Feeding response and growth performance of Atlantic halibut fed diets with varying macronutrient levels

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Abstract

Atlantic halibut, *Hippoglossus hippoglossus,* is a highly valued and well-established fish species that has gotten its reputation from being a high-end product from the fishery industry. Because of halibut overfishing and its fishery sustainability issues, the demand for a steady supply creates opportunities for aquaculture of halibut to succeed. In Norway, the halibut aquaculture industry has been active for the last couple of decades, and it aims to be a substantial niche in the Norwegian aquaculture industry. On its road towards success many challenges have been identified and, to some extent solved. However, today one of the major remaining problems is the fish's lack of interest in commercial feed. Thus, farming of Atlantic halibut will be more efficient if the feed intake and feed efficiency is improved by optimizing the feed composition. In this thesis the appetite response and growth performance of grow-out halibut (317.7 ± 80.5 g) was studied when groups of fish were fed with 12 different feed compositions and varying macronutrient levels over a period of 60 weeks. Supporting previous findings, grow-out halibut grow better when given lower protein diets. Elevated dietary lipid (levels up until 25%) induced high growth, even though the apparent appetite was lower for these diets. However, increasing dietary lipid up to 30% resulted in major growth reduction. This supports previous findings for an upper tolerable limit for lipid content in formulated diets. Further, for carbohydrates, increased levels up to 25% of dietary inclusion was tolerated while maintaining the growth performance, contradicting previous findings. Furthermore, a correlation between appetite and elevated carbohydrate content was found, as well as with decreased dietary lipid. The results indicate that lower protein diets, down to 45% dietary inclusion, with higher lipid and carbohydrate contents can be used in the grow-out stages for halibut. Since feed protein usually is the highest cost feed ingredient, the results of this thesis can serve as a basis for the industry to produce more affordable diets for Atlantic halibut, enhancing its profitability. Also, the role of carbohydrates and lipid as appetite modulators in fish species is a research area that warrants further investigation in the formulation of an optimal feed that encourage feed intake. Thus, this thesis contributes to supplying further knowledge regarding ideal macronutrient gradients for enhancing appetite, growth, and fish welfare for the aquaculture of Atlantic halibut.

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1 Introduction

1.1 Status of halibut farming in Norway

Atlantic halibut is traditionally a highly valued food fish, with a good reputation from being a high-end product from the fishery industry. The fishery industry is under increasing pressure with issues surrounding environmental impact through overfishing and other sustainability problems. This high demand of halibut as a marine product has drawn the interest of the aquaculture industry. Production in Norway has shown a positive trend over the recent years. While through its time many challenges in the halibut production cycle have been identified, also solutions have been found, securing a stable aquaculture production (Glover *et al*., 2006). However, a challenge that remains to be solved is a weak response and interest of the fish to the formulated feed currently available at the grow-out stage. The low appetite is stalling the growth significantly, and consequently, the time needed to reach final slaughter size is increased. The high investment needed to keep the fish for longer periods until slaughter, limits the industry's growth potential. Thus, identifying formulated diets that are optimal for the appetite and growth efficiency is crucial for the halibut aquaculture growth and success.

1.2 Feed for halibut production

To achieve optimal growth and health conditions in the grow-out stage, a correct feed composition is essential. Like for all other forms of industrial fish farming, feed is the major cost in the production, and a cost-efficient feed with a maintained and optimized growth rate is therefore the desirable target for the industry. In modern farming, the appetite response of Atlantic halibut to the commercial fish feed has been remarkably lower than what's seen for other aquaculture species such as Atlantic salmon, *Salmo salar*, or rainbow trout, *Oncorhynchus mykiss*. This results in a significant amount of feed-waste. Feed waste represents a significant cost, both economically and environmentally, and reducing this waste to a minimum will positively influence the overall efficiency and profitability, along with the public reputation of the halibut industry.

1.2.1 Macronutrient composition

Most of the work done in formulating commercial fish feed for the Norwegian aquaculture has been aimed towards the needs of the main aquaculture species Atlantic salmon and Rainbow trout. However, there are inter-species differences regarding the nutritional composition of feeds used in aquaculture (Teles *et al.*, 2020). Finding the optimal macronutrient composition, with correct ratios between protein, lipid and carbohydrates is therefore essential. In addition to stimulating appetite, the digestive properties of feed represent one of the crucial aspects of feed formulation. As growth, feed efficiency and fish health are the major measurements of success, the digestibility should be high, with minimum waste of nutrients evacuated as feces.

1.2.1.1 Protein

Commercial fish feed diets often include higher levels of dietary protein compared to terrestrial farmed animals. A major reason for this is the lower energy requirements of fish related to their ectotherm nature, making the energy needs in the formulated feed to be significantly lower compared to endothermic animals. With a reduced need for high-energy feed components like lipids and carbohydrates, the relative protein content naturally is increased (Teles *et al.*, 2020). In aquaculture, due to the cost of protein ingredients, protein and amino acid dietary composition targets the amount needed to promote good growth, while being economically sustainable. For halibut the ideal content of feed crude protein seems to vary through the life stages and in relation to the fish size (Hatlen *et al.*, 2005; Árnason *et al.*, 2009). Thus, research so far done on different fish sizes and life stages has only given fragmented results and non-validated assumptions regarding the ideal protein content.

The protein requirements for small, juvenile halibut are high. Hamre *et al.* (2003 and 2005) reported minimum crude protein requirements at 58% and 63% for juvenile fish growing from 0.5 g to 6-7 g. Further, Aksnes *et al.*, (1996) described that the requirement of protein for fish between 6 to 556 g was 61.8%. Weight increments within this range showed lower requirements, at 56% and 51% dietary protein for fish in the ranges of 58 to 126 g and 140 to 266 g, respectively (Grisdale-Helland and Helland, 1998; Hatlen *et al.*, 2005). For larger fish the requirement of protein seems to drop (Árnason *et al.*, 2009). It is estimated that fish between 559 and 877 g only required 41% protein (Árnason *et al.*, 2009), supporting the results obtained by Hatlen *et al.*, (2005). Árnason *et al.* (2009) also included fish starting at 980 grams growing up to 1493 g, and reported a requirement estimated to 35% protein.

