Feeding frequency in commercial farming of Atlantic halibut: effects on appetite and growth performance

Thesis submitted for partial fulfilment of the degree

Master of Aquaculture

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Abstract

Atlantic halibut (*Hippoglossus Hippoglossus)* is one of the few marine fish species being farmed in Norway. A challenge impairing further growth of the halibut aquaculture industry is slow growth of individuals above 1 kg (grow-out halibut). In order to understand some of the factors underlying growth in this species this study aimed at investigating feed intake, appetite, and growth performance in relation to feeding frequencies in a commercial setting. Four tanks (á 150 m x 6.5 m x 1.2 m) with five feeding stations were used in the experiment. Two different feeding frequencies were used: feeding every second day (d2) and feeding every third day (d3). Further, two separate trials were conducted, a growth trial (trial A) and a feed-intake trial (trial B). For trial A, externally tagged fish were followed from May 2022 to March 2023, and during this period, specific growth rate (SGR) was calculated for three growth periods. There was a trend towards higher SGR in fish fed every third day, but no significant differences were observed, which gave the conclusion that feeding fish every second or every third day had the same effect on growth. However, there were significant differences in SGR between female and male individuals during the winter. All males had become sexually mature at the last sampling and had reallocated energy into development of gonads with a lower SGR over the winter. The males had not able to catch up with the females at the end of the trial in March 2023 (average weight males; d2: 2.4 (± 0.5) kg, d3: 2.6 (± 0.9) kg, and females; d2: 4.0 (± 1) kg, d3: 4.5 (± 2) kg). For trial B, feed intake was measured by sampling fish after a meal, and quantifying content in stomach and gut. There were no significant differences in the size of the meal between fish fed every second or third day. However, there was differences between females and males, due to all males being mature and generally having lower appetite. In addition to measuring feed intake, gene expression of hormones related to appetite control in the digestive tract (*ghrl*, *pyya*, *pyyb*, *cck1,* and *cck2*) was examined, but no significant differences in relation to treatment was observed. The conclusion for trial B is therefore that feeding every second or every third day did not affect feed intake or expression of appetite related genes in the gut.

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1. Introduction

1.1 Atlantic halibut (*Hippoglossus Hippoglossus*)

Atlantic halibut (*Hippoglossus Hippoglossus)* is a cold-water species found in the northern Atlantic and is one of the few fish species being farmed in Norway. It is the largest species of flatfish (Pleuronectiformes) and has a long-life span of up to 50 years (*Atlantic Halibut | NOAA Fisheries*, n.d.; Haug, 1990). Because of their long-life span wild halibut mature late, average around 10 years. Halibut fillet is considered a delicacy with a high price yield, but late maturation makes the wild stocks vulnerable under heavy fishing pressure. To keep the stock at a sustainable level, especially during the spawning period, fishing of halibut is regulated (*Atlantic Halibut | NOAA Fisheries*, n.d.; *Kveite | Havforskningsinstituttet*, n.d.). The halibut fillet is semi-fatty, and the long life expectancy increases the risk of environmental fat-soluble compounds like PCBs and mercury uptake in the fillet (B. Nilsen et al., 2020; B. M. Nilsen et al., 2016). Thus, wild individuals over 40 kg should not be consumed. Farmed halibut, on the other hand, is fed with formulated diets and it is documented to be free from harmful fillet-parasites like anisakis (NIFES, 2009) and can be consumed raw. Atlantic halibut is, therefore, a good aquaculture candidate due to its low wild stocks, minimal risk of environmental toxins and parasites and the high price yield of the fillet.

1.2 Atlantic halibut in Norwegian aquaculture

In the 1980s, halibut became a candidate for the Norwegian aquaculture industry. Its large size and high value made the Atlantic halibut a sought out choice for farming (Kristiansen et al., 2004). However, it took some time to introduce halibut to the industry, as the production of juveniles was challenging to establish due to their long and vulnerable larval stage (Kristiansen et al., 2004; Kristiansen & Fernö, 2007). In the early 2000s, the production of halibut was still relatively small, but investments were made to increase production and help solve problems in the industry. Today, the production of juveniles has become well-established, with much lower mortality rates (Hamre et al., 2020). However, one of the challenges remaining is the slow growth of individuals above 1 kg (grow-out halibut).

1.2.1 Broodstock and larvae

Atlantic halibut are batch spawners and, in nature, they spawn at several hundred meters depth, and the eggs are pelagic. In aquaculture, the eggs and milt are manually stripped from

the fish (Hamre et al., 2019). The larval stage is relatively long, with the yolk-sac stage alone lasting a little over a month (Hamre et al., 2019). During the yolk-sac stage, the fish is kept in large dark silos with weak upwelling currents (Harboe et al., 1994). Larvae are moved to smaller tanks when ready for exogenous feeding, where they are initially fed *Artemia* spp. until weaning at 28 dpff (days post first feeding) (Hamre et al., 2019). Halibut larvae start to go through metamorphosis at approximately 50 dpff (Hamre et al., 2019), where they undergo major morphological changes, including pigmentation on the right side, eye migration, and development of the digestive system (Gomes et al., 2014).

1.2.2 Grow-out phase of Atlantic halibut

The grow-out stage is the last and longest period in the fish production cycle. Fish are either reared for the whole period on land-based facilities or transferred to sea cages at a minimum of 200 g. Tanks used at land-based facilities are shallow (about 1 m) as halibut is a bottom dwelling species. In sea cages, shelves are installed to increase surface area for the halibut to rest on. Halibut usually stays in the grow-out facility for around 2-3 years until they reach a minimum slaughter weight of 5 kg. During this stage, there are some problems with early maturation in males (Norberg et al., 2001). This is a major problem for farmers because it affects the quality and properties of the fish fillet, and promotes a poor growth (Norberg et al., 2001; Roth et al., 2007). During maturation the growth will halter due to the fish investing all its energy to the development of gonads (Norberg et al., 2001; Roff, 2011a). After maturation the growth will likely continue to be slow, and generally mature Atlantic halibut in aquaculture rarely reaches more than 5 kg (Roth et al., 2007). Males usually mature earlier than females, which is likely due to a small male being able to produce enough sperm to ensure maturation whereas for the female fecundity increases with body weight (Björnsson, 1995; Roff, 2011b).

1.2.3 Growth rate

For wild halibut, the growth rate is relatively low during the first years of their life, with an average weight of about 2 kg after 4 years (Haug, 1990). However, after maturation the growth rate decrease, even more so for males (Björnsson, 1995; Haug, 1990).

For farmed halibut, previous feeding experiments have shown that they have a potential for rapid growth (Tuene & Nortvedt, 1995) . However, the growth rate in most commercial farms are still considerably low compared to what is desired (*Håndbok i Kveiteoppdrett*, n.d.).

The low growth rates lead to a long and expensive production period. The main reason for slow growth is presumed to be due to low appetite and high stocking densities (Björnsson, 1994; Holm et al., 1998; Kristiansen et al., 2004). Observations of halibut in the wild indicate that juvenile individuals lay on the bottom by themselves and are rarely in contact with each other. However, in aquaculture, there are high stocking densities and the fish seems to have an increased swimming activity, likely due to stress from high contact levels (Kristiansen et al., 2004). A typical stress response in fish is reduced feed intake, and it can also impact feeding behavior in relation to the search and capture of food (Beitinger, 1990; Kristiansen & Fernö, 2007).

Halibut naturally have large metabolic reserves and a long life span, which allows them to tolerate extended periods of starvation (Foss et al., 2009). There is limited knowledge of wild halibut behavior, but observations indicate that halibut primarily utilize a "sit-and-wait" hunting strategy. This has also been observed in studies with farmed halibut, where they respond to moving pellets and rarely swim around searching for food (Kristiansen & Fernö, 2007). It is also suspected that feeding frequency influences feeding behavior and appetite, and that an empty stomach gives a stronger appetite signal than a full one (Kulczykowska & Sánchez Vázquez, 2010). This has been observed in studies on rainbow trout (*Oncorhynchus mykiss*) where the rate of appetite return showed close correlation to the rate of gastric emptying (Grove et al., 1978). How food is presented in combination with an optimal feeding schedule could influence feeding activity and behavior and help keep feeding motivation high, which could further increase growth rates and, thus, reduce the cost of production.

The effect of feeding frequency on growth has been explored in several species, and there seem to be a lot of variations between different species. A study of feeding frequency on growth and reproduction in zebrafish (*Danio reiro*) (Lawrence et al., 2012) found that feeding once per day was sufficient enough for good growth and reproduction performance. However, a study on one-year-old rainbow trout (Ruohonen et al., 1998) fed two different diets, showed that for both diets at least three feedings per day was necessary. Further, for juvenile olive flounder (*Paralichthys olivaceus*) optimal feeding frequency was two or three times per day (Lee et al., 2000). For halibut it has been found large variability in feeding among individuals (Tuene & Nortvedt, 1995), however, generally it has been observed that they eat a larger meal every second or third day (Mangor-Jensen & Holm, 2004; Rønnestad, 1988).

1.3 Feed intake and digestion in vertebrates

Feed intake and energy metabolism are vital for the growth and survival of an organism. In general, feed intake is affected by external factors, such as temperature, photoperiod, stress, predators, and food availability, and internal factors, like genetics, life stage, gut content, and stored energy (Rønnestad et al., 2017).

1.3.1 Appetite-control signals

For vertebrates, the hypothalamus is the center that controls appetite and energy balance and incorporate signals related to food intake and digestion, metabolism and energy storage (Rønnestad et al., 2017). Endocrine signals, i.e., hormones and neuropeptides produced and released in the central nervous system and peripheral organs, can either be orexigenic (stimulate appetite) or anorexigenic (inhibit appetite (Rønnestad et al., 2017). Other signals, such as nutrient levels in the blood, and content in the gastrointestinal tract also play a role in the appetite control (Rønnestad et al., 2017).The signals can either be orexigenic (stimulate appetite) or anorexigenic (inhibit appetite (Rønnestad et al., 2017). The physiological mechanisms that control appetite are similar among vertebrates, and many of the neuropeptides and hormones found in mammals have also been found in fish (Rønnestad et al., 2017).

1.3.2 The gastrointestinal tract

In most vertebrates, the gastrointestinal tract (GIT) is a large organ responsible for food storage, digestion, and absorption of nutrients. A large diversity in feeding habits among teleosts has resulted in different structures of the GIT, as mentioned in the review by Wilson & Castro (2010) (Wilson & Castro, 2010), therefore, much is still unknown about the full effects of the various parts of the GIT in different fish species (Rønnestad et al., 2017; Wilson & Castro, 2010).

Most fishes go thru a larval period before developing into juveniles with a fully functional GIT (Wilson & Castro, 2010). In Atlantic halibut the stomach becomes fully functional after metamorphosis, and its efficiency in digesting and processing proteins increases (Gomes et al., 2015; Hamre et al., 2019; Rønnestad et al., 2007). The primary role of the stomach in vertebrates is food storage, secretion of enzymes and stomach acid, and breakdown and

mixing of food (Stevens & Hume, 1989). Further, following the stomach is pyloric caeca and midgut, and the final section of the GIT is the hindgut (Wilson & Castro, 2010).

1.3.3 Other digestive organs in vertebrates

The liver, pancreas, and gallbladder are also key organs in the digestion process. For all vertebrates, the liver is a distinct and compact organ (Stevens & Hume, 1989). The liver has several functions, one of them being storage of fat, but in relation to digestion, the liver is mainly associated with bile secretion (Stevens & Hume, 1989). The bile, generally stored in the gallbladder, is important for digestion of lipids. The release of bile into the intestine happens when food is present in the intestine (Rønnestad et al., 2007; Stevens & Hume, 1989). In fish, the form and distribution of pancreatic tissue varies between species. In some species it is diffusely distributed across the intestinal wall; for others, it is a more compact organ (Stevens & Hume, 1989). The pancreas produces and secretes enzymes that contribute to digestion (Rønnestad et al., 2013).

1.4 Gastrointestinal hormones involved in appetite and digestion control

As mentioned, several hormones that affect appetite are produced in the GIT. Many of these peptides function in a way that they are sensitive to gut content and act in relation to gut filling and emptying. Thus, the food movement throughout the GIT affects appetite with hunger and satiety signals (Rønnestad et al., 2017). Some of the gastrointestinal hormones found in fish that affects appetite and regulate digestion are ghrelin, peptide yy (pyy), and cholecystokinin (cck) (Rønnestad et al., 2007, 2017).

1.4.1 Ghrelin

For mammals, ghrelin is the only orexigenic hormone originating from the GIT. Ghrelin is mainly produced in the stomach, influencing both digestion and feeding behavior. Therefore, it is commonly known as the “hunger hormone” (Higgins et al., 2009). Fasting and/or starvation activates ghrelin production in the stomach, and typically ghrelin plasma levels are high before feeding and decline after feeding (Higgins et al., 2009). Studies have shown that ghrelin is a well-conserved hormone among vertebrates, and several of its functions have been preserved during evolution (Breves et al., 2009; Kaiya et al., 2008). *Ghrelin* has been found in several teleost species, including Atlantic halibut (Manning et al., 2008). A study by Unniappan et al. (2004) showed that short- and long-term fasting lead to increased

transcription and peptide secretion of ghrelin in goldfish (*Carassius auratus*); however, the same effect was not observed in a study on tilapia (*Oreochromis niloticus*) (Parhar et al., 2003), suggesting that ghrelin's influence on appetite may differ among teleost species. In halibut, it is known that ghrelin is present during first feeding, and its expression increase during development and after metamorphosis (Gomes et al., 2015; Hamre et al., 2019), but there are still many uncertainties about its function on appetite control in this species (Gomes et al., 2015).

1.4.2 Peptide YY

Peptide YY (PYY) is a member of the neuropeptide Y (NPY) protein family. *Npy* is a well-conserved gene among vertebrates, and it provides one of the strongest orexigenic signals in mammals. Unlike NPY, PYY act as an anorexigenic signal in mammal, and it is an important factor in regulating food intake and energy use (Ueno et al., 2008). The *pyy* has been shown to have an anorexigenic effect in goldfish, but not in Atlantic salmon (*Salmo salar*) (Gonzalez & Unniappan, 2010; Murashita et al., 2009), which suggest that its role in appetite differ among teleost species. Two *pyy* genes have been identified in several teleosts species, including Atlantic halibut, *pyya* and *pyyb* (Gomes et al., 2022). During evolution of the vertebrate lineage, two rounds of whole genome duplication occurred, resulting in several paralogous genes (Meyer & Van De Peer, 2005; Zhang, 2003). In addition, a third whole genome duplication (the fish-specific genome duplication) occurred in the fish lineage, making their genome even more complex (Meyer & Van De Peer, 2005). PYY is assumed to have evolved during the duplication of a single ancestral gene, from the neuropeptide Y family, in early vertebrates (Sundström et al., 2008), and later during the fish-specific genome duplication it duplicated again into two paralogs now known as *pyya* and *pyyb* (Sundström et al., 2008). In Atlantic halibut larvae, *pyya* is mainly expressed in the brain and *pyyb* in the gut (Gomes et al., 2022). In the brain of these larvae, the expression of *pyya* and *pyyb* mRNA was affected by feeding, which supports the premise that these genes play a role in central feeding regulation (Gomes et al., 2022).

1.4.3 Cholecystokinin

Cholecystokinin (*cck*) has been also observed in the intestine of most fish groups. In higher vertebrates, CCK is important for stimulating pancreatic enzyme secretion, contraction of the gallbladder, and intestinal peristalsis, but it also plays a role in gastric emptying and feed intake control (Rønnestad et al., 2007). Research suggests that Cck has similar functions in

teleosts, acting as a short term satiety factor and promoting digestion (Rønnestad et al., 2017). A study on the effects of Cck on gut motility in ballan wrasse (*Labrus bergylta)* (Le et al., 2019) showed that *cck* promotes contractions in the gallbladder in this fish. The same effect has also been observed in rainbow trout (Aldman & Holmgren, 1987). Although the role of *cck* is established for some fish species, there is still much unknown about its function in Atlantic halibut (Gomes et al., 2022).

There has been identified to types of *cck* in fish, named *cck1* and *cck2* (Kurokawa et al., 2003). Cck likely evolved from a common ancestor with gastrin, another neuroendocrine peptide (Kurokawa et al., 2003). Further, the occurrence of two *cck* genes happened during the fish-specific genome duplication (Kurokawa et al., 2003). Research has shown that both *cck* genes are present in the brain and gut of halibut larva; however *cck2* seem to be more abundant in the brain whereas *cck1* has a similar expression in both tissues (Gomes et al., 2022). For Atlantic halibut larvae the main location of *cck*-producing cells appears to be the anterior midgut (Rønnestad et al., 2007). The anterior location of Cck-producing cells in halibut larvae supports the theory of its physiological role in controlling digestion processes. Anterior-located Cck-producing cells sense the presence of chyme as it enters the intestine from the stomach and can, thus, give immediate feedback for digestion control (Rønnestad et al., 2007).

1.5 Aims of the thesis.

The main aim of this thesis is to find a best-fitting feeding schedule to help increase feed intake and, consequently, growth in Atlantic halibut. This was investigated by looking on how different feeding frequencies affect feed intake (by looking at gut content), expression of appetite related genes and growth. Thus, two different feeding schedules were applied, feeding every second day and every third day.

Objective 1 – Investigate differences in growth between females and males by looking at specific growth rate (SGR) from different growth periods.
$H0_1$: No difference in growth between female and male halibut.

Objective 2 – Investigate differences in growth between fish following different feeding regimes by looking at specific growth rate (SGR) from different growth periods.
$H0_2$: No differences in growth between fish following different feeding regimes.

Objective 3 – Investigate differences in feed intake, by examining gut content, in Atlantic halibut when exposed to different feeding regimes:
$H0_3$: No difference in feed intake between fish following different feeding regimes.

Objective 4 – Investigate difference in expression of appetite-related controlling hormones (*ghrl*, *pyya*, *pyyb*, *cck1*, and *cck2*)
$H0_4$: No difference in expression of appetite-related hormones in response to meal.

Objective 5 – Investigate difference in feed intake between females and males (matured):
$H0_5$: No difference in feed intake between females and matured males

2. Material and Methods

2.1 Trial A

2.1.1 Experimental design

The Atlantic halibut growth trial was conducted in the commercial facility Sogn Aqua in Ortnevik (Sogn, Norway) from May 2022 to March of 2023. Four tanks were used in the experiment. Each tank was 150 m long, 6.5 m wide and 1.2 m deep (0.8 m water column) and had 5 different feeding stations (Figure 1).

