	0.5875	0.5082
12°C	R	0.04906	0.007018	0.09766	0.3452	0.2610	0.2292	-0.2363
	R squared	0.002407	0.00005	0.009538	0.1192	0.06810	0.05253	0.05586
	P value (two tailed)	0.6934	0.9554	0.4317	0.0042 **	0.0329 *	0.0621	0.0542
15°C	R	-0.09346	-0.09886	-0.07650	0.2086	0.5111	0.3861	-0.4007
	R squared	0.008734	0.009773	0.005853	0.04351	0.2612	0.0011	0,1605
	P value (two tailed)	0.4450	0.4190	0.5321	0.0854	<0.0001***	0.0011 **	0.0006 ***