Protein digestibility for Atlantic halibut is usually quite high. Berge and Storebakken (1991) reported a protein apparent digestibility coefficient (ADC) of 82 to 86% for fish meal-based diets with varying protein and lipid content. In Grisdale-Helland and Helland (1998) fish meal-based diets with varying amounts of dietary protein (48-61%), lipid (20-28%) and carbohydrates (9-16%) also resulted in an ADC in the 84-86% range.

1.2.1.2 Lipid

Previous research shows that halibut seems to tolerate quite a wide range of dietary lipid levels. However, most studies have focused on the smaller fish and larval stages. Varying lipid content from 12 to 21% (Berge and Storebakken, 1991) and 5 to 25% (Hamre et al., 2003) for halibut ranging from 6 to 12 g and 0.5 to 6-7 g respectively, has been proved to not result in any significant differences in growth. However, a lipid content of 30% resulted in a major growth reduction was reported in one study (Hamre et al., 2003). Further, for halibut starting at 33 g growing to approx. 100 g, a lipid content range from 14% to 25% did not seem to affect growth performance, but had a significant increasing effect on the whole body lipid content when fed higher lipid content diets (Martins *et al.*, 2007). A study mapping this requirement for fish weighing 600 to 1500 g has shown no weight differences for varying lipid levels, ranging from 8 to 20% (Berge and Storebakken, 1991).

Lipid digestibility for halibut was investigated in two separate studies(Berge and Storebakken, 1991; Grisdale-Helland and Helland, 1998), where it proved to be high and quite stable with the varying compositions used, ranging from 84.9 to 94.1% and 96.7 to 97.5% ADC respectively for marine, fish meal-based diets.

1.2.1.3 Carbohydrates

Carnivorous fish like Atlantic halibut tend to have a low degree of carbohydrates in its natural diet, thereby follows a lowered capacity for digestion, Thus, it is believed that carbohydrates should be included in low levels in formulated diets. The tolerance for dietary carbohydrates at the early life stages seem to be low. Indeed, two studies have reported a maximum limit of carbohydrate inclusion at 5% for fish growing from 0.5 g to 6-7 g (Hamre *et al.*, 2003, 2005). In a macronutrient trial with carbohydrate feed content ranging from 3.1 to 26.9 % (fish weight from around 5-7 g to 400-600 g), results showed significant increased growth and feed

efficiency as the carbohydrate content in the feed decreased indicating a low optimal carbohydrate inclusion (Aksnes *et al.*, 1996).

For carbohydrates, there is a clear relationship between inclusion of starch in the diet and its ADC of starch. This is described for halibut in Grisdale-Helland and Helland (1998), where increasing the quantity of added starch negatively affected the ADC of starch from 83-86% for the normal starch diets to 52-55% when starch content is elevated.

1.3 Appetite control

1.3.1 Central and peripheral appetite control

Appetite control in fish is a complex process that is modulated by intricate physiological systems. The overall role of appetite-control is to secure efficient and sufficient energy and nutrient uptake to support the fish development and growth. In natural conditions, feed intake is a tradeoff, where the fish needs to take into account the costs and rewards of feed search and intake. In an aquaculture environment, even though the food supply is high and dangers like predation absent, the physiological patterns controlling behavior remain somewhat the same. Fish need energy to maintain health, to move and forage, to support somatic growth and, after puberty, invest in reproduction, as well as for surviving periods of low food availability (Jönsson, 2013; Rønnestad *et al.*, 2017). The center of appetite control is in the brain. The main part of this activity occurs in the hypothalamus, however other brain regions might also be involved (Volkoff, 2016; Rønnestad *et al.*, 2017; Norland *et al.*, 2023). This central control system receives information from the body regarding energy status, blood nutrient levels and gut content. The signaling mechanisms delivering this information to the central control are central nutrient sensing systems, vagal afferents monitoring gut filling as well as endocrine signals in the form of gut peptides from the Gastrointestinal (GI) tract (Rønnestad *et al.*, 2017).

Endocrine signals from the GI-tract are major modulators of appetite, with different peptide hormones being secreted and communicating with the central control system. One of the key GI-tract hormones believed to be involved in teleost appetite control is ghrelin (Rønnestad *et al.*, 2017). In mammals, ghrelin is the only known hormone with an orexigenic effect from the peripheral system. Ghrelin is believed to be involved in several physiological processes related to metabolism, physical activity and, feed intake (Higgins *et al.*, 2009; Jönsson, 2013). In the

GI-tract, the anorexigenic peptide hormones Cck (Cholecystokinin) and Pyy (Peptide YY) are also present in Atlantic halibut. These hormones has shown to respond to meal ingestion in several teleost species (Rønnestad *et al.*, 2017), among these Atlantic halibut larvae (Gomes *et al.*, 2015, 2022), suggesting involvement in appetite/digestion control. This does however not necessarily mean that the appetite controlling functions are alike for the adult life stage, as it is expected to find some differences in these physiological processes through an individual's life stage development (Rønnestad *et al.*, 2017). In the appetite central control system, Npy (Neuropeptide Y), Pomc-c (proopiomelanocortin) and Cart (cocaine-amphetamine-regulated transcript) have been identified in Atlantic halibut at larval stages (Gomes *et al.*, 2015). These neuropeptides are important in teleost appetite control, and are secreted based on peripheral cues, promoting or inhibiting feed intake (Rønnestad *et al.*, 2017).

1.3.1 Gut transit

Gut content and digestive status are important factors in the appetite control. The gut transit, especially regarding stomach filling and evacuation of stomach content has long been a focus for research to understand digestion, but also feed intake (Grove *et al.*, 1978). Gut transit for adult Atlantic halibut (454-2334 g) was reported in Davenport *et al.*, (1990), by use of a colored marker (chromic oxide) in the feed. The time from ingestion to the marker was first observed in the feces was 24—33 h. The total gut clearance time, meaning when the last sign of the marker was evacuated, was 120 h after feeding. The gut filling and its relation to appetite around meal ingestion has been studied and shown to correlate for many teleost species, such as rainbow trout (Grove *et al.*, 1978), sockeye salmon (Brett, 2011), Nile tilapia (Azaza and Dhraief, 2020), turbot (Grove *et al.*, 1985) and common dab (Gwyther and Grove, 1981). There is described clear relationships between this stomach filling and the return of appetite after feeding. For rainbow trout, after ingesting a meal, appetite has shown to steadily return as stomach content is transferred to midgut, and is near maximized at a 80-90% stomach emptying rate (Grove *et al.*, 1978). Whether or not this relation can be translated to halibut is not known.