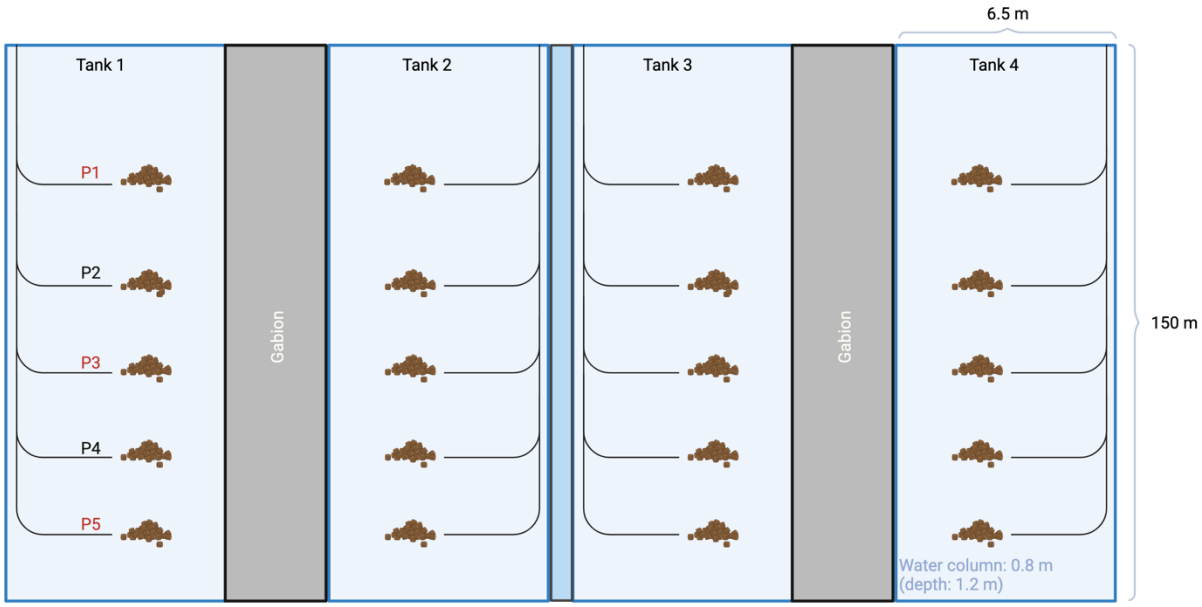

Figure 1: Tank design including the feeding stations in each tank. (Created with BioRender.com)

Feeding took place every second day (d2) in tank 1 and 2, and every third day (d3) in tank 3 and 4, and the fish is fed Hippo Express from Skretting (Skretting, Stavanger, Norway). Feeding started at station 1, where feed was let out for approximately 50 minutes before stopping and then continued directly at the next station (1-2 minutes to change between points), finishing with station 5. The fish followed a natural light regime, water temperature varied with sea water temperature and oxygen levels also varied over the trial. For specific mean temperature and oxygen levels each day see Appendix 1.

Externally tagged fish were measured in length and weight at the beginning of the trial (May 2022), again in October 2022, and at the end of the trial in March 2023. These measurements were used to calculate the specific growth rate for each fish.

2.2 Trial B

2.2.1 Experimental design

2.2.1.1 Sampling

The feed intake trial followed the same design as trial A, and it took place from from June 2022 to October 2022. The fish samples were collected in October 2022. A total of 120 fish, distributed among four different tanks, were collected. From each tank 10 fish were taken at three different locations, feeding point 1,3 and 5, to account for possible differences, a total of 30 fish per tank. Fish was collected approximately 2-5 minutes after feeding had taken place at each point, and sampling was done in between each collection of fish.

Two tubs with water and anesthetics (5-10 mL/L, dose depending on effect, Aqui-S, New Zeland ltd.) were prepared for holding the fish. A hand net was used to capture the fish, and 5 fish were put in each of the tubs with anesthetics. The fish were left for a few minutes until the anesthetics took effect, then the tubs with fish were carried along the gabion over to the sampling station. Here the fish were transferred to a bigger tub, where they were given an overdose of anesthetics (1 mL/L, dosed depending on effect, Benzoak vet., ACD Pharma, Leknes, Lofoten, Norway).

After the fish was killed, a check was done to score morphological welfare indicators. Then, the fish were cut open and the gastrointestinal tract was removed, using clamps to hold the content of the different segments. Content from the stomach, midgut, hindgut and pyloric caeca was collected in tubes and stored at -20 ºC. Tissue samples for gene expression analyses were taken from stomach, anterior- and posterior midgut and stored in RNAlater (Thermo fisher scientific,), stored at 4 ºC for at least 24 h before being transferred into the -80 ºC. Gonads, liver and heart were collected and weigh recorded using a scale (VWRI611-3353, model: LPC-213i, VWR international, Italy). Weight and color of the bile was noted.

2.2.2 Gastrointestinal tract compartments content weight

The wet and dry weight of the stomach, midgut, hindgut, and pyloric caeca content was recorded using a scale (ENTRIS623I-1S, Sartorius Lab Instruments GmbH & Co. KG, 37070 Goettingen, Germany). Before getting the wet weight, the gut tubes were taken out of the freezer and left at room temperature for a couple of minutes while setting up the scale. The lid was removed from the tube before weighing. If there was content stuck to the lid a tweezer was used to transfer the content back into the tube. For the tubes containing midgut, hindgut, and pyloric caeca it was noted down if the content had a dark or light color. After wet weight was taken the samples were put in a dehydrator (Excalibur, EXC10ELF, USA) for 48 to X h, until content was completely dried. The dry weight was then recorded using the same scale. For the stomach content, the number of pellets was also registered. But if the pellets were too dissolved to differentiate, only weight was taken.

2.2.3 Gene expression analysis

Gene expression analysis was conducted at the Marine Development Biology laboratory at the Department of Biological Sciences at the University of Bergen. Tissue from sampling, kept in RNA-later, were used for the gene expression analysis. A total of 240 samples were used, 120 from anterior midgut and 120 from stomach. From alle the tissue collected, these samples were chosen for analysis as it was suspected that these tissues would be of most interest to the master thesis.

2.2.3.1 RNA-isolation

Before starting RNA-isolation the samples were taken out from the freezer and thawed on ice. While the samples were thawing, tubes with stainless steel beads (5mm, 200/pk, Quiagen, Inc. 69989, Waltham, Massachusetts, USA) and 1 mL of TRI-reagent (Thermo Fisher Scientific, Waltham, Massachusetts, USA) were prepared on an ice block.

After the samples had thawed, they were dried off to remove excess RNAlater and transferred to the tubes with TRI-reagent. The samples were left for 5 minutes, before being homogeneized in a Precellys 24 tissue homogenizer (Bertin technologies, Montigny-le-Bretonneux, France) 2 times at 5000 rpm for –15 sec. The samples were taken out of the homogenizer and left in room temperature for 5 minutes. Further, 200 ml of chloroform (Sigma Aldrich, Saint-Louis, Missouri, USA) was added to each sample, and the samples were then shaken vigorously for 1 minute. The samples were centrifuged for 15 minutes at

4°C at 13200 rpm in an Eppendorf 5415R Refrigerated Centrifuge (Eppendorf, Hamburg, Germany). While centrifuging, new tubes (Eppendorf tubes 3810X, Hamburg, Germany) was prepared. Then, 400 µL of the aqueous phase was then transferred to the new tubes, before adding 500 ml of isopropanol (Sigma Aldrich, Saint-Louis, Missouri, USA). Each tube was inverted five times to mix the content. The tubes were then left at room temperature for 10 minutes. RNA was precipitated by centrifuging for 10 minutes at 4°C at 13200 rpm. The supernatant was removed, and the RNA was washed using 1 mL 80% cold EtOH. Samples were centrifuged for 10 minutes at 4 °C at 12000 rpm. The supernatant was then removed by pouring it in a waste container, followed by a quick spin in a centrifuge to help get the last drop out using a pipette. The samples were air dried on ice for 10 – 15 minutes, before nuclease-free water was added. The amount of water was dependent on the size of the pellet, ranging from 80 – 180 µL. If the pellet was difficult to dissolve, the tube was placed on a heating block (VWR International, no. 13259-062, Radnor, Pennsylvania, USA), at 55 °C, for 5-10 minutes before mixing again.

2.2.3.2 RNA concentration and purity analyses

The total RNA concentration was measured using a NanoDrop One spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The 260/280 and 260/230 absorbance ratio were used as indicators of sample purity. If the sample had a 260/280 or 260/230 absorbance ratio below 1.8, then 1/10 volume of 3 M NaAc with pH 5.2 and 2.5 volume of cold 100% EtOH were added, and samples were stored in the freezer at -80°C overnight. After, the samples were centrifuged (Eppendorf centrifuge 5424 R, Hamburg, Germany) for 30 minutes at 4 °C at 12000 rpm. The supernatant was gently removed into a waste container. Then, the pellet was washed with 200 ml 80% EtOH and centrifuged for 5 minutes. The supernatant was removed, and a quick spin in the centrifuge was used to get the last drop of EtOH out with a pipette. The pellet was left to air dry for 5-10 minutes before being resuspended in nuclease-free water, the amount depending on the pellet size. The concentration of RNA was measured again on the NanoDrop spectrophotometer.

2.2.3.3 DNase treatment

Any traces of genomic DNA contamination were removed using TURBO DNA-free kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

A total of 10 µg of total RNA in a volume of 30 µL with 0.1 volumes of turbo DNase buffer and 1 mL turbo DNase were added to a tube (Eppendorf tube 3810X- Microtube, Hamburg, Germany), and incubated at 37°C for 30 minutes in a thermal cycler (2720 Thermal Cycler, Applied Biosystems, Waltham, Massachusetts, USA). After, 3 mL of DNase inactivation reagent was added to each tube. The content was mixed well by gently flicking the tubes and incubating them for 5 minutes at room temperature, mixing occasionally. The samples were then centrifuged at 6000 rpm (C12XX-220V, Galaxy Mini Centrifuge, VWR International, Pennsylvania, USA) for 1.5 minutes. The supernatant containing the DNase-treated total RNA was transferred to a new tube (PCR tube, strip of 8 tubes, VWR) using a pipette. The samples were then stored at -80°C for later use.

2.2.3.4 RNA integrity analysis

DNase-treated total RNA integrity was accessed on 25% of the stomach and anterior midgut samples using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, California, USA) with Agilent RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, California, USA) followed by the manufacturer's protocol. The samples were run using the program Eukaryote total RNA Nano assay. RNA Integrity Number (RIN, values range from 1 to 10) was used to determine the quality of the RNA.

2.2.3.5 cDNA synthesis

DNase-treated samples were used for cDNA synthesis. Samples were thawed on ice before their RNA concentration and purity was quantified using a NanoDrop spectrophotometer. There was used 2µg of RNA for cDNA synthesis reaction. The RNA concentration was then used to calculate the amount of RNA needed.

The final volume of the cDNA reaction was 20 µL. First, for each reaction tube, water, 25 ng of RNA and 1 µL of Oligo dT (50 µM) and 1 µL dNTPs (10 mM) and the reaction heated at 65 °C for 5 minutes before being incubated on ice for at least 1 minute. After, 4 µL of 5x First-Strand Buffer, 1 µL of 0.1 M DDT, 1 µL RNaseOUT (40 U/µL) and 1 µL SuperScript III RT (200 U/µL) were added to each sample. The content of each tube was mixed by gently pipetting up and down. The tubes were then centrifuged briefly to collect the content, before being incubated at 50 °C for 60 minutes, and then heated to 70 °C for 15 minutes to inactivate the reaction. After this the cDNA was finished and could be used as a template for

amplification in PCR. The cDNA was stored at -20°C until further use. A minus reverse transcriptase (-RT) was also done using RNA and water from a random selected sample, but on those, RNase free water was added instead of SuperScript III RT.

2.2.3.6 Real-time RT-qPCR

cDNA stored at -20°C was taken out of the freezer to thaw. While the samples were thawing, tubes (PCR tubes, 8 strips) were prepared for diluted cDNA. Samples from stomach tissue were diluted to contain 12.5 ng per reaction and samples from anterior midgut tissue were diluted to contain 25 ng per reaction. Water was added to the tubes first and then cDNA was mixed in with a pipette. Samples were vortexed and spun down. In addition to diluted cDNA there was also made tubes with diluted -RT for each tissue and aliquot of between plate control (BPC). Hard-shell 96-well PCR plates (Bio-Rad laboratories, Hercules, California, USA) was used for the real-time qPCR analysis.

For each gene there was made a standard curve using a two-fold dilution series, ranging from 50-1.5625 ng for stomach and 100-3.125 ng for anterior midgut. A pool of all samples, for each tissue, was used to make the dilution series. The standard curve for each gene were run in triplicates.

When preparing the qPCR plates, the first steps were done in a sterile fume hood to avoid contamination. A master mix was prepared, containing 2Xitaq Universal SYBR Green Supermix (Bio-Rad laboratories, Hercules, California, USA), two primers (one forward and one reverse) and water. Per sample there was used 10 mL 2XiTaq, 0.8 mL of each primer an 0.4 mL water. 12 µL of master mix was pipetted into each well in the qPCR plate. The plate was spun down and covered with aluminum foil. The foil was removed and 8 µL of diluted cDNA was added to each well, each sample of cDNA was added in duplicates. For each plate there were also added duplicates of -RT (NRT), BPC and water (NTC). -RT was used as control for possible genomic DNA contamination, BPC was used to make out possible differences between plate runs, and water was used as control for contamination
The plate was spun down and run in a C1000 Thermal Cycler (CFX96 Real-Time System, Bio-Rad Laboratories, Hercules, California, USA) using the following conditions:

95 °C for 30 s, followed by 35 cycles at 95 °C for 5 s to 60 °C for 25 s. A melting curve analysis was used in all qPCR assays to ensure the absence of non-specific products and primer dimer formation, beginning at 65 °C to 95 °C, increment of 0.5 °C for 2 s.

Relative mRNA expression was calculated by dividing real-time PCR efficiency (E) of a target gene on the geometric mean (GEOMEAN) of the efficiency of two reference genes (Pfaffl, 2004) (See equation 1).

Equation 1:

$$relative\ expression\ ratio = \frac{\left(E_{target}\right)^{-Cq_{sample}}}{GEOMEAN(E^{-Cq_{ref1}}: E^{-Cq_{ref2}})}$$

The reference genes *elongation factor 1 alpha* (*ef1a)* and *40S ribosomal protein S30* (*fau*) was chosen as their expression has shown to be stable across tissue samples (Fernandes et al., 2008; Gomes et al., 2014, 2015). The expression of genes was normalised to control for internal errors in the qPCR analysis.

Table 1:List of primers used for gene expression, including gene bank accession no., primer sequence, amplication size, R^2 (R2) and efficiency (E%).

Gene	GeneBank accession no.	Sequence (5′ → 3′)	Amplicon (bp)	R2	E %
ef1a	XM_034600905.1	F: CGCAGAAACACCGCAACTACAA R: GCCCTTGCCCATCTCGGCAG	180	0.99	92.5%
fau	XM_034580234.1	F: GACACCCAAGGTTGAAAAGCAG R: GGCATTGAAGCATTTAGGAGTTG	149	0.99	87.6%
ghrl	XM_034586479.1	F: GGCTGCTGGTTGTTCTACTCTG R: TCCTCGGTGGGTTGATTCTG	154	0.99	101.1%
pyya	XM_034594388.1	F: GTGTGTCTGGGAACGCTGGC R: TTTCCATACCTCTGCCTTGTGAT	140	0.99	90.5%
pyyb	XM_034570163.1	F: TCATCACCAGACAGAGGTATG R: GGCTTGAATCGCCTCCGAAC	81	0.97	88.5 %
cck1	XM_034612377.1	F: CCAGGAGGACACAGACCCTA R: CTGCGTCTCCCAAAGTCCAT	177	0.99	81.4%
cck2	XM_034600627.1	F: CAGAAACTCAGCGGCGTACA R: TCCAGCCCAAGTAGTCCCTG	74	0.99	84.9%

2.2.4 Statistics

The statistical analysis and plots were conducted in RStudio (Version 2022.12.0+353, RStudio, Inc.) using R (version 4.2.3, R Core Team) with the following packages: tidyverse (Wickham et al., 2019), emmeans (Searle et al., 1980), and ggplot2 (Wickham, 2016).

Gene expression and gut content data were first visualized by using density and boxplot. If outliers were identified, those were excluded. Both gene expression and gut content data were modelled with a generalized linear mixed model (GLMM) with, Gamma distribution (log-link function) for the gene expression data and t-family distribution (identity-link function) for gut content data, with treatment (d2: feeding every second day, d3: feeding every third day) and gender (female and male) as categorical explanatory variables. An interaction between these two fixed variables was also included. Tank and feeding location were added as random intercepts to account for potential within-cluster correlation. The contrast between groups was explored using Tukey HSD post-hoc tests, where a P-value < 0.05 indicated significance.

Spesific growth rate (SGR) data was modelled with a generalized linear mixed model (GLMM) with gaussian distribution (identity-link-function) with treatment (d2: feeding every second day, and d3: feeding every third day), gender (female and male, and growth period (sgr 1-2, sgr 2-3, sgr 1-3) as categorical explanatory variables. Tank was added as a random intercept to account for potential within-cluster correlation. Contrast between treatment group and gender was explored using Tukey HSD post-hoc tests, where P-value < 0.05 indicated significance.

All figures were made using jitterplots, where the mean of predicted values and the ± 95% confidence interval was plotted including the raw data points in the background.

3. Results

3.1 Trial A

The mean weight for individuals at the start of the trial was 2.3 (± 0.4) kg for males in the d2 group, 2.5 (± 0.8) kg for males in the d3 group, 3.4 (± 1) kg for females in the d2 group, and 3.3(±1) kg for females in the d3 group. The mean weight at the end of the trial was 2.4 (± 0.5) kg for males in the d2 group, 2.6 (± 0.9) kg for males in the d3 group, 4.0 (± 1) kg for females in the d2 group, and 4.5 (± 2) kg for female in the d3 group. Total 62 fish was used for calculations of SGR in trial A, however, not all fish in the trial was captured during the second measuring (October 2022), and sgr 1-2 and sgr 2-3 was calculated with less individuals than sgr 1-3 (See Figure 2).