1.3.2 External environmental impact on appetite

External cues and factors play an important role in the control of feed intake, both by environmental factors directly affecting the fish and by sensory perceptions. Atlantic halibut, like most fish are ectothermic. Thus, environmental temperature directly affects the rates of

physiological processes such as the standard metabolic rate, digestion and gut transit time (Sandblom *et al.*, 2014; Volkoff and Rønnestad, 2020). Higher temperatures will in general speed up these physiologic processes. Thus, moderate increases in temperature usually result in increased feed intake, while elevated temperature outside of the fishes' optimal range will cause discomfort and decrease feed intake (Volkoff and Rønnestad, 2020). There is a strong relationship between stress and disruption of feeding behavior in fish, thus, feeding behavior can be used as an indicator of stress. Other potential environmental stressors include poor or fluctuating water quality or oxygen saturation. Stress can impact multiple aspects of feeding behavior in aquaculture, such as appetite, search for feed, detection and ingestion of feed (Beitinger, 1990).

During feed intake the sensory perceptions come to play regarding identification and location of food. A combination of senses is involved in these processes, including vision, olfaction, gustation, touch and by using the lateral line sensory system. Halibut has been described as a visual predator (Evans, 1937). Thus, visibility of the feed in the aquaculture environment is of importance. Olfaction also plays a major part in detection of feed. The fish detect small molecules (attractants) from the feed diffusing in water, identifying, and locating the available food. Attractants can be added to feed to make it more attractive for the halibut. Some are already present in the feed used in fish farming. An example of this is specific free amino acids (FAA) that has been shown to induce feed intake in teleost's (Kolkovski *et al.*, 1997; Jafari Shamushaki *et al.*, 2007). This suggests that protein levels have positive effects on appetite and feed intake. Chemical stimuli can also be perceived through gustation, which will affect the fishes' response to the food. Through direct contact and water movement fish can also detect and locate food using touch and the lateral line sensory system.

1.4 Feeding behavior

At the adult stage and in natural conditions, halibut are "sit-and-wait" predators mostly located at the sea bottom at varying depths (Gibson, 2007). Its feeding strategy relies on camouflage, using its flat body shape and the upper pigmented dark colored side and waiting at the bottom until a potential prey appears. In the ocean the access to prey varies, making halibut a solitary, opportunistic feeder (Best and St-Pierre Seattle, 1986), that is also capable of waiting long periods between each instance of feed intake. In aquaculture systems the surrounding

environmental conditions change drastically, there is a high fish density, and an almost constant and abundant availability of food. In aquaculture halibut display shoaling behavior (Kristiansen *et al.*, 2004). The fish form clusters on the floor of the rearing units, and feed intake take place either on the bottom, or through swimming movements and feed intake up in the water column. The halibut uses its large mouth and protrusion of the jaws to create a suction effect, often combined with a lunge, to capture prey into its mouth (Gibson, 2007).

Social interactions also play a part in the behavioral patterns around feeding. Large in-tank fish size differences are often seen in aquaculture. With these differences follows some sort of feeding hierarchy that will further expand the differences in growth (Davenport *et al.*, 1990). These hierarchal interactions have shown to play a part in the feed intake regulation and feeding behavior to a varying level based on the significance of in-tank size differences (Beitinger, 1990). Also higher fish densities is known to result in an elevated swimming activity in the tank (Kristiansen *et al.*, 2004), using more energy for locomotion, and therefore less feed energy is allocated towards growth.

1.4.1 Anticipatory behavior

Fish feeding behavior follows rhythmic patterns. The reasons for this are many, and stem from natural instincts that have evolved in relation to what feeding strategies are ideal for the survival of the species (López-Olmeda *et al.*, 2012). In an aquaculture environment where feeding regimes are quite rigid and standardized, the fish has cues that they use to adapt and learn when to expect feed. This involves the internal clock and light conditions (Houlhihan, 2012). It has been shown that halibut has significant learning capabilities, associating different light signal cues with feeding, and as a result showing an elevated feed response (Nilsson *et al.*, 2010). The halibut's response differed from what's seen in other fish species, where halibut fish seemed to prepare for its signature "sit-and-wait" feeding behavior ahead of scheduled meals.

1.5 Growth efficiency

Measuring the performance of feed for aquaculture, as mentioned earlier, growth is the indicator of success. More specifically, growth in relation to feed eaten. Usually, this is measured through Feed Conversion Ratio (FCR) or Feed conversion Efficiency (FCSE). FCR is calculated by dividing feed intake with the total weight gain, giving a ratio of feed needed

per unit of growth. FCSE is calculated by dividing total weight gain with the feed intake. Feed conversion is affected by many factors, with one of the main being waste of feed, something that is a problem in halibut farming as the interest for the feed is low. Further, after pellet intake takes place, through varying rate of digestive processes and nutrient uptake, the nutritional and digestive properties of the feed is of importance for feed conversion.

1.6 Aims

This master work is part of a feeding trial that aimed to map the nutritional needs of Atlantic halibut done by the Institute of Marine Research. The main objective of this thesis is to characterize the feeding behavior and appetite response of Atlantic halibut to different diets that vary in macronutrient compositions, comparing this with the growth performance recorded throughout the trial period. Appetite was monitored through video and day-to-day observations, along with analyses of gut compartmental filling at specific sampling points. The primary objective is to determine the essential factors that influence appetite regulation in regards to the macronutrient content of the feed, ensuring optimal growth and health conditions for the fish. There was made 12 diets varying in macronutrient composition with gradients of protein, lipid and carbohydrates to investigate the effects on the fish. Further, the thesis aims to examine the behavioral characteristics surrounding feeding and its influence on feed intake. Consequently, aims can be summarized as follows:

Objective 1: Map **appetite** response to **diets** with different macronutrient content:
$H0_1$: Appetite is not affected by the macronutrient level of the diets
$H1_1$: Appetite is affected by the macronutrient level of the diets

Objective 2: Map **feeding behavior** for the different macronutrient diets.
$H0_2$: Feeding behavior is not affected by the macronutrient level of the diets
$H1_2$: Feeding behavior is affected by the macronutrient level of the diets

Objective 3: Determine correlation between **feed intake** and **growth**.
$H0_3$: The relationship between feed intake and growth does not vary for different macronutrient diet compositions.
$H1_3$: The relationship between feed intake and growth varies from diet to diet

Objective 4: Investigate which diet performed best in the **growth** trial.

$H0_4$: There will be no difference in growth performance based on dietary protein, lipid, and carbohydrate levels

$H0_4$: There will be measurable difference in growth performance based on dietary protein, lipid, and carbohydrate levels

Objective 5: Investigate the role of filling of the **GI-tract** on appetite.

$H0_5$: There is no particular relationship between stomach filling and appetite for Atlantic halibut.

$H0_5$: There is a clear relationship between stomach filling and appetite for Atlantic halibut, similar to what is previously described for Rainbow trout.

2 Material and methods

2.1 Ethics statement

The animal handling and procedures described in this thesis was approved by the National Animal Research Authority in Norway (FOTS ID 23999).

2.2 Fish and rearing facility

Atlantic halibut females, 317.7 ± 80.5 g, were provided from Sterling White Halibut AS. Rørvik and Imsland facilities, to the Institute of Marine Research, Austevoll. The fish were fed a commercial diet before the start of the trial. At arrival, the fish were randomly distributed into 15 tanks (120 fish per tank) with a diameter of 2.5 meters, a total depth of 1.2 m and 0.6 m of water level. The bottom of the tanks was slightly inclined towards the drainage at the bottom to improve the removal of waste from the tanks.

The tanks were organized in six rows, each row consisting of three tanks (**Figure 2**). Water entered the tank from an inlet by the tank wall, facing the clockwise direction, thereby resulting in a clockwise water flow. The waterflow was initially set to 3000 L/h for each of the tanks, from the start of the trial the 20th of October 2021, before being adjusted to 4000 L/h the 29th of April. This flow rate remained constant until the end of the trial the 9th of November. The fish were reared under artificial light mimicking the natural light regime at the location (60.088 N).

Each tank was fed a specific experimental diet from the start of the trial. The feeding schedule throughout the project timeline was adjusted like shown in **Table 1,** in relation to the light regime. Thus, feeding started shortly after light was turned on and continued throughout the day, ending before the light was turned off. The feed was administered by automatic feeders close to the water outlet dispensing fixed doses of pellets at specific time intervals evenly spread over the active feeding hours. The doses were calibrated and timed to supply a specific amount of feed (g) daily for each of the tanks, adjusted throughout the trial timeline based on

the biomass in each tank. Details regarding the feeding schedule and regime are shown in **Table 1** in the Appendix.

In addition to the automatic feeding, operators at the research station did daily rounds of manual feeding to ensure that the halibut were fed to apparent satiation. The feeder's assessment of appetite in each tank was scored during this operation (See section 2.6). Other daily routines included measuring oxygen levels and temperature and flushing the tanks. Some light scrubbing and washing of the tanks were also done on a regular basis.

Table 1: The feeding schedule throughout the trial timelime.

Period	Start of feeding	End of feeding
1st of October 2021 to 4th of April 2022	09:00	18:00
4th of April 2022 to 24th of October 2022	08:00	18:00
24th of October 2022 to 10th of November 2022	09:00	17:30

2.3 Dietary composition

A total of 12 different diets were prepared for the trial. The composition of the 12 diets was based on a mixed gradient design, further described in the statistics section, containing gradients of the three macronutrients (**Figure 1**). Briefly, protein ranged from 45 to 77 %, lipid from 5 to 30 % and carbohydrates from 5 to 25 %. The exact diet compositions are presented in **Table 2**. The compositions were made using varying amounts of fish meal, wheat gluten, wheat, tapioca starch, fish oils and marine lecithin.