Post-hoc analyses on SGR revealed significant difference between the first (sgr 1-2) and second (sgr 2-3) growth period for both females and males in each treatment group (d2 and d3) (Figure 2) (Appendix 3 Figure 23). There was also significant difference (p-value < 0.05) between females and males in both treatment groups during the second growth period (sgr 2-3) (Figure 2) (Appendix 3 Figure 24). For the whole trial period (sgr 1-3) there was a significant difference (p-value < 0.01) between males and females in the d3 treatment group (Figure 2) (Appendix 3 Figure 24). The predicted mean for SGR for all individuals in both treatment groups was lowest during the second growth period (sgr 2-3) (Figure 2).

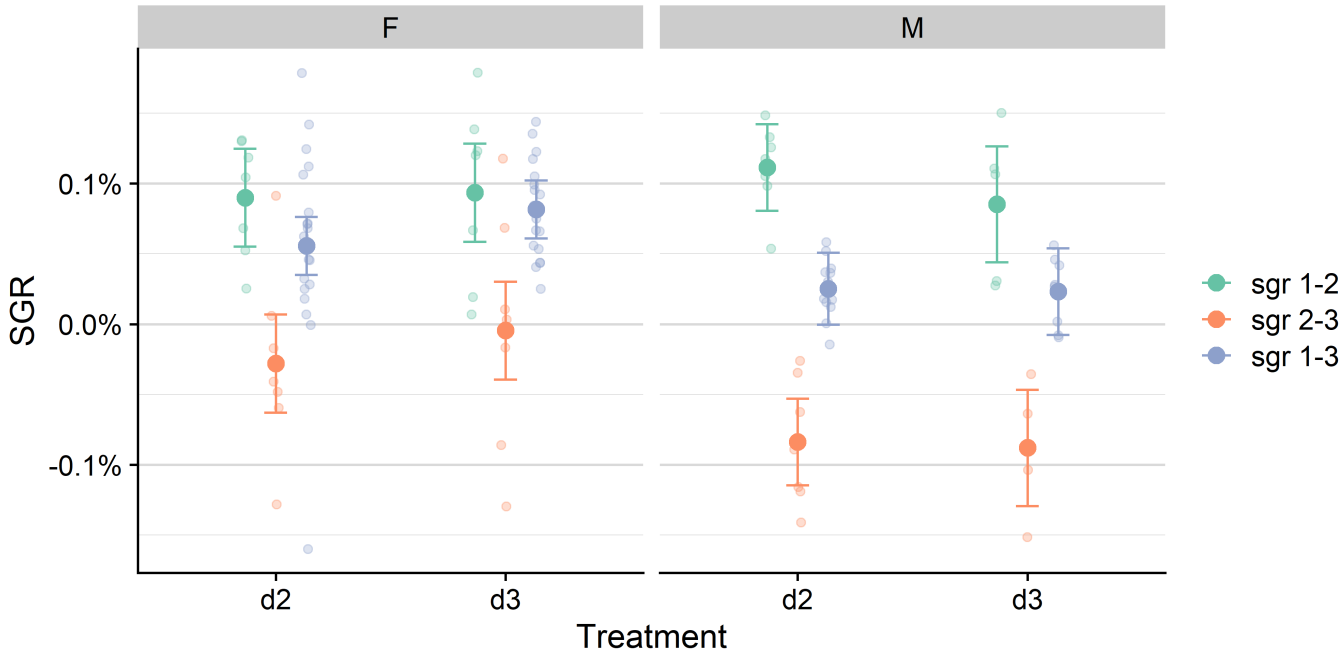

Figure 2: Mean predicted values for specific growth rate (SGR) for female (F) and male (M) halibut for two different growth periods (sgr 1-2:May 2022 – October 2022 sgr 2-3: October2022 – March 2023), including SGR for the whole trial (sgr 1-3: May 2022 – March 2023) following two treatments (d2: feeding every second day, d3: feeding every third day). The ± 95% confidence interval is indicated (lines), and raw data is plotted (smaller transparent dots). Number of individuals: sgr 1-2 and sgr 2-3 (males: d2: 9, d3: 5, females: d2: 7, d3: 7); sgr 1-3: (males: d2: 13, d3: 9, females: d2: 20, d3: 20).

3.2 Trial B

All male individuals were sexually mature.

3.2.1 Gut content analysis

GLMM analysis revealed gender (mostly maturation in males) had an effect on the stomach content (See Appendix 3 Table 2). The post-hoc analysis revealed that there was a significant difference (p-value < 0.05) between males and females in each treatment group (Appendix 3 Table 3). No effect from tank or feeding location (5.4e-05, and 2.4e-01).

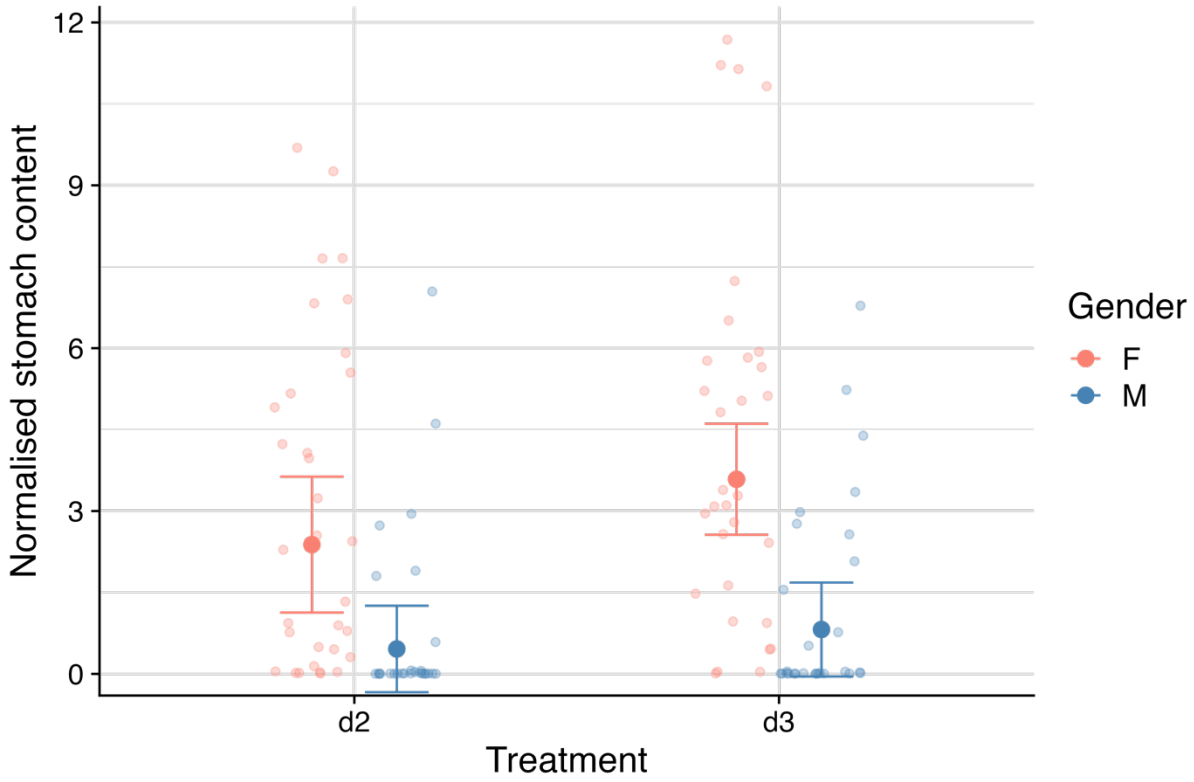

Figure 3: Mean predicted values for stomach content for males (M) and females (F) in two treatment groups (d2: feeding every second day, and d3: feeding every third day). The ± 95% confidence interval is indicated (lines), and raw data is plotted (smaller transparent dots). Number of individuals included in the figure: d2: males:27, females:33; d3: males:28, females:32.

The statistical analysis showed that both treatment and gender (mostly mature males), and the interaction between these variables had an effect on midgut content (See Appendix 3 Table 4). Significant differences (p-value < 0.05) were observed between females in the different treatments, and between males and females in the d2 treatment group (Figure 4, Appendix 3 Table 5). For hindgut, the fixed variable gender had a marginally significant effect (See Appendix 3 Table 6) and, indeed, the post-hoc test revealed a significant difference (p-value < 0.05) between females and males in the d3 treatment group (Appendix 3 Table 7). No effect from tank or feeding location for both midgut (2.7e-07, and 1.7e-07) or hindgut (6.1e-07, and feeding points: 1.7e-02).

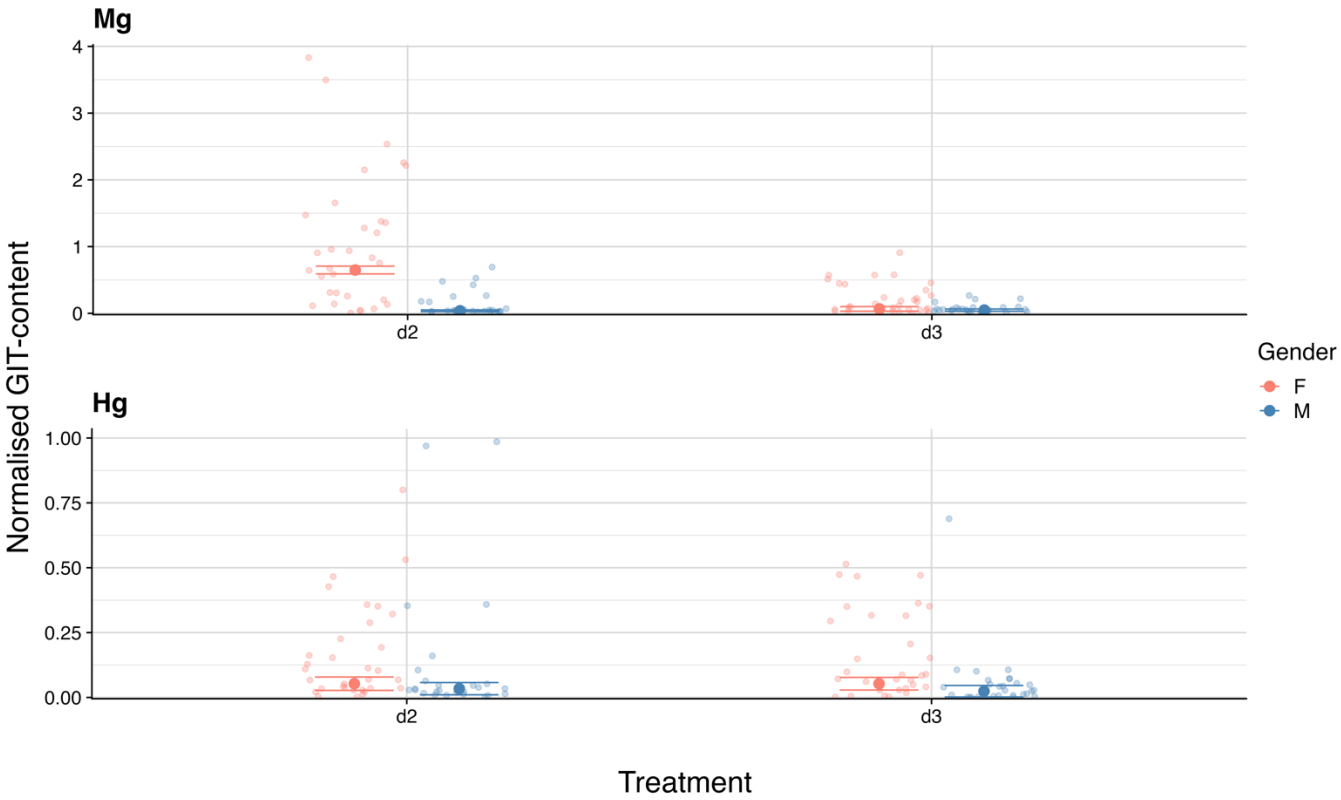

Figure 4: Mean predicted values for midgut (Mg) and hindgut (Hg) content for males (M) and females (F) in two treatment groups (d2: feeding every second day, and d3: feeding every third day). The ± 95% confidence interval is indicated (lines), and raw data is plotted (smaller transparent dots). Number of individuals included in the figure: d2: males:27, females:33; d3: males:28, females:32.

3.2.1 Gene expression analysis

The GLMM analyses revealed that none of the fixed variables had an effect on the *ghrelin* gene expression (See Appendix 3 Table 8), as also revealed by the post-hoc analyses. There was no significant (p-value > 0.05) difference in the relative mRNA expression of *ghrelin* Figure 5, Appendix 3 Table 9).The feeding location random intercepts were negligible (standard deviation of 2.2e-05, see Appendix Figure 18), while there was a weak tank effect (standard deviation of 2.4e-01, see Appendix Figure 18).

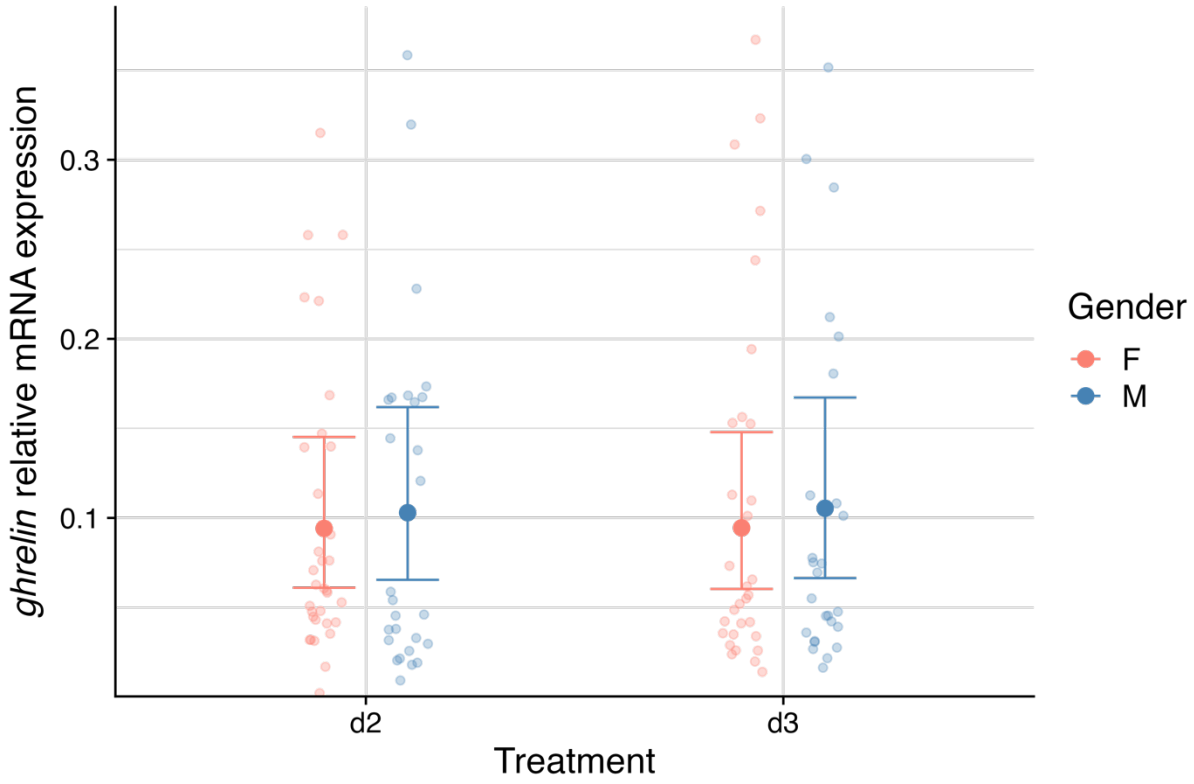

Figure 5: Mean predicted values for relative mRNA expression of ghrelin in female (F) and male (M) halibut following to different feeding regimes (d2: feeding every second day and d3: feeding every third day), The ± 95% confidence interval is indicated (lines), and raw data is plotted (smaller transparent dots). No significant difference in relative expression of ghrelin. Number of individuals included in the figure: d2: males:27, females:33; d3: males:28, females:32.

The statistical analysis revealed gender (or most probably maturation) is important to explain the *pyya* mRNA expression, while treatment (feeding regime) and the interaction between both fixed variables did not have a significant effect (Appendix 3Table 10). Thus, no significant difference in the relative mRNA expression of *pyya* between treatments was observed (Figure 6, Appendix 3 Table 11). Additional post hoc analyses showed that there is significant ($p < 0.0001$) difference between males and females in the d2 feeding group, but not in the d3. The tank random intercepts were negligible (standard deviation of 7.4e-06, see

Appendix Figure 19), while there was a very weak feeding location effect (standard deviation of 3.5e-02, see Appendix Figure 19).

As for *pyyb*, the fixed variable gender (maturation) is important to explain its expression (Figure 6, Appendix table 12). There was a significant ($p < 0.05$) difference between males and females within each feeding group (Figure 6, Appendix table 13). The effects of tank (standard deviation of 9.8e-07, see Appendix Figure 20) and feeding location (standard deviation of 1.4e-05, see Appendix Figure 20) were negligible.

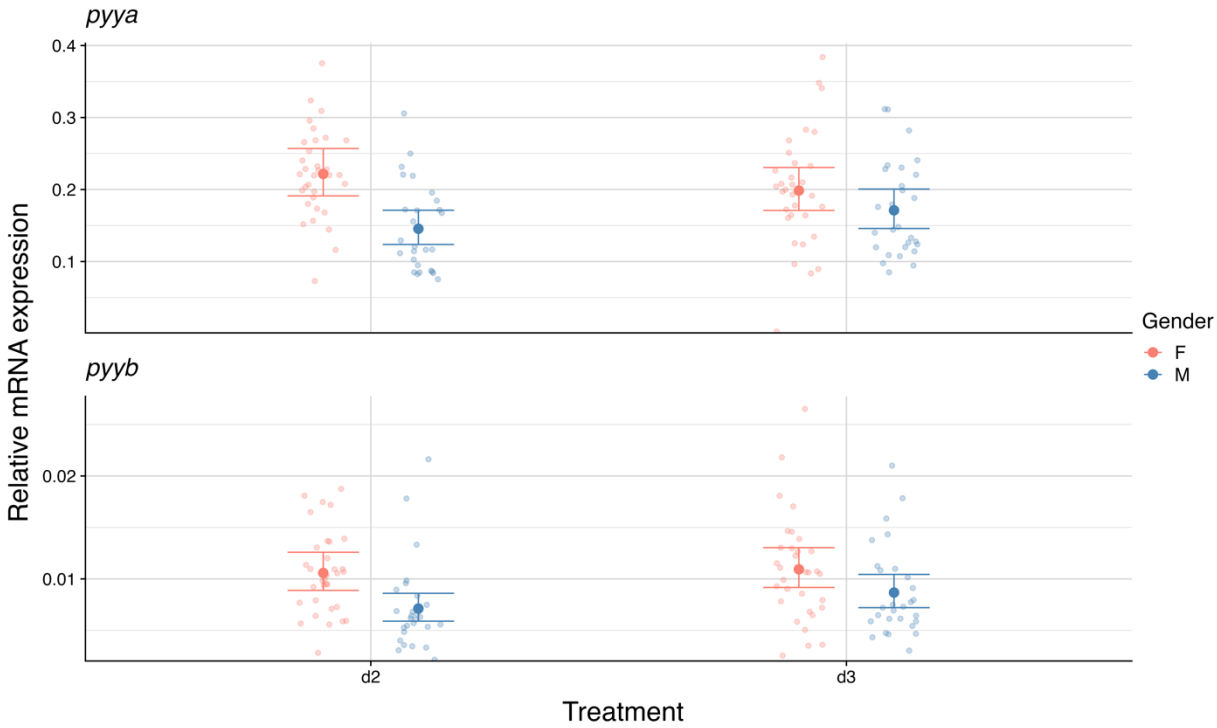

Figure 6: Mean predicted values for relative mRNA expression of pyya and pyyb in female (F) and male (M) halibut following to different feeding regimes (d2: feeding every second day and d3: feeding every third day). The ± 95% confidence interval is indicated (lines)l, and raw data is plotted (smaller transparent dots). Number of individuals included in the figure: d2: males:27, females:33; d3: males:28, females:32.

The mRNA expression of cck1 was mainly explained by the variable gender (maturation), while the other variables had no effect (Appendix Table 14, Figure 7). Additionally post hoc analyses revealed there is no significant difference (p-value > 0.05) for the *cck1* mRNA expression between treatments (Figure 7, Appendix 3 table15) groups, while there were significant changes (p-value < 0.0097) between males and females in group d2, but not in d3 (Figure 7, Appendix 3 Table 15). There was no random effect of tank or feeding location (standard deviations of 3.3e-5 and 7.3e-29, respectively, Appendix Figure 21).

Similar results were obtained from the GLMM analyses of *cck2* mRNA expression as (Appendix 3 Table 16, Figure 7). No significant (p-values > 0.05) differences between

treatment groups, but there is a significant (p-value < 0.01) difference between females and males within each group (Figure 7, Appendix 3 Table 17). There was a small effect of feeding location (standard deviation of 0.15, Appendix Figure 22), but the tank random intercepts were negligible (standard deviation of 5.5e-05, see Appendix Figure 22).

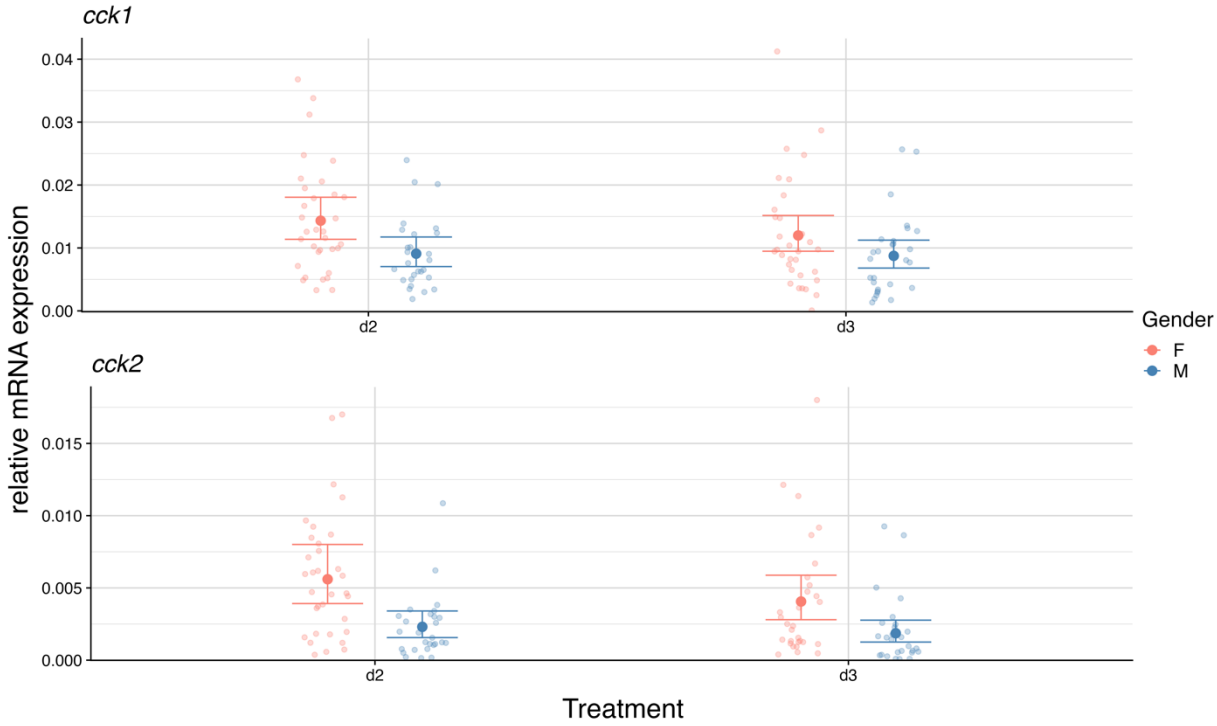

Figure 7: Mean predicted values for relative mRNA expression of cck1 and cck2 in female (F) and male (M) halibut following to different feeding regimes (d2: feeding every second day and d3: feeding every third day). The ± 95% confidence interval is indicated (lines), and raw data is plotted (smaller transparent dots). Number of individuals included in the figure: d2: males:27, females:33; d3: males:28, females:32.

4. Discussion

4.1 Trial A

The SGR for the tagged Atlantic halibut was calculated for two periods, March - October (1-2) and October – April (2-3), in addition to the whole trial 1-3. The highest mean SGR for all fish was observed in the first growth period (1-2) (Figure 2). During the second growth period the SGR decreased notably. This is probably due to the fish having gone through the winter season. Fish often have increased growth during the summer when days are longer, and reduced growth during the winter when there is less natural light (Norberg et al., 2001), and the fish in this study followed a natural light regime. These seasonal variations correlate with what was observed in this study.

In addition, the males had a lower SGR compared to females during the second growth period, which is likely a result of males going into and/or having matured during this period. The 2nd weight control took place during the autumn and based on the results from trial B, all males sampled were matured at this time, therefore it can be assumed that this was also the case for males in this trial as well. Atlantic halibut decreasing growth due to maturation is well-documented (Imsland & Jonassen, 2005; Norberg et al., 2001). Previous studies have shown that matured males had reduced growth compared to immature individuals. Further, in the study by Imsland & Jonassen (2005), it was also found that under commercial rearing conditions, due to more feed available and thus, better growing conditions, the age for first maturity for males seemed to be lower than in nature (Imsland & Jonassen, 2005). The reduced growth during maturation is likely a combination of appetite loss and the relocation of energy into the development of gonads (Norberg et al., 2001; Taranger et al., 2010). The energy investment into gonad development has been studied most extensively in salmonids, where it has been clearly shown that somatic weight decreases with the growth of gonads (Fleming, 1998; Kadri et al., 1996; Taranger et al., 2010).

Overall, in this study, the feeding frequencies, i.e., every 2 or 3-days, did not have any significant effect on growth as the SGR was similar within gender (Figure 2). Females had the better growth in total (sgr 1 – 3) (Figure 2), likely due to males having a larger decrease in SGR over the winter (sgr 2 – 3) (Figure 2) when they were matured, and therefore males were not able to catch up with the females before the last sampling.

4.2 Trial B

4.2.1 Gut content results

In this study gut content was used as a proxy to know the feed intake. All GIT-segment samples were visually inspected in the lab and it was clear that all pellets found in the stomach were fresh, from which it can be assumed that the stomach was empty before feeding. The stomach generally had more content compared to the other GIT-segments (Figure 3 & 4) which is expected as this is where food is initially stored as digestion starts. There was significantly (p-value < 0.05) less content in the stomach of male individuals, which is likely an effect of all males being mature while females were not. Reduced appetite is a common response in sexual maturing fish (Jobling et al., 2012), and reduced feed intake for maturing fish has been also observed in Atlantic salmon (Kadri et al., 1996) and Arctic charr (*Salvelinus alpinus*) (Tveiten et al., 1996). Looking into the different feeding groups (d2 and d3), it was expected to be a clear difference in stomach content, with the d3 group having more content than the d2 group. Since the fish in the d3 group would have had more time to empty their gut, it was expected that this would give a stronger appetite, as found for rainbow trout (Grove et al., 1978), where it was shown that appetite was correlated with gastric evacuation. However, no significant differences (p-value > 0.05) were found for stomach content between treatment groups.

A study by Davenport (1990) showed that no food left the stomach of halibut until 12 hours after a meal, and food remains was observed in the stomach up until 4 days after a large meal (Davenport et al., 1990). This contradicts what was found in this study, as no individuals had remains from a previous meal after 2 days. However, in the Davenport (1990) study, gut transit time was measured using x-ray for which the fish had to be sedated (Davenport et al., 1990). There is a possibility that x-raying same fish over time put stress on the digestive processes in the fish, thus explaining why the findings were different from this study. In addition, there was used a different type of feed in the Davenport (1990) study than in this study, which could also have impact results (Davenport et al., 1990)

Interestingly, a large variation was found in midgut content between females in the different treatment groups (Figure 4, Appendix 3 Table 4), and since the stomach was empty before feeding, we can assume that the remaining midgut content is from the previous feeding.

Females in the d2 group had significantly (p-value < 0.0001) more midgut content compared to the d3 group, which is in accordance with what would be expected, in that the fish in the d3 group would have evacuated more content from a previous meal compared to the fish in the d2 group. There was also a significant difference between males and females in the d2 group. Although, all male fish had little to no content in midgut (Figure 4), which is likely an effect of the males having eaten less due to maturation. In hindgut there was generally little content, and no significant differences between either treatment or gender.

4.2.2 Appetite-related genes expression

Relative gene expression of *ghrelin* in the stomach, and of *pyya*, *pyyb*, *cck1*, and *cck2* in the anterior midgut tissue was done using real-time qPCR. The gene expression levels were compared within the treatments and gender due to the big differences in appetite within groups. In addition, gene expression results were correlated with gut content data to assess for a possible relationship.

4.2.2.1 Ghrelin

The relative expression of *ghrelin* was similar for both treatments with no significant difference (Figure 5), suggesting that feeding regime did not affect the expression levels of *ghrelin*. It was expected that *ghrelin* mRNA expression would be different between treatments, due the d3 group having had longer time to evacuate gut content, thus anticipating stronger hunger signal for fish in this group and therefore some higher expression of *ghrelin*. There was also no significant difference in the *ghrelin* mRNA expression between males and females, which was a bit unexpected as there was thought to be a distinct difference due to males being mature, hence having reduced appetite. Halibut males generally had slightly higher expression levels of *ghrelin*, although these differences were not significant, and it should be emphasized that there is high individual variation. Nonetheless, *ghrelin* levels have been found to decrease relatively quick after feed intake in humans (Cummings et al., 2001) and rodents (Tschop et al., 2000), and it is possible that this also is the case for halibut, which then could have affected the results and contributed to the similar expression between treatment groups and gender.

Basically, no correlation between stomach content and expression of *ghrelin* was found, as there is no clear trend following expression in relation to gut content, adverting to no

significant p-values and low R^2 values (Figure 10). The *ghrelin* mRNA expression levels vary a lot between individuals with little to no stomach content, especially among males (Figure 10), and the expression levels also varies among individuals with near the same quantity of stomach content. In addition, many of the individuals with no content have lower *ghrelin* expression than individuals with content. This contradicts the expected result that an empty stomach would give a higher expression level of *ghrelin* than a full one, considering *ghrelin* being orexigenic (Higgins et al., 2009). However, for females in the d2 group, and males in the d3 group, there is a weak indication of a decreasing trend towards lower expression following higher stomach content, although this trend is not significant (p-value > 0.05). Relative expression of *ghrelin* was also compared to content in the other segments of the gut to check for trends, but no significant results were found.

4.2.2.2 Pyy

In this study, both *pyy* paralogs, i.e. *pyya* and *pyyb*, were expressed in the anterior midgut of halibut, as has been described in Atlantic halibut larvae (Gomes et al., 2022), and other teleost species (Sundström et al., 2008). The relative expression of *pyyb* was lower than the expression of *pyya* (Figure 5). For both *pyya* and *pyyb* there were no significant differences between treatment groups. However, *pyya* expression was significantly higher in females compared to males in the d2 group, and, although not significant, the same trend was observed in the d3 group. Similar to *pyya*, *pyyb* expression was significantly higher in females compared to males and this difference was observed for both feeding frequencies.

For males in both feeding frequencies and females in the d3 group a significant correlation (p-value < 0.05) between midgut content and expression levels of *pyya* and *pyyb*, with a positive correlation in expression following more content (Figure 11 & 12). However, it must be noted that the R^2 values are not very strong, but the results are significant. Females in the d2 group are the only ones with no significant correlation between midgut content and expression levels of *pyya* and *pyyb*, and they do not seem to follow the same trend as the other experimental groups as the results are more disperse There is also a significant correlation between hindgut content and the mRNA expression of *pyya* and *pyyb* for males in the d2 group, with a positive correlation in expression following more content. However, it should be noted that only a few male individuals influenced this trend, as most males had no content in hindgut.

Previous studies on teleost species (Chen et al., 2013; Gonzalez & Unniappan, 2010; Velasco et al., 2018) have supported an anorexigenic function of PYY, therefore it was expected that *pyy* mRNA expression levels would increase with more content in GIT-segments. In this study, this trend was found for midgut content in this study, but not for the other GIT-segments. Increase of *pyya* after feeding has also been observed in goldfish (Gonzalez & Unniappan, 2010), and the increase of *pyyb* levels in fed fish has been observed in Nile tilapia (*Oreochromis niloticus*) (Yan et al., 2017) and grass carp (*Ctenopharyngodon idellus*) (Chen et al., 2014), but not in yellowtail (*Seriola quinqueradiata*) (Murashita et al., 2006).

4.2.2.3 Cck

In this study, both *cck* paralogs, i.e., *cck1* and *cck2,* were expressed in the gut of halibut, as previously has been found in halibut (Gomes et al., 2022) and other teleost species (Kurokawa et al., 2003). Relative expression of both *cck1* and *cck2* was generally low, and there was no significant difference between treatment groups (Figure 7). This suggests that feeding regime did not have an impact on the relative expression of *cck*. Similar to the results of *pyy*, *cck1* expression was significantly higher in females, compared to males, in the d2 group, while *cck2* expression was significantly higher in females for both treatment groups. These results can be linked to males having generally less content due to reduced appetite from sexual maturation.

It was expected an increasing expression of the *cck* paralogs following more GIT-segment content, being that CCK is a satiety signal (Rønnestad et al., 2017) and has been observed to promote digestion in other teleosts (Aldman & Holmgren, 1995; Micale et al., 2012; Murashita et al., 2009). However, no correlation was found between expression of *cck1* and *cck2* in relation to gut content (Figure 13 & 14). The only significant correlation was found between stomach content and expression of both *cck* types in males in the d2 group, where increased expression followed higher content levels. This can indicate the involvement of *cck* in a feed forward mechanisms and its anorexigenic function (Rønnestad et al., 2017). However, most males in the d2 group had not eaten, thus, only a few individuals influenced the increased expression trend following stomach content, and therefore these results should not blindly be trusted.

In a study on *cck* in white sea bream by Micale V. et al (2012) it was observed that *cck*2 levels decreased after 72 h of fasting (Micale et al., 2012). In Atlantic halibut larvae it was also

shown that levels of *cck2* were low in fasted fish compared to fed, however, the levels of *cck1* was found to be higher in fasted than fed fish (Gomes et al., 2022). In addition, *cck2* also seem to be higher expressed in the brain than gut of Atlantic halibut larvae (Gomes et al., 2022). These findings could explain why the expression of *cck2* was generally low in this study, as many individuals had not eaten, meaning they would have had a short fasting period given that the fish are fed either every second- or every third day. However, as there is no clear difference in expression between fish with no content and those with content, no conclusions can be made in relation to difference between fish that have had a short fasting period (not eaten) and those who have eaten.

4.2.2.4 Pyy and Cck

There would be expected to see some opposing trends in expression of *pyy* and *cck* in relation to gut content, as PYY and CCK has been found to operate in feedback control in mammals, where CCK stimulate release of PYY, and PYY inhibit release of CCK (Guan et al., 1993; Lin et al., 2000). However, there is little knowledge about whether this antagonistic relationship between PYY and CCK is present in fish.

From previous studies it has been shown that in the gut, CCK and PYY are involved in local digestion control, like gastric movements, stimulation of gallbladder contraction, and secretion of pancreatic enzymes (Murashita et al., 2008, 2009; Murphy & Bloom, 2004). This makes it difficult to draw complete conclusions from the correlations between gut content and expression of *pyy* and *cck* found in this study as there could be more factors influencing expression levels that was not accounted for in this trial.

4.2.2.5 Methodical consideration

During the growth trial external tags were used to locate and recapture fish. The tags were initially easy to spot because they had different bright colors, however, they were quickly covered with algae. The algae growth made the tags similar color to the halibut skin, which made tagged fish very hard to locate. The algae covered tags in combination with less daylight during the second measuring (October 2022), resulted in less fish being recaptured.

In addition, there was expected to be some loss of tags due to the external location, although this loss resulted in being a more than initially thought. The algae growth in combination with the loss of tags resulted in not all fish measured at the start of the trial making it through to the end of the trial. For future studies this is something that could be improved when using external tags to identify fish.

When sampling from the gut, it is difficult to collect content without any loss. In this study collection of content was mostly manageable with only minor loss, however, collection of content from the pyloric caeca was difficult to manage without loss, due to the shape of this tissue (finger-like arms). Therefore, content from pyloric caeca was excluded from the data, due to high margin of error.

Conclusions

The result from growth trial showed that feeding every second day or every third day did not have any effect on SGR, however there was some differences related to lower specific growth rate in males during winter, due to sexual maturation. Therefore, the null hypothesis in objective 1 is rejected and the null hypothesis from objective 2 can be accepted.