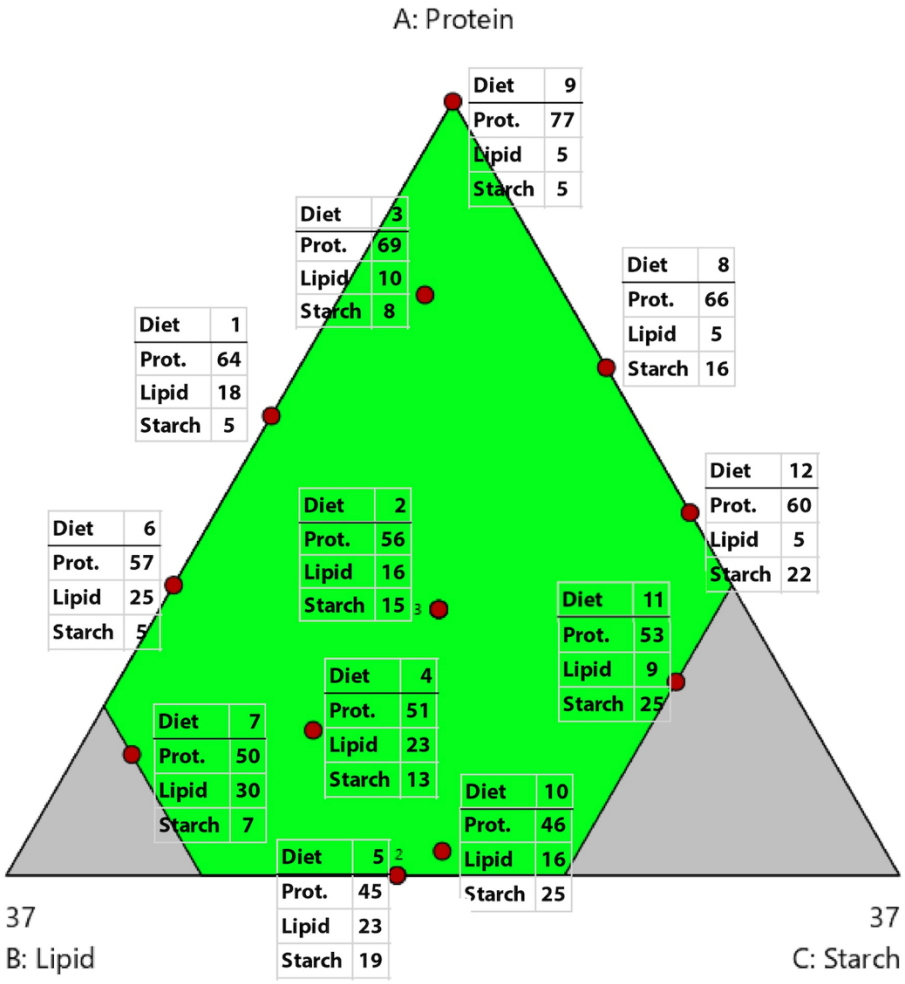


Figure 1: The setup for the 12 experimental diets and their compositions shown in the macronutrient gradient triangle

The diets were prepared and produced by NOFIMA. Because of the varying compositions there were some variances in the production methods. Diets 1, 3, 8, 11 and 12 proved to be too hard for normal extruding and were therefore pelleted. This resulted in harder pellets, with a slightly differing shape for these diets.

Table 2: Dietary composition (% of pellet weight) and respective macronutrient energy (kJ/g).

Diet	Protein (%)	Lipid (%)	Carbohydrate (%)	Energy (kJ/g)	Diet name
1	64	18	5	1839	P60L20C05
2	56	16	15	1799	P60L20C20
3	69	10	8	1679	P70L10C10
4	51	23	13	1939	P50L20C10
5	45	23	19	1939	P50L20C20
6	57	25	5	1979	P60L30C05
7	50	30	7	2079	P50L30C05
8	66	5	16	1579	P70L05C20
9	77	5	5	1579	P80L05C05
10	46	16	25	1799	P50L20C30
11	53	9	25	1659	P50L10C30
12	60	5	16	1477	P60L05C20

Due to the different production processes and the varying physiochemical properties of the ingredients there were also significant size variations between diets. The pellets had a varying degree of powdering, caused by the different production methods used. For each diet, 50 pellets were individually weighed giving us an average mass with standard deviation (**Table 3**).

Furthermore, due to production challenges, Diet 12 had to be tweaked during the project, resulting in a new feed with a different size. The new diet variation was introduced to the fish on the 7th of July. The diet composition remained the same.

Table 3: Pellet mass for each of the diets. Diet 12* is the revised formulation and production of Diet 12 that was used from seventh of July until the end of the trial

Diet	Pellet weight (g)	Standard deviation	Composition name
1	0.22	0.07	P60L20C05
2	0.61	0.05	P60L20C20
3	0.26	0.10	P70L10C10
4	0.41	0.09	P50L20C10
5	0.53	0.06	P50L20C20
6	0.36	0.13	P60L30C05
7	0.54	0.02	P50L30C05
8	0.31	0.13	P70L05C20
9	0.44	0.06	P80L05C05
10	0.70	0.06	P50L20C30
11	0.75	0.10	P50L10C30
12	0.68	0.07	P60L05C20
12*	0.19	0.07	P60L05C20

2.3.1 Macronutrient content groups

Further, to manage macronutrient levels categorically, diets were separated into high/mid/low for protein, lipid and carbohydrate levels, respectively. Grouping was done based on the macronutrient content, dividing the gradient range for each macronutrient in three equal parts. For protein the groups are as follows; low from 45 to 53%, mid from 56 to 60% and high from 66 to 77%. For lipid; low from 5 to 10%, mid from 16 to 18% and high from 23 to 30%. For carbohydrates: low from 5 to 8%, mid from 13 to 16% and high from 19 to 25%. This macronutrient grouping is presented in **Table 4**.

Table 4: Overview of the diets categorized into macronutrient level groups.

Diet	Protein group	Lipid group	Carbohydrate group	Composition name
8	high	low	mid	P70L05C20
3	high	low	low	P70L10C10
9	high	low	low	P80L05C05
6	mid	high	low	P60L30C05
2	mid	mid	mid	P60L20C20
1	mid	mid	low	P60L20C05
12	mid	low	high	P60L05C20
5	low	high	high	P50L20C20
4	low	high	mid	P50L20C10
7	low	high	low	P50L30C05
10	low	mid	high	P50L20C30
11	low	low	high	P50L10C30

2.3.2 Environmental data

Temperature and oxygen were measured regularly throughout the trial period, using a YSI Pro20 Dissolved Oxygen Meter. This was done every other day, at a varying hour. However, at some point during the trial there were some issues with the data managing systems at IMR, resulting in loss of data and an incomplete dataset. The missing data was mainly the first half of 2022. Remaining data for oxygen levels and temperature is presented in the results section. The water used was collected from 160 m (termed raw, unheated water) with a quite constant temperature (Yvonne Rong, IMR, pers. comm., 2022).

2.4 Experimental design and sampling

The 60 week-long trial was conducted in a land-based flowthrough facility. Here the fish were distributed in 15 tanks, as seen in **Figure 2**. Different diets were randomly assigned to the tanks, resulting in the setup shown in **Table 5**. Throughout the trial timeline the fish was

weighed and measured five times, and two samplings took place at the mid-way point (Week 31) and at the end of the trial (Week 60).