The gut content analysis showed no significant differences in regard to treatment for stomach and hindgut content, therefore in relation to stomach and hindgut the null hypothesis ($H0_3$) is confirmed. However, midgut revealed a significant difference between female individuals in relation to treatment, in which for this GIT-segment the null hypothesis ($H0_3$) is rejected.
In relation to differences between females and the mature males, there was significant difference for stomach content and midgut content, which confirm the alternative hypothesis in objective 5. For hindgut there was no difference in gut content between gender and $H0_5$ is accepted. In investigation of expression of appetite-related hormones, the null hypothesis ($H0_4$) can be accepted for *ghrl*. However, for *cck1*, *cck2*, *pyya*, and *pyyb* the alternative hypothesis can be accepted, due to significant difference related to gender.

Since there is overall little effect of feeding frequency for both trials, the conclusion is that the only thing that effected growth and appetite was the sexually mature males. For future studies it might be interesting to examine possible differences in expression of *ghrl*, *pyya*, *pyyb*, *cck1*, and *cck2* in the gut, both before and after feeding when feeding every second and every third

day. As, more knowledge is needed to understand the effects of how these hormones and other factors influence appetite in Atlantic halibut.

In regard to the industry, since both feeding regimes did not seem to have any different effect on either growth or appetite, the most economical and least labor-intensive alternative can be utilized when feeding grow-out halibut. That would be to feed every third day.

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Appendix 1 – Oxygen and temperature

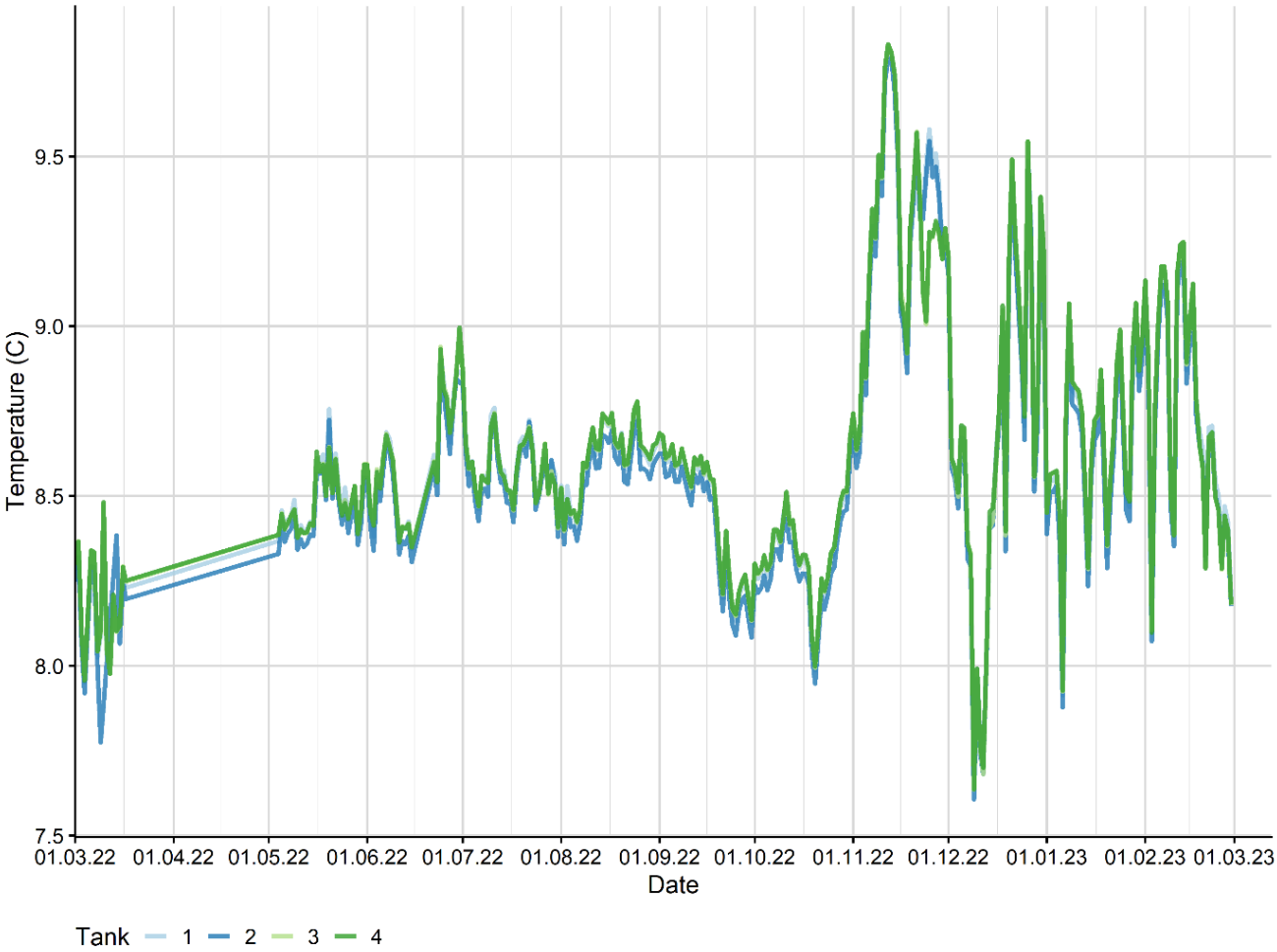

Figure 8: Mean temperature each day in the four fish rearing tanks (1-4) during trial period.

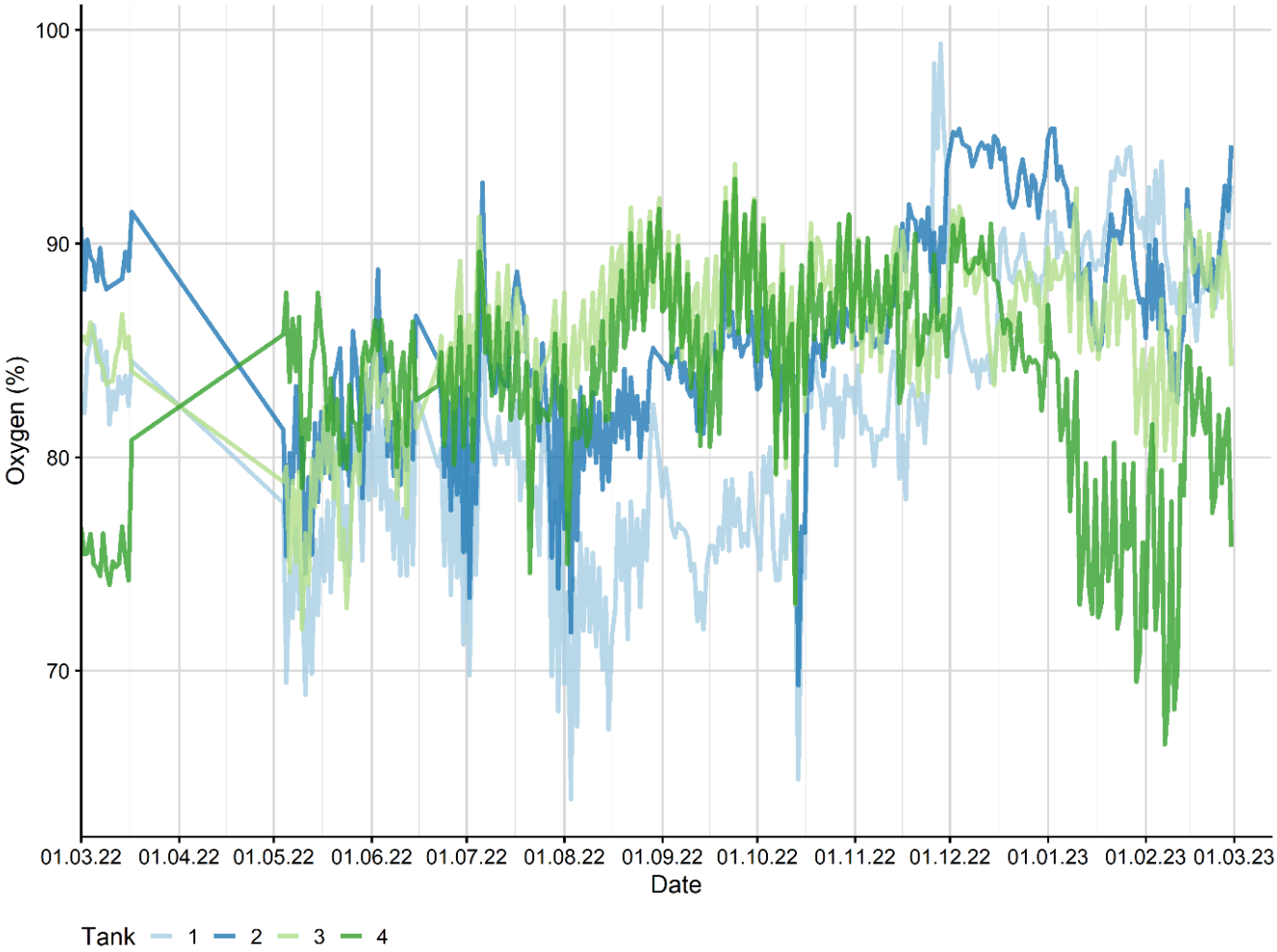

Figure 9: Mean oxygen levels each day in the four fish rearing tanks (1-4) during the trial period.

Appendix 2 – Expression and gut correlation figures

Mean normalised expression of *ghrelin*, *pyya*, *pyyb, cck1*, and *cck2* in correlation with normalized GIT-segment content. P-value < 0.05 was used to determine significance, R^2-value (marked as R2 in the figures) was used to determine how well the model fitted the data.

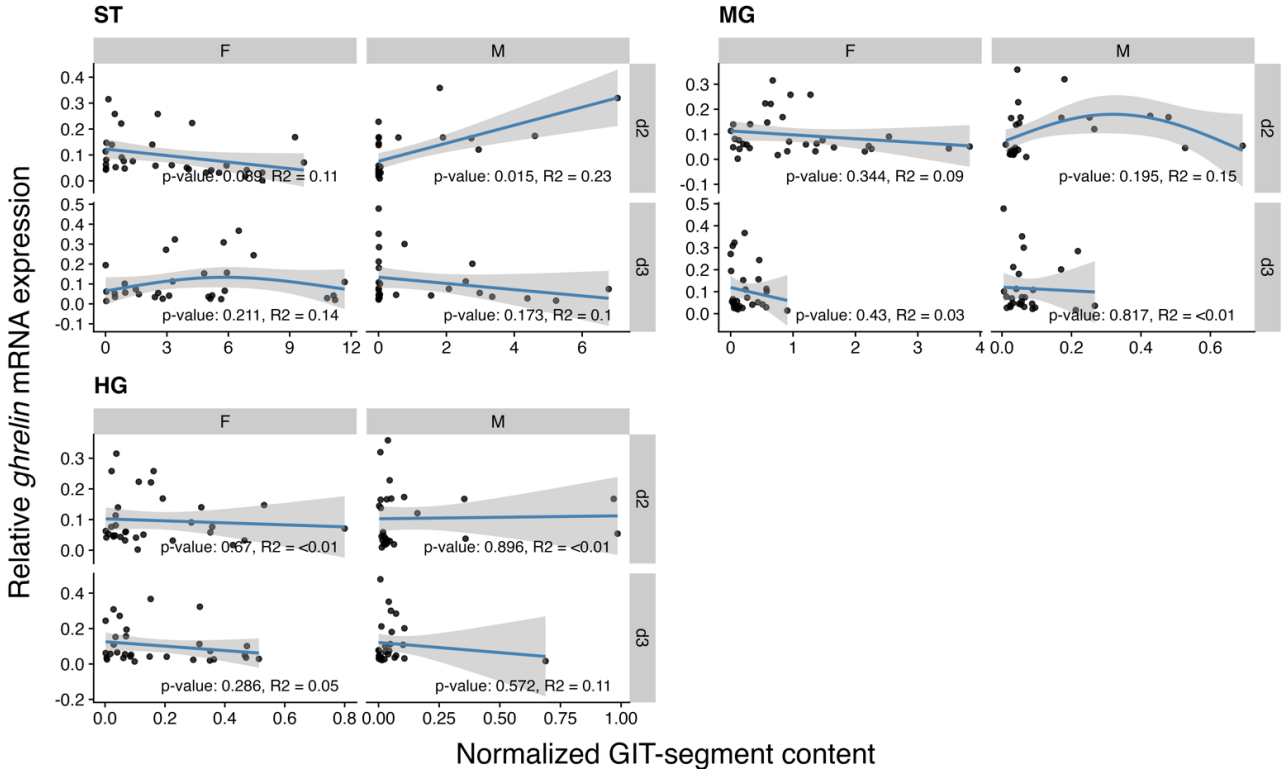

Figure 10: Correlation between mean normalised ghrelin expression and normalized GIT- segment content (ST: stomach), MG: midgut, and HG: hindgut) in male (M) and female (F) Atlantic halibut following two different treatments (d2: feeding every second day, d3: feeding every third day).

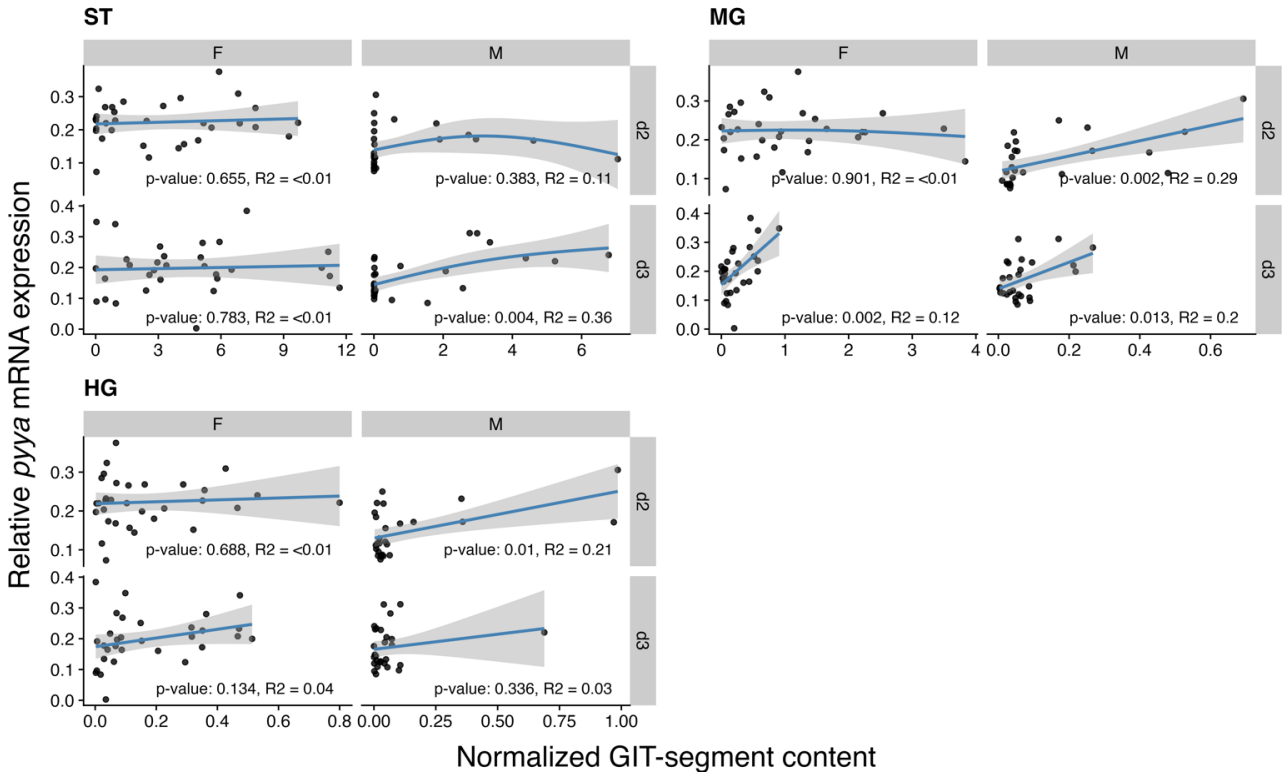

Figure 11: Correlation between mean normalised pyya expression and normalized GIT- segment content (ST: stomach), MG: midgut, and HG: hindgut) in male (M) and female (F) Atlantic halibut following two different treatments (d2: feeding every second day, d3: feeding every third day).

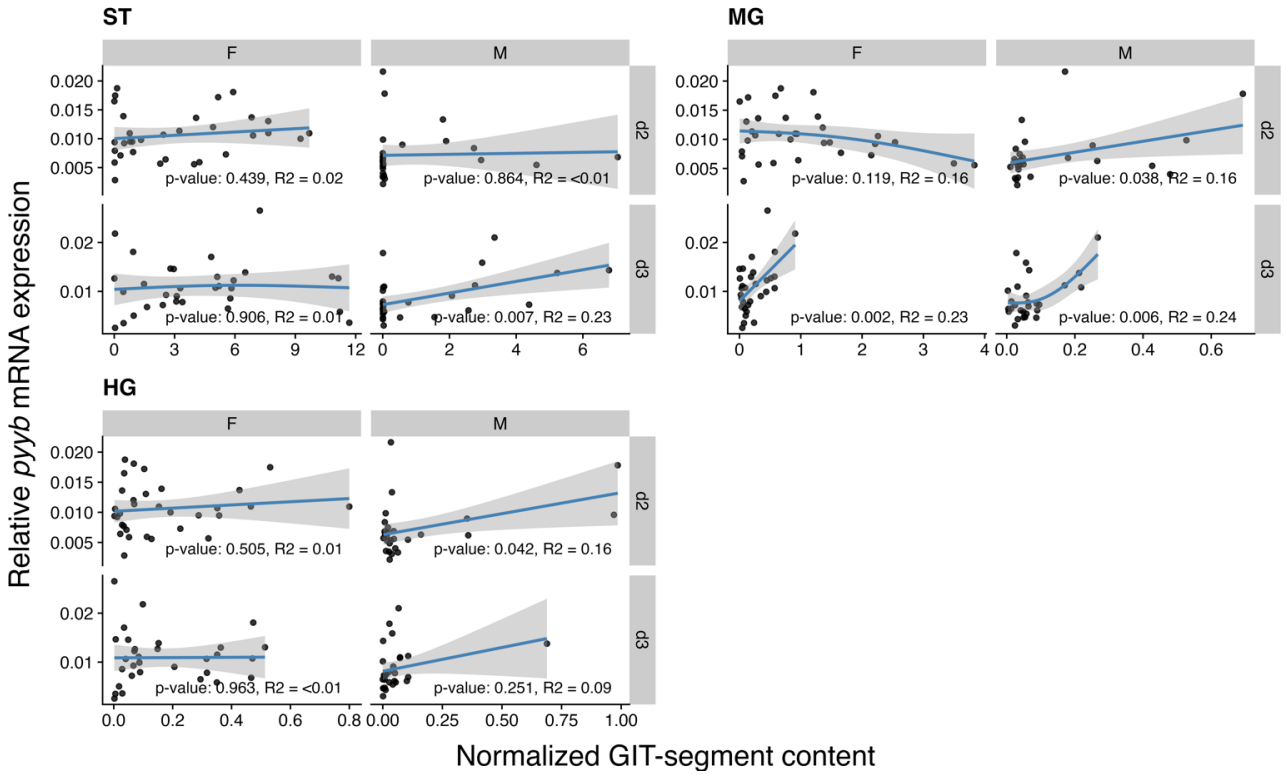

Figure 12: Correlation between mean normalised pyyb expression and normalized GIT- segment content (ST: stomach), MG: midgut, and HG: hindgut) in male (M) and female (F) Atlantic halibut following two different treatments (d2: feeding every second day, d3: feeding every third day).

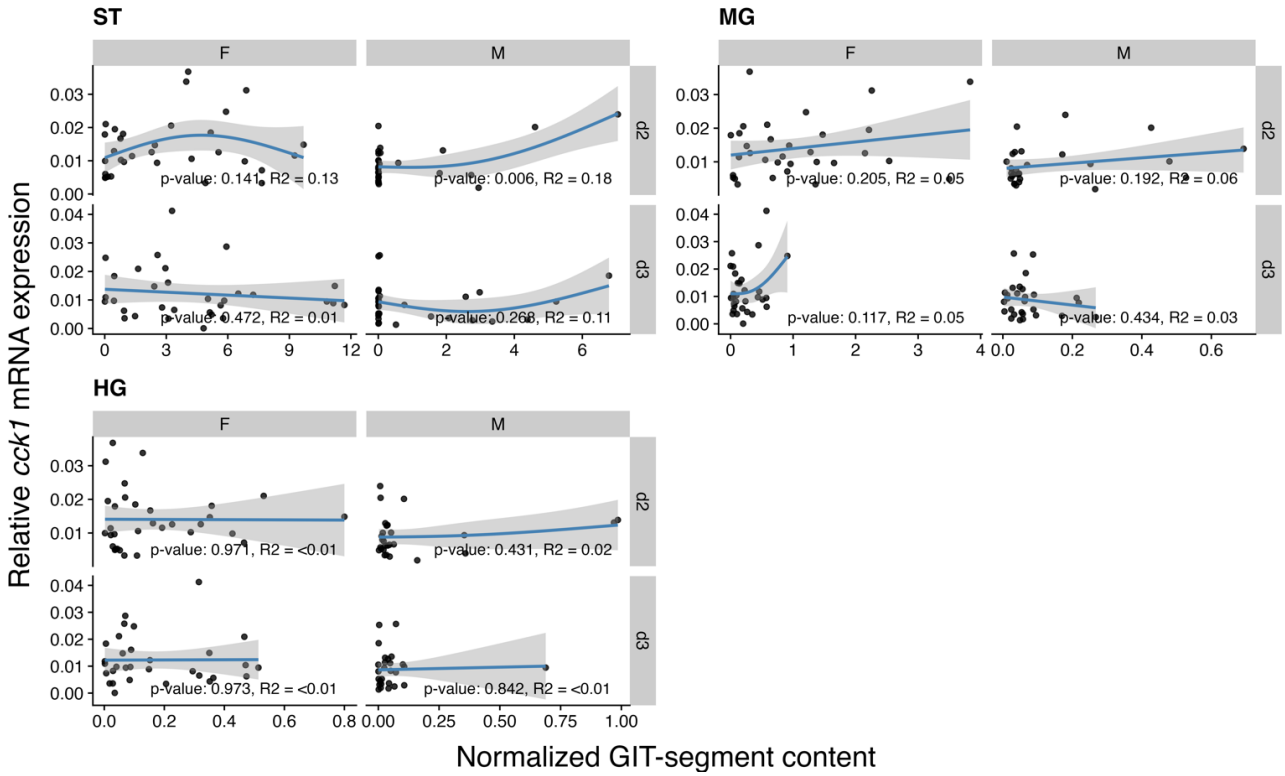

Figure 13: Correlation between mean normalised cck1 expression and normalized GIT- segment content (ST: stomach), MG: midgut, and HG: hindgut) in male (M) and female (F) Atlantic halibut following two different treatments (d2: feeding every second day, d3: feeding every third day).

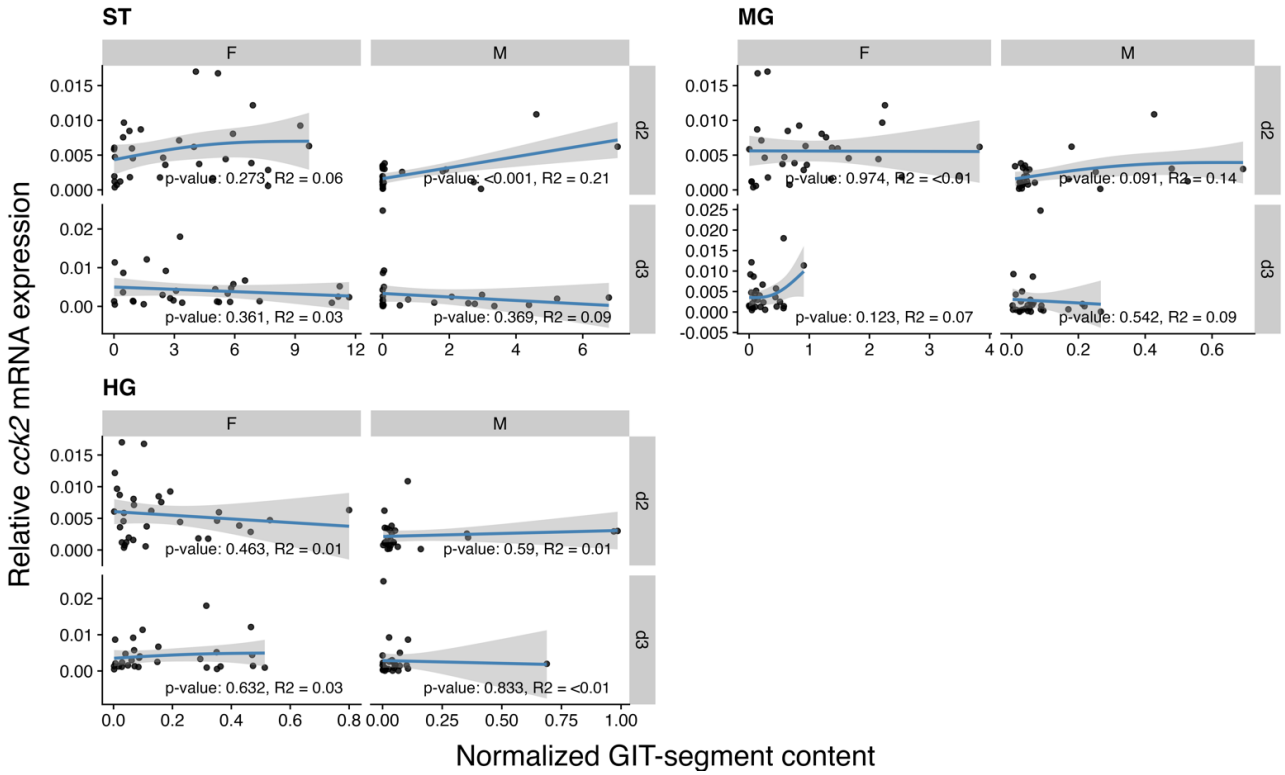

Figure 14: Correlation between mean normalised cck2 expression and normalized GIT- segment content (ST: stomach), MG: midgut, and HG: hindgut) in male (M) and female (F) Atlantic halibut following two different treatments (d2: feeding every second day, d3: feeding every third day).

Appendix 3 – Supplementary figures

Results from statistical analyses on stomach content:

Table 2: Estimate, standard error (std. Error), z-value, and p-value on stomach content using a generalized mixed model (GLMM) with a t-family distribution (identity-link-function) with treatment (d2:feeding every second day, d3: feeding every third day) and gender (female and male) as categorical explanatory variables, including the interaction between these fixed variables.

	Estimate	Std. Error	z-value	p-value
ffeeding_groupd2	2.3820	0.6385	3.731	< 0.001
ffeeding_groupd3	3.5851	0.5217	6.872	< 0.001
sexM	-1.9231	0.6697	-2.871	< 0.01
ffeeding_groupd3:sexM	-0.8445	0.9289	-0.909	0.3632

Table 3: Estimate, associated standard errors (SE), z-ratio, and p-value from post-hoc analysis on stomach content of male and female Atlantic halibut, using a generalized mixed model (GLMM) with a t-family distribution (identity-link-function) with feeding regime (d2: feeding every second day, and d3: feeding every third day) and gender (female and male) as categorical explanatory variables.

Contrast	Etimate	SE	z.ratio	p-value
Feeding regime| Females	-1.203	0.735	-1.638	0.1015
Feeding regime | Males	-0.359	0.532	-0.673	0.5007
Sex | Feeding regime d2	1.920	0.670	2.871	0.0041
Sex | Feeding regime d3	2.770	0.617	4.484	< 0.0001

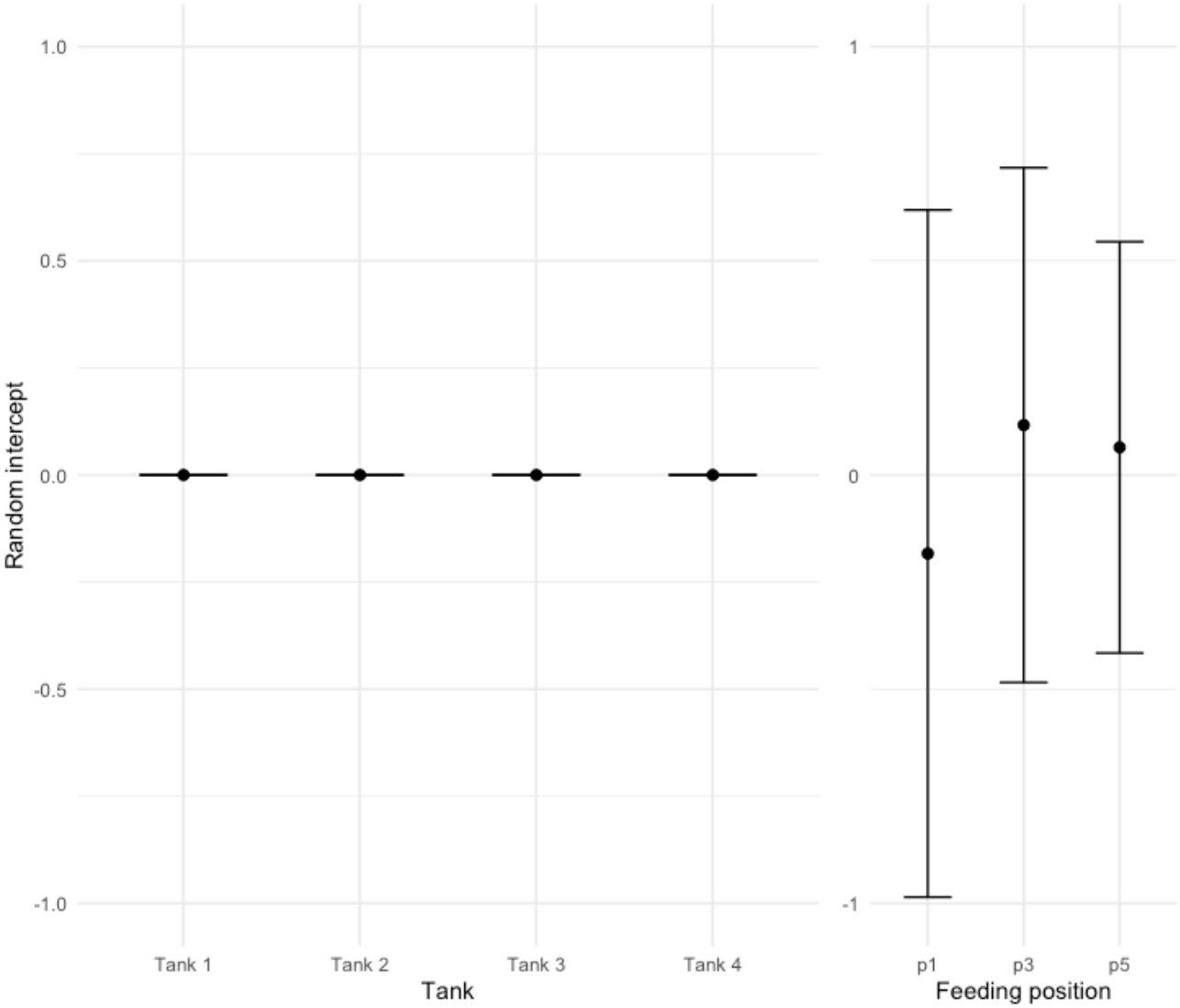

Figure 15: Random intercepts and ± 95 % confidence intervals for tank and feeding points estimated in a generalized mixed linear model with treatment (d2: feeding every second day, d3: feeding every third day) and gender (female and male) as categorical explanatory variables on stomach content of Atlantic halibut.

Results from statistical analyses on midgut content:

Table 4: Estimate, standard error (std. Error), z-value, and p-value on midgut content using a generalized mixed model (GLMM) with a t-family distribution (identity-link-function) with treatment (d2:feeding every second day, d3: feeding every third day) and gender (female and male) as categorical explanatory variables, including the interaction between these fixed variables.

	Estimate	Std. Error	z-value	p-value
ffeeding_groupd2	0.6489	0.0297	21.848	< 0.001
ffeeding_groupd3	0.0662	0.0175	3.778	< 0.001
sexM	-0.6111	0.0304	-20.112	< 0.001
ffeeding_groupd3:sexM	0.5932	0.0363	16.338	< 0.001

Table 5: Estimate, associated standard errors (SE), z-ratio, and p-value from post-hoc analysis on midgut content of male and female Atlantic halibut, using a generalized mixed model (GLMM) with a t-family distribution (identity-link-function) with feeding regime (d2: feeding every second day, and d3: feeding every third day) and gender (female and male) as categorical explanatory variables.

Contrast	Estimate	SE	z.ratio	p-value
Feeding regime| Females	0.5828	0.0345	16.899	< 0.0001
Feeding regime | Males	-0.0105	0.0106	-0.989	0.3228
Sex | Feeding regime d2	0.6111	0.0304	20.112	< 0.0001
Sex | Feeding regime d3	0.0179	0.0195	0.0919	0.3583

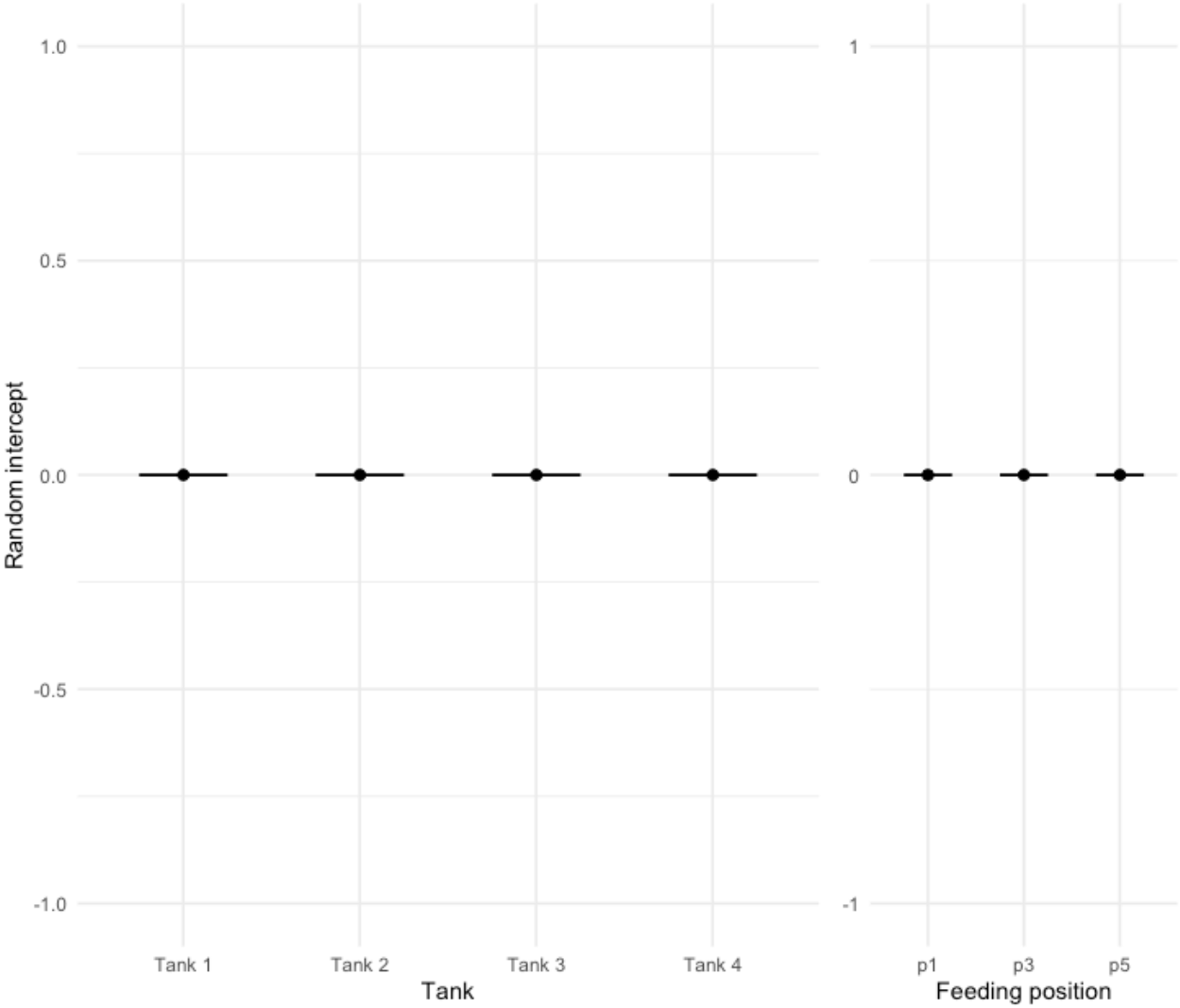

Figure 16: Random intercepts and ± 95 % confidence intervals for tank and feeding points estimated in a generalized mixed linear model with treatment (d2: feeding every second day, d3: feeding every third day) and gender (female and male) as categorical explanatory variables on midgut content of Atlantic halibut.