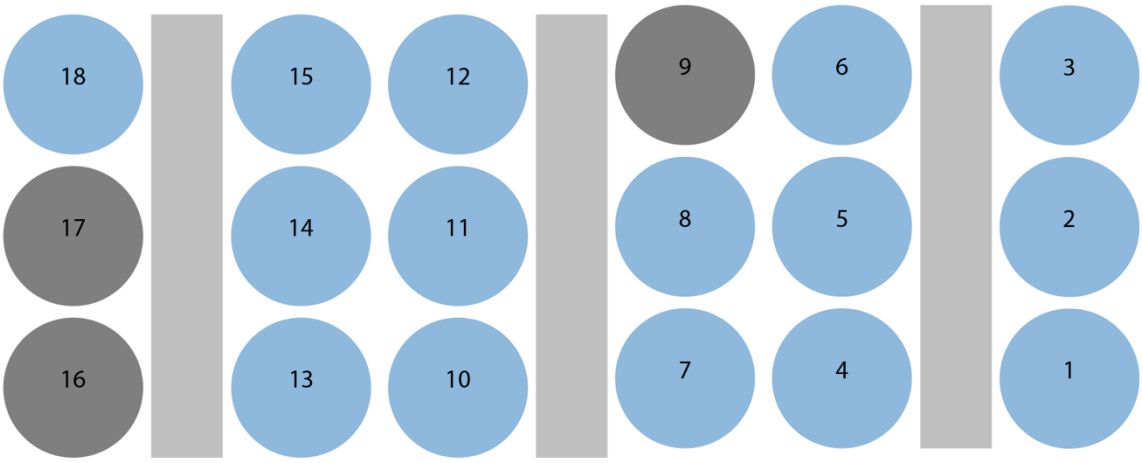


Figure 2: The tank setup in the facility, separated by footbridges. Grey tanks were involved in a separate, parallel trial.

Table 5: Allocation of different diets to the 15 tanks.

Diet	Tank number
1	15
2	2, 5, 7
3	12
4	4
5	6, 18
6	8
7	10
8	11
9	3
10	13
11	14
12	1

2.4.1 Biometric data

At trial start the fish were PIT-tagged to enable tracking of fish individually through the trial. Fish weight and length were measured a total of five times throughout the trial period: when

fish arrived at the station and then at 20, 31, 46 and 60 weeks from start of the trial. Length was measured to the nearest half-centimeter and weight to the nearest gram.

Two samplings were performed, one at the midway-point (31 weeks into the trial) and one at the end of trial (week 60). Sampling was done from 9:00 until 15:00, with the automatic feeders running as usual. For the first sampling, six fish from each tank were sampled, while for the second sampling eight fish from each tank. Fish were killed by an overdose of MS-222. Multiple samples were taken from each fish, of which used in this thesis were sampled GI-tract content and liver weight.

2.4.2 GI-tract content

The GI-tract content samples were taken to assess to what extent presence of feed and digesta in different GI-tract sections affected feed intake. The abdominal cavity of the halibut was opened, and by use of clamps the GI-tract was separated as shown in **Figure 3** and taken out. The gut content was then collected from the stomach, midgut and hindgut and stored in a -20ºC freezer. Gut content samples from the different gastrointestinal sections were later thawed and wet weight was measured using a TOLEDO MS6002TS scale in a laboratory at IMR. Thereafter the samples were freeze-dried, before dry weight was measured. Freeze-drying was done in a LABCONCO FreeZone freeze dryer.

ST = Stomach
MG = Midgut including Pyloric Caeca
HG = Hindgut

Figure 3: The GI-tract of Atlantic halibut. Named segments are separated by clamps at the marked locations. (Lygre, E. (2022). [Picture of dissected Atlantic halibut GI-tract], Unpublished)

2.5 Data collection

2.5.1 Feeding behavior - Video analysis

Video analysis was used to capture feeding behavior and assess appetite on four occasions throughout the 60-month trial period. This was always done the week before scheduled weighing, measuring and samplings, securing that the fish behavior remained undisturbed by the handling related to sampling. In each time point, two video recordings of 25 minutes each were done around feeding, capturing ten minutes before feeding started and the first fifteen minutes of feeding. To get this synchronized to the start of feeding, the feeding start was overruled manually and started 10 minutes into the recording.

Video recordings were done in a randomized order over a period of 3 to 4 days with up to six videos filmed each day. When filming two rounds of video recordings per day there was a slight offset timewise in feeding start from the normal feeding schedule. This usually resulted in a time offset of less than 30 minutes.

2.5.1.1 Camera setup

A camera rig was used to capture feeding behavior, feed ingestion and general tank activity from two angles. One submerged GoPro Hero 8 (marked B in **Figures 4 and 5**) was angled 40 degrees forward from the tank wall capturing the area where the majority of fish were located. From the top a GoPro Hero 10 action camera (marked A in **Figures 4 and 5**) was used to get an overhead view of the tank. The camera rig was positioned 70 cm left of the automatic feeder to capture the area of the tank where most of the pellets landed and most of the feed intake took place. Camera A monitored the activity within the tank. Camera setup is visualized in **Figure 4** and pictured in **Figure 5.**

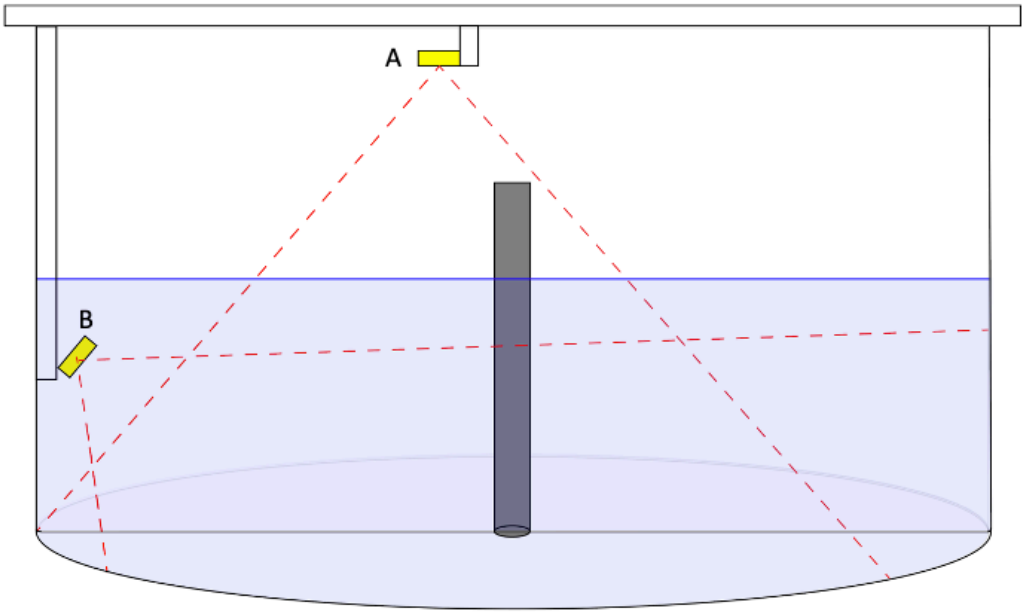


Figure 4: Visualization of the camera setup used for the video recording, Red stapled line indicating the field of view of the two cameras.