Results from statistical analyses on hindgut content:

Table 6: Estimate, Standard error (std. Error), z-value, and p-value on hindgut content using a generalized mixed model (GLMM) with a t-family distribution (identity-link-function) with treatment (d2:feeding every second day, d3: feeding every third day) and gender (female and male) as categorical explanatory variables, including the interaction between these fixed variables.

	Estimate	Std. Error	z-value	p-value
Ffeeding_groupd2	0.0529	0.0132	4.021	< 0.001
Ffeeding_groupd3	0.0529	0.0125	2.265	< 0.001
sexM	-0.0191	0.0099	-1.910	0.0561
Ffeeding_groupd3:sexM	-0.0099	0.0143	-0.701	0.4832

Table 7: Estimate, associated standard errors (SE), z-ratio, and p-value from post-hoc analysis on hindgut content of male and female Atlantic halibut, using a generalized mixed model (GLMM) with a t-family distribution (identity-link-function) with feeding regime (d2: feeding every second day, and d3: feeding every third day) and gender (female and male) as categorical explanatory variables.

Contrast	Estimate	SE	z.ratio	p-value
Feeding regime| Females	-0.0001	0.0122	-0.005	0.9963
Feeding regime | Males	0.0099	0.0008	1.203	0.2289
Sex | Feeding regime d2	0.0191	0.0099	1.910	0.0561
Sex | Feeding regime d3	0.0291	0.0098	2.955	0.0031

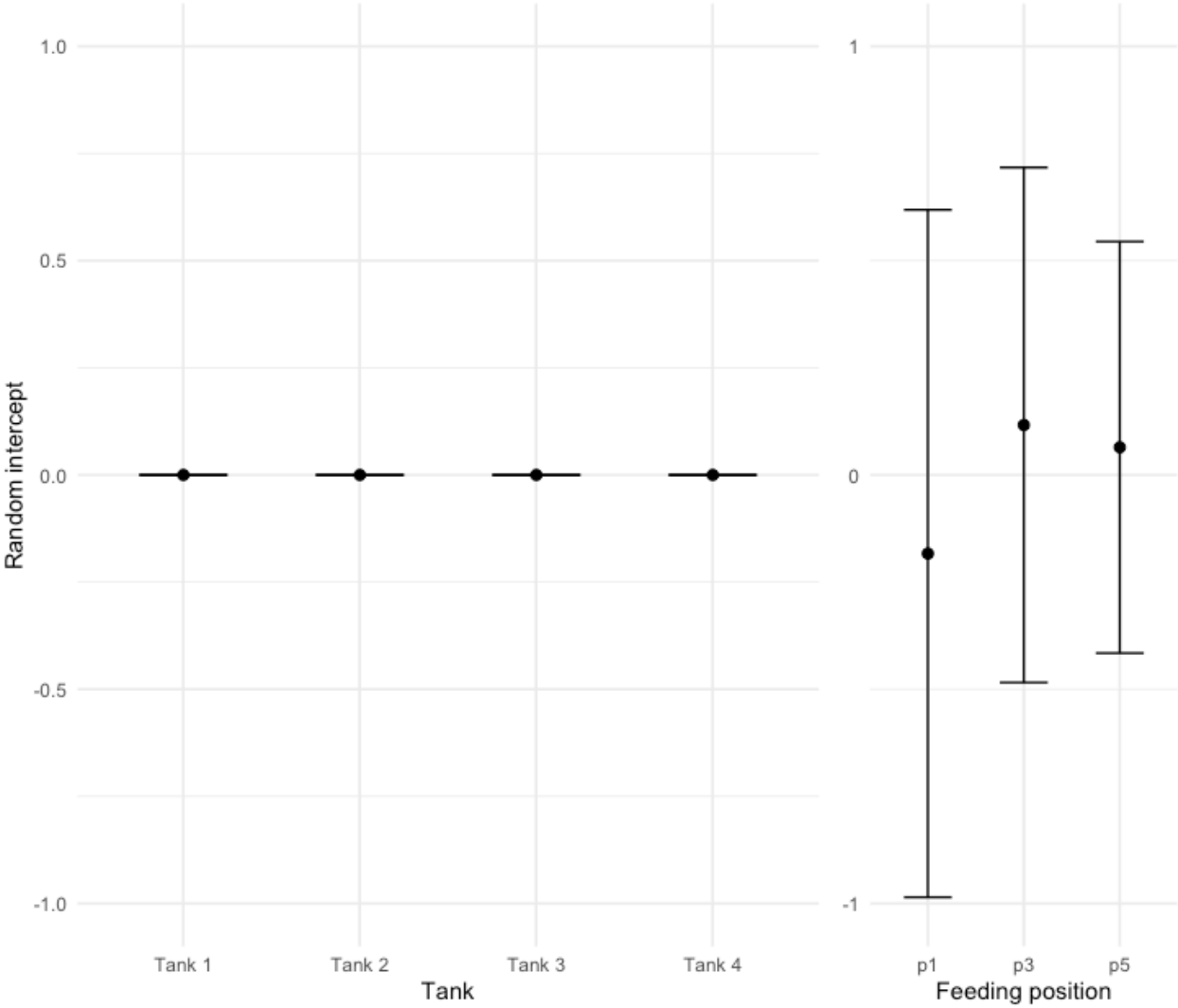

Figure 17: Random intercepts and ± 95 % confidence intervals for tank and feeding points estimated in a generalized mixed linear model with treatment (d2: feeding every second day, d3: feeding every third day) and gender (female and male) as categorical explanatory variables on hindgut content of Atlantic halibut.

Results from statistical analyses on *ghrelin* expression:

Table 8: Estimate, standard error (std. Error), z-value, and p-value on relative expression of ghrelin in stomach tissue of female and male Atlantic halibut using a generalized mixed model (GLMM) with a gamma distribution (log-link-function) with treatment (d2:feeding every second day, d3: feeding every third day) and gender (female and male) as categorical explanatory variables, including the interaction between these fixed variables.

	Estimate	Std. Error	z-value	p-value
fFeedingd2	-2.3629	0.2212	-10.683	< 0.001
fFeedingd3	-2.3595	0.2287	-10.317	< 0.001
fSexM	0.0893	0.2110	0.423	0.672
fFeedingd**3:fSexM**	0.0199	0.3128	0.064	0.949

Table 9: Contrast ratio, associated standard errors (SE), z-ratio, and p-value from post-hoc test on relative mRNA expression of ghrelin in stomach tissue of female and male Atlantic halibut using a generalized mixed model (GLMM) with a gamma distribution (log-link-function) with treatment (d2: feeding every second day, d3: feeding every third day) and gender (female and male) as categorical explanatory variables.

Contrast	Contrast ratio	SE	z.ratio	p -value
Feeding regime | Females	0.997	0.317	-0.011	0.9913
Feeding regime | Males	0.977	0.322	-0.071	0.9434
Sex | Feeding regime d2	0.915	0.193	-0.423	0.6722
Sex | Feeding regime d3	0.897	0.207	-0.473	0.6364

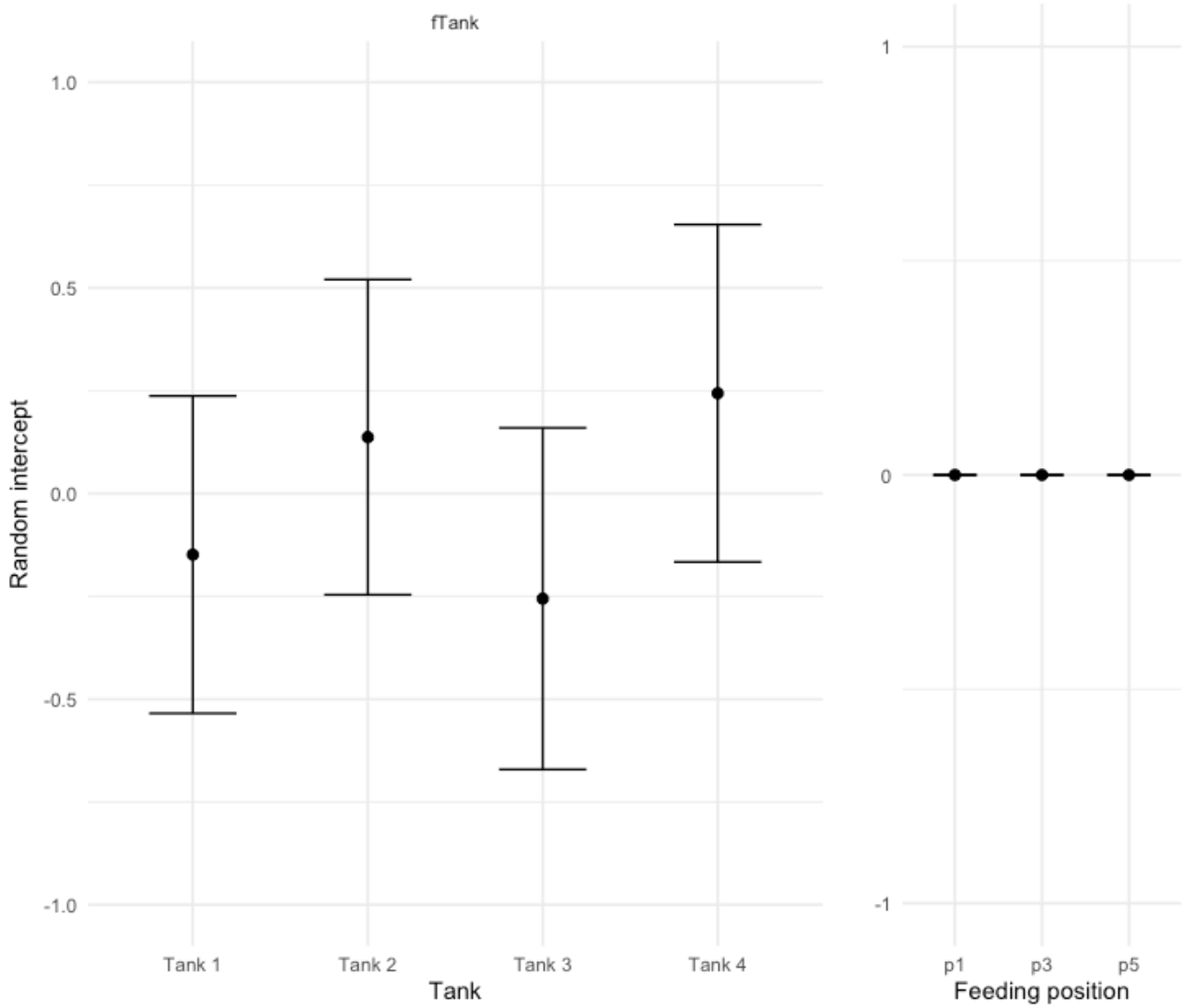

Figure 18: Random intercepts and ± 95 % confidence intervals for tank and feeding points estimated in a generalized mixed linear model with treatment (d2: feeding every second day, d3: feeding every third day) and gender (female and male) as categorical explanatory variables on relative mRNA ghrelin expression in Atlantic halibut.

Results from statistical analyses on *pyya* expression:

Table 10: Estimate, standard error (std. Error), z-value, and p-value on relative expression of pyya, in anterior midgut tissue of female and male Atlantic halibut, using a generalized mixed model (GLMM) with a gamma distribution (log-link-function) with treatment (d2:feeding every second day, d3: feeding every third day) and gender (female and male) as categorical explanatory variables, including the interaction between these fixed variables.

	Estimate	Std.Error	z-value	p-value
fFeedingd2	1.5068	0.0754	-19.976	< 0.001
fFeedingd3	-1.6166	0.0764	-21.171	< 0.001
fSexM	-0.4206	0.1084	-3.879	< 0.001
fFeedingd3:fSexM	0.2717	0.1528	1.778	0.0754

Table 11: Contrast ratio, associated standard errors (SE), z-ratio, and p-value from post-hoc test on relative mRNA expression of pyya, in anterior midgut tissue of female and male Atlantic halibut, using a generalized mixed model (GLMM) with a gamma distribution (log-link-function) with treatment (d2:feeding every second day, d3: feeding every third day) and gender (female and male) as categorical explanatory variables.

Contrast	Contrast ratio	SE	z.ratio	p -value
Feeding regime | Females	1.116	0.115	1.063	0.2879
Feeding regime | Males	0.851	0.095	-1.441	0.1497
Sex | Feeding regime d2	1.520	0.165	3.879	0.0001
Sex | Feeding regime d3	1.160	0.125	1.381	0.1673

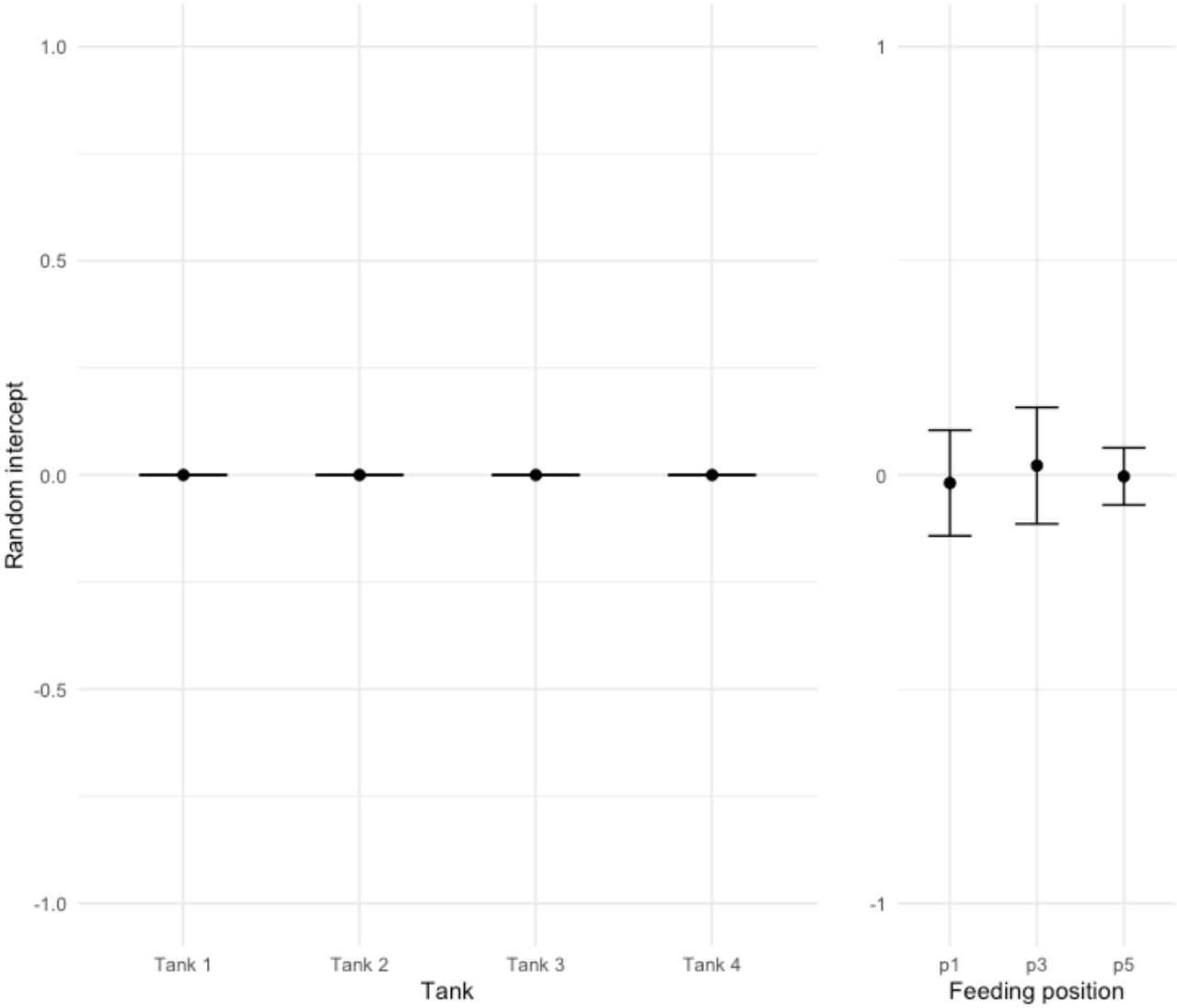

Figure 19: Random intercepts and ± 95 % confidence intervals for tank and feeding points estimated in a generalized mixed linear model with treatment (d2: feeding every second day, d3: feeding every third day) and gender (female and male) as categorical explanatory variables on relative mRNA pyya expression in Atlantic halibut.

Results from statistical analyses on *pyyb* expression:

Table 12: Estimate, standard error (std. Error), z-value, and p-value on relative expression of pyyb, in anterior midgut tissue of female and male Atlantic halibut, using a generalized mixed model (GLMM) with a gamma distribution (log-link-function) with treatment (d2:feeding every second day, d3: feeding every third day) and gender (female and male) as categorical explanatory variables, including the interaction between these fixed variables.

	Estimate	Std.Error	z-value	p-value
fFeedingd2	-4.5491	0.0888	-51.19	< 0.001
fFeedingd3	-4.5159	0.0897	-50.31	< 0.001
fSexM	-0.3955	0.1183	-3.35	< 0.001
fFeedingd3:fSexM	0.1638	0.1667	0.98	0.3256

Table 13: Contrast ratio, associated standard errors (SE), z-ratio, and p-value from post-hoc test on relative mRNA expression of pyyb, in anterior midgut tissue of female and male Atlantic halibut, using a generalized mixed model (GLMM) with a gamma distribution (log-link-function) with treatment (d2:feeding every second day, d3: feeding every third day) and gender (female and male) as categorical explanatory variables.