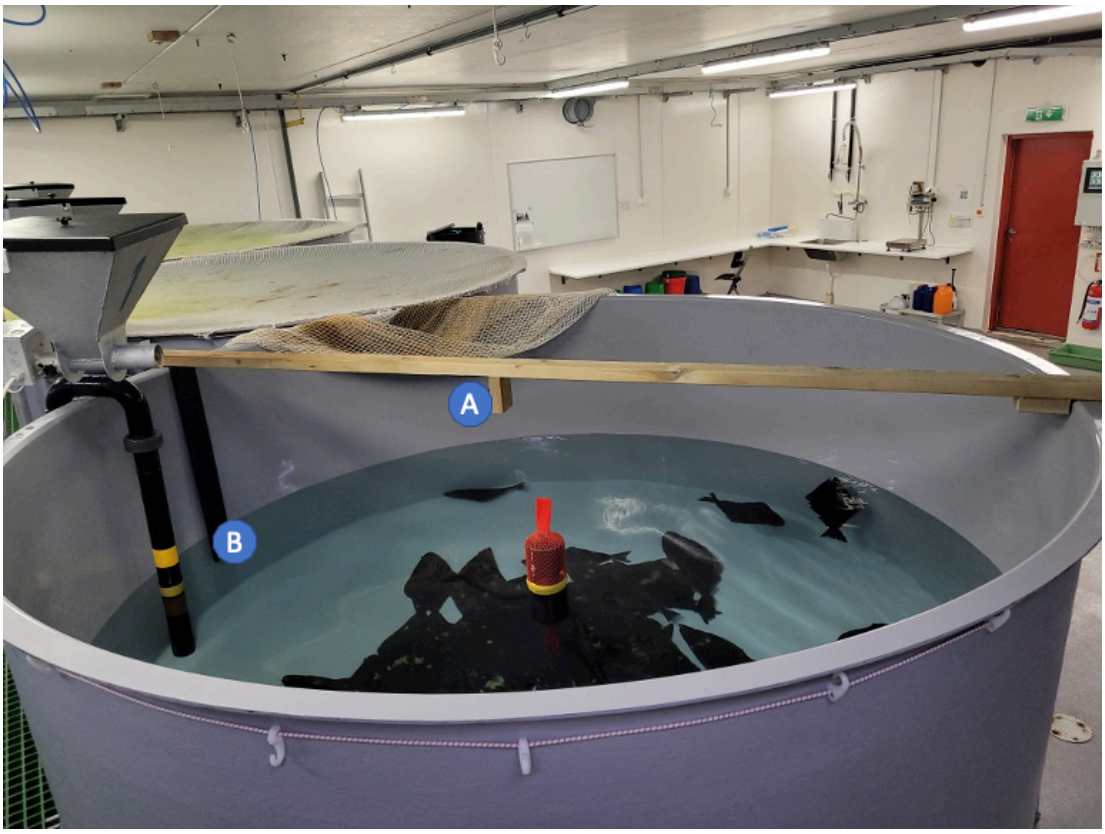


Figure 5: The camera rig pictured in a tank at the facility, with camera positions marked as A and B. Photo: Yvonne Rong, IMR.

2.5.1.2 Video editing and analyses

Videos were edited using the free video editing software ShotCut (Version 22.03.30, https://shotcut.org/). The videos from the two camera angles were synchronized and placed next to each other in a new video file (mp4) to maximize the observability of the activity and feed intake. The tanks were divided into four quarters like shown in **Figure 6** to help the quantification of activity.

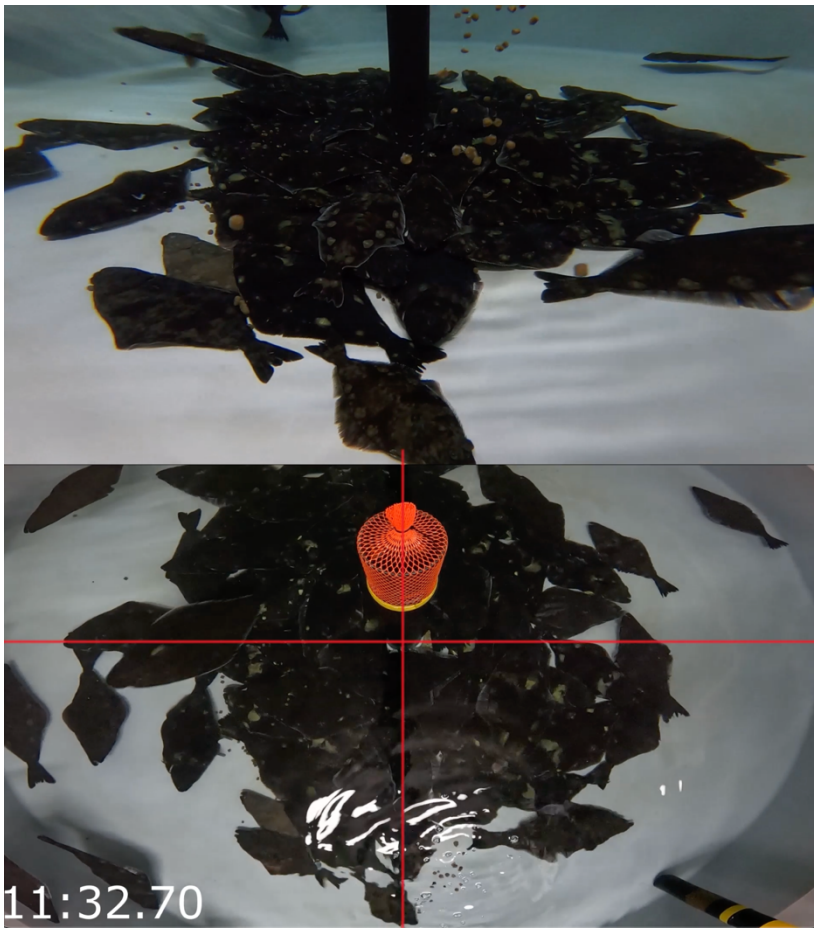


Figure 6: Screen capture of an edited video recording showing the pellet falling into frame where each instance of ingestion was observed.