Contrast	Contrast ratio	SE	z .ratio	p -value
Feeding regime | Females	0.967	0.109	-0.294	0.7688
Feeding regime | Males	0.821	0.100	-1.614	0.1065
Sex | Feeding regime d2	1.49	0.176	3.345	0.0008
Sex | Feeding regime d3	1.26	0.148	1.978	0.0479

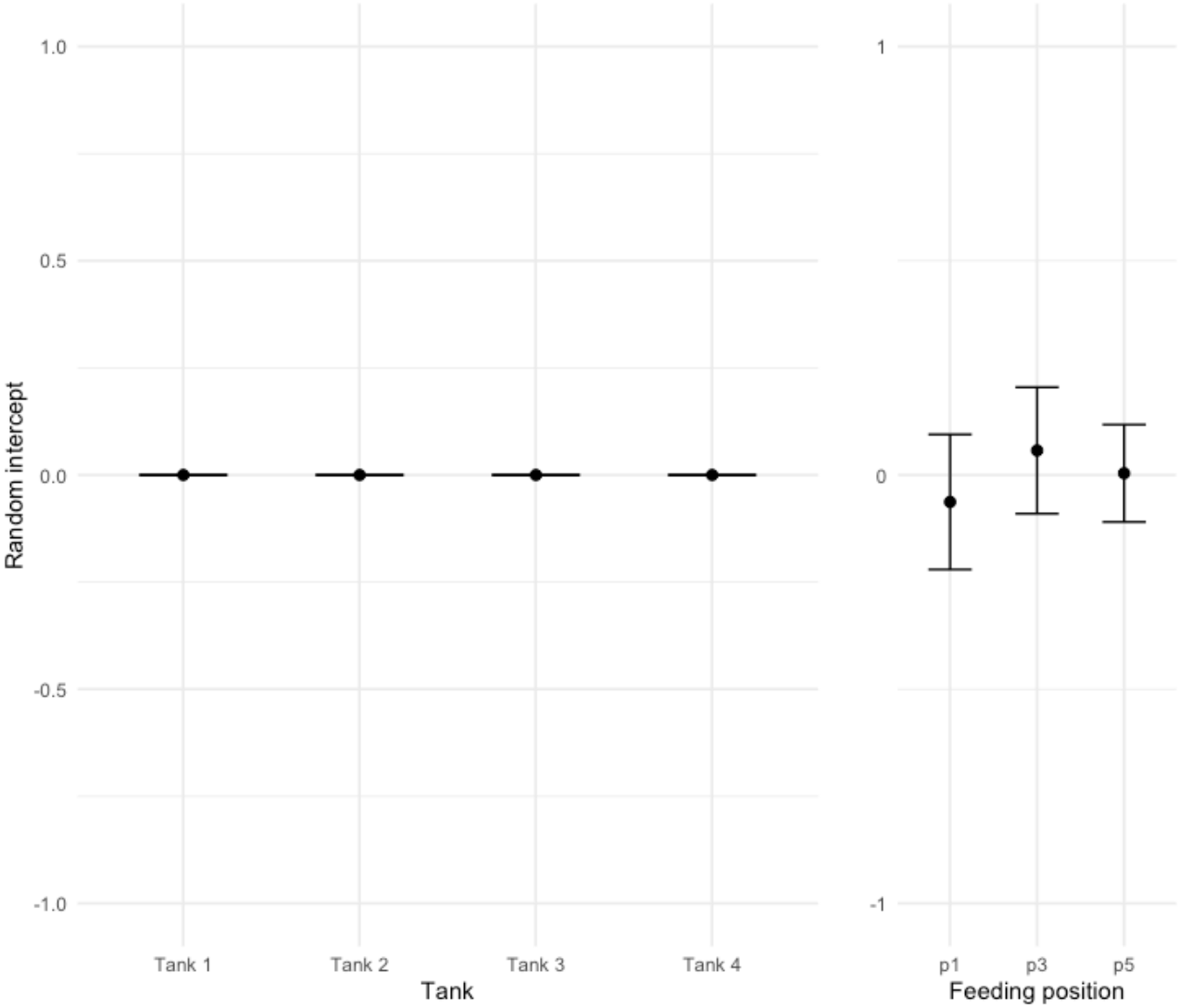

Figure 20: Random intercepts and ± 95 % confidence intervals for tank and feeding points estimated in a generalized mixed linear model with treatment (d2: feeding every second day, d3: feeding every third day) and gender (female and male) as categorical explanatory variables on relative mRNA pyyb expression in Atlantic halibut.

Results from statistical analyses on *cck1* expression:

Table 14: Estimate, standard error (std. Error), z-value, and p-value on relative expression of cck1, in anterior midgut tissue of female and male Atlantic halibut, using a generalized mixed model (GLMM) with a gamma distribution (log-link-function) with treatment (d2:feeding every second day, d3: feeding every third day) and gender (female and male) as categorical explanatory variables, including the interaction between these fixed variables.

	Estimate	Std. Error	z-value	p-value
fFeedingd2	-4.245	0.118	-35.99	< 0.001
SfFeedingd3	-4.423	0.119	-36.93	< 0.001
fSexM	-0.455	0.175	-2.59	< 0.01
fFeedingd3:fSexM	0.139	0.248	0.56	0.5742

Table 15: Contrast ratio, associated standard errors (SE), z-ratio, and p-value from post-hoc test on relative mRNA expression of cck1, in anterior midgut tissue of female and male Atlantic halibut, using a generalized mixed model (GLMM) with a gamma distribution (log-link-function) with treatment (d2:feeding every second day, d3: feeding every third day) and gender (female and male) as categorical explanatory variables.

Contrast	Contrast ratio	SE	z.ratio	p -value
Feeding regime | Females	1.20	0.201	1.062	0.2882
Feeding regime | Males	1.04	0.190	0.214	0.8309
Sex | Feeding regime d2	1.58	0.277	2.587	0.0097
Sex | Feeding regime d3	1.37	0.240	1.799	0.0720

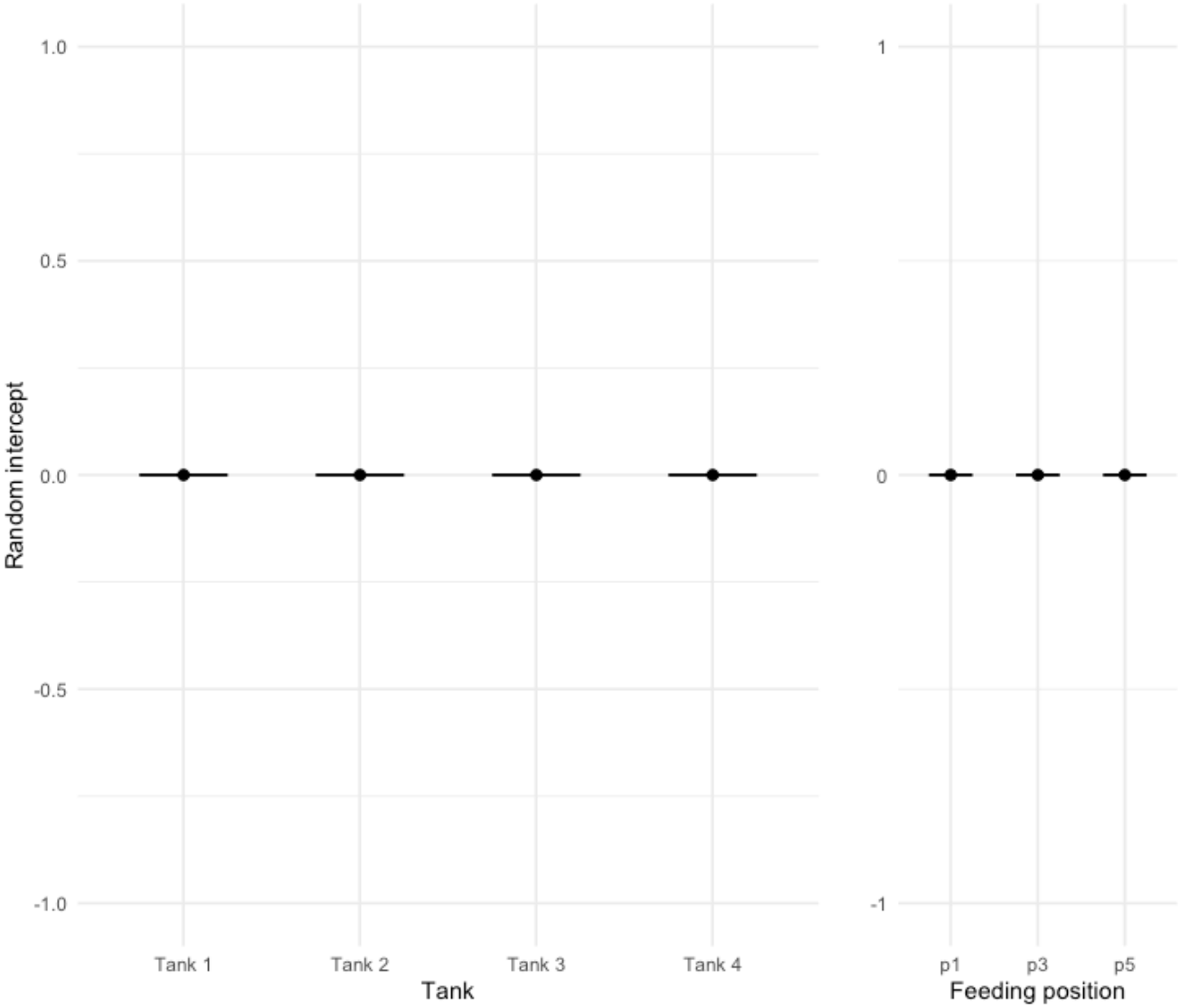

Figure 21: Random intercepts and ± 95 % confidence intervals for tank and feeding points estimated in a generalized mixed linear model with treatment (d2: feeding every second day, d3: feeding every third day) and gender (female and male) as categorical explanatory variables on relative mRNA cck1 expression in Atlantic halibut.

Results from statistical analyses on *cck2* expression:

Table 16: Estimate, standard error (std. Error), z-value, and p-value on relative expression of cck2 using a generalized mixed model (GLMM) with a gamma distribution (log-link-function) with treatment (d2: feeding every second day, d3: feeding every third day) and gender (female and male) as categorical explanatory variables, including the interaction between these fixed variables.

	Estimate	Std. Error	z-value	p-value
fFeedingd2	-5.1848	0.1824	-28.433	< 0.001
fFeedingd3	-5.5069	0.1891	-29.122	< 0.001
fSexM	-0.8871	0.2390	-3.712	< 0.001
fFeedingd3:fSexM	0.1082	0.3500	0.309	0.7571

Table 17: Contrast ratio, associated standard errors (SE), z-ratio, and p-value from post-hoc test on relative mRNA expression of cck2, in anterior midgut tissue of female and male Atlantic halibut, using a generalized mixed model (GLMM) with a gamma distribution (log-link-function) with treatment (d2:feeding every second day, d3: feeding every third day) and gender (female and male) as categorical explanatory variables.

Contrast	Contrast ratio	SE	z.ratio	p -value
Feeding regime | Females	1.38	0.317	1.404	0.1604
Feeding regime | Males	1.24	0.320	0.829	0.4071
Sex | Feeding regime d2	2.43	0.580	3.712	0.0002
Sex | Feeding regime d3	2.18	0.537	3.161	0.0016

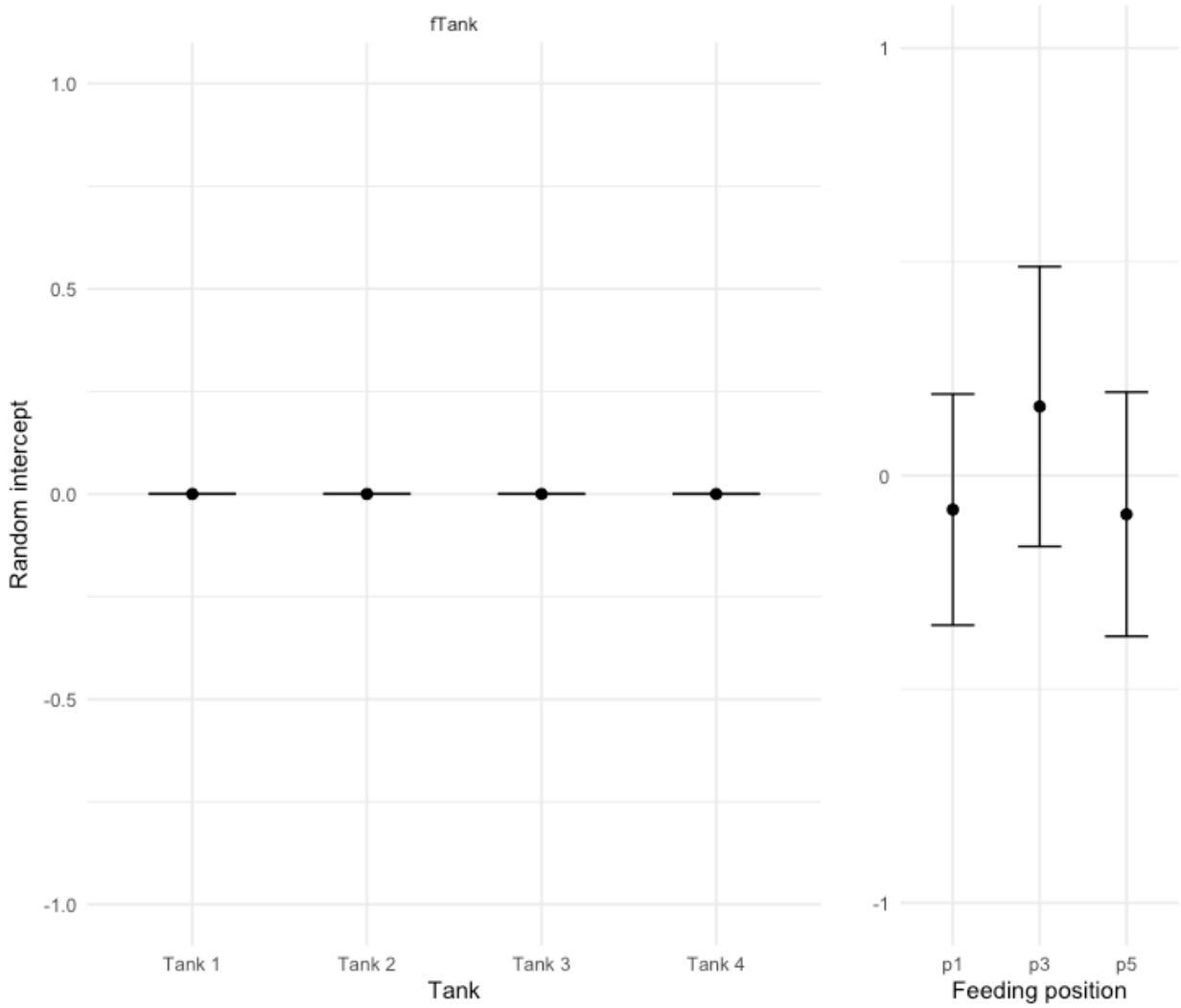

Figure 22: Random intercepts and ± 95 % confidence intervals for tank and feeding points estimated in a generalized mixed linear model with treatment (d2: feeding every second day, d3: feeding every third day) and gender (female and male) as categorical explanatory variables on relative mRNA cck2 expression in Atlantic halibut.

Results from statistical analyses on *SGR*:

```
## treatment = d2, sex = F:
##  contrast               estimate       SE  df t.ratio p.value
##  (sgr 1-2) - (sgr 2-3)  0.001180 0.000252 104   4.688  <.0001
##  (sgr 1-2) - (sgr 1-3)  0.000343 0.000207 104   1.657  0.2269
##  (sgr 2-3) - (sgr 1-3) -0.000837 0.000207 104  -4.050  0.0003
##
## treatment = d3, sex = F:
##  contrast               estimate       SE  df t.ratio p.value
##  (sgr 1-2) - (sgr 2-3)  0.000980 0.000252 104   3.893  0.0005
##  (sgr 1-2) - (sgr 1-3)  0.000119 0.000207 104   0.574  0.8341
##  (sgr 2-3) - (sgr 1-3) -0.000861 0.000207 104  -4.164  0.0002
##
## treatment = d2, sex = M:
##  contrast               estimate       SE  df t.ratio p.value
##  (sgr 1-2) - (sgr 2-3)  0.001951 0.000222 104   8.790  <.0001
##  (sgr 1-2) - (sgr 1-3)  0.000862 0.000204 104   4.221  0.0002
##  (sgr 2-3) - (sgr 1-3) -0.001089 0.000204 104  -5.334  <.0001
##
## treatment = d3, sex = M:
##  contrast               estimate       SE  df t.ratio p.value
##  (sgr 1-2) - (sgr 2-3)  0.001731 0.000298 104   5.813  <.0001
##  (sgr 1-2) - (sgr 1-3)  0.000621 0.000263 104   2.363  0.0518
##  (sgr 2-3) - (sgr 1-3) -0.001111 0.000263 104  -4.229  0.0001
##
## P value adjustment: tukey method for comparing a family of 3 estimates
```

Figure 23: Contrast, estimate, associated standard errors (SE), t.ratio, and p-values from post-hoc analysis on specific growth rate (SGR) during two growth periods (sgr 1-2, sgr 2-3) including for the whole trial period (sgr 1-3) for female (F) and (M) Atlantic halibut following two different treatments (d2: feeding every second day, and d3: feeding every third day), using a generalized linear model (GLMM) with gaussian distribution (identity-link-function) with treatment, gender and growth periods as categorical explanatory variables.

```
## treatment = d2, name = sgr 1-2:
##  contrast  estimate       SE  df t.ratio p.value
##  F - M    -2.14e-04 0.000237 104  -0.903  0.3687
##
## treatment = d3, name = sgr 1-2:
##  contrast  estimate       SE  df t.ratio p.value
##  F - M     8.24e-05 0.000276 104   0.299  0.7657
##
## treatment = d2, name = sgr 2-3:
##  contrast  estimate       SE  df t.ratio p.value
##  F - M     5.57e-04 0.000237 104   2.347  0.0208
##
## treatment = d3, name = sgr 2-3:
##  contrast  estimate       SE  df t.ratio p.value
##  F - M     8.34e-04 0.000276 104   3.024  0.0031
##
## treatment = d2, name = sgr 1-3:
##  contrast  estimate       SE  df t.ratio p.value
##  F - M     3.05e-04 0.000168 104   1.819  0.0718
##
## treatment = d3, name = sgr 1-3:
##  contrast  estimate       SE  df t.ratio p.value
##  F - M     5.84e-04 0.000189 104   3.091  0.0026
```

Figure 24: Contrast, estimate, associated standard errors (SE), t.ratio, and p-values from post-hoc analysis on differences in specific growth rate (SGR) between female (F) and male (M) Atlantic halibut following two different treatments (d2: feeding every second day, and d3: feeding every third day during two growth periods (sgr 1-2, sgr 2-3) including for the whole trial period (sgr 1-3), using a generalized linear model (GLMM) with gaussian distribution (identity-link-function) with treatment, gender and growth periods as categorical explanatory variables.