2.5.1.2 Feed intake analysis

The quantification of feed intake was based on the 15 minutes of video recording from the time where feeding started. Analyses were done using the multimedia player QuickTime, (v. 10.5, Apple Inc, Cupertino, CA, USA). The videos were analyzed thoroughly to follow each instance of feed intake. This was possible since the number of pellets eaten was quite low. All pellets eaten were counted, and the time of feed intake was noted to the nearest ten seconds. The fish feed intake movement was classified into the following three categories:

Ingestion behavior 1 (IB1): when no, or close to no movement (displacement) of the fish was observed leading up to feed intake. Some small movements would usually occur as a result of hydrodynamics related to gaping and ingestion.

Ingestion behavior 2 (IB2): fish displacement was noticeable, but less than a body length, for catching the pellet. Often, IB2 was seen as a fish first positioning in proximity to a pellet, waiting for a second and then starting the eating movement.

Ingestion behavior 3 (IB3): fish displacement was more than one body length to ingest the pellet. This included swimming vertically in the water column, as well as long movements along the bottom, or over the cluster of fish usually seen in the tanks.

2.5.1.3 Quantification of feeding activity response

A system for quantifying the fish activity and behavior around morning feeding was created. The activity observed was based on movements greater than one body length. To capture activity development throughout the recordings, five increments of one minute each were selected, as shown in **Figure 7**. The first increment is from eight minutes to seven minutes before feeding starts. Next is two minutes before feeding, meant to capture a possible anticipatory behavioral response before feeding. After feeding is started the increments looked at are two minutes after, 5 minutes after and 10 minutes after the feeding started.

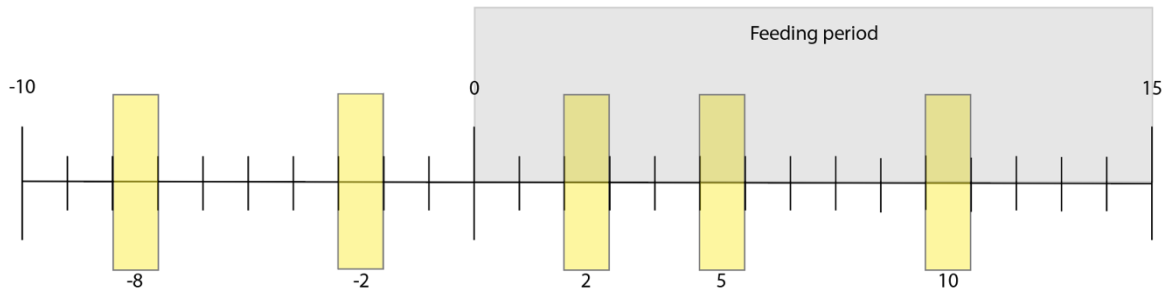


Figure 7: Video timeline showing the time intervals where activity analysis takes place. Time unit is minutes before/after feeding start.

2.6 Appetite scoring system

A complementary system to score appetite was based on assessment by the technician who tended the tanks during the whole trial. The appetite was assessed daily for each tank based on that the fish was hand-fed to satiation i.e., until the fish stopped ingesting pellets, which was monitored by visual observation, and the appetite was scored. This was done on a daily basis, starting 22 weeks into the trial (February 2022). Appetite scoring was done on a scale from zero to five, with a score of 0 indicating no appetite, and 5 indicating extreme and voracious appetite-response from the fish. Scores for each tank were registered daily and merged into a dataset mapping the development of appetite over time.

Fish were deprived from feed, thus not scored on days of weighing, sampling, or other days where the feeding was paused. 27th and 28th of February 2022 the fish were not fed because of a formalin treatment due to suspected, and since confirmed costia disease.

2.7 Calculations

Specific growth rate (SGR): was calculated individually per fish for the whole trial period as well as for periods in between weight measurements using the following equation:

$$\text{SGR} = \frac{(\ln(weight_{final}) - \ln(weight_{initial}))}{days} \cdot 100$$

Hepatosomatic index (HSI) was measured in relation to the samplings, and was calculated as shown here:

$$\text{HSI (\%)} = \frac{Liver\ weight}{Fish\ weigth} * 100\%$$

An **anticipatory index** was used to identify and quantify the change in fish activity leading up to start of feeding. The reference point is the minute of activity analyzed eight minutes before feeding, while the anticipation is expected to be observable at the time interval two minutes before feeding:

Anticipatory index = $\frac{Activity\ in\ increment\ 2\ (2\ \mathrm{min}\ before\ feeding)}{Activity\ in\ increment\ 1\ (8\ \mathrm{min}\ before\ feeding)}$

Post-feeding activity: The quantified **activity after feeding started** was summed to quantify and look at the behavioral response to feeding:

Post-feeding activity =

$$Activity\ in\ increment\ 3\ (2\ \mathrm{min}\ after\ feeding) + Activity\ in\ increment\ 4\ (5\ \mathrm{min}\ after\ feeding) + Activity\ in\ increment\ 5\ (10\ \mathrm{min}\ after\ feeding)$$

Mortality and following differences in **population** in the tanks made it necessary to **normalize** the data:

Population normalization = $\frac{data}{population} \cdot initial\ population\ (120)$

Feed ingested (g): Because of varying pellet size all data related to count of pellets eaten were calculated based on mass:

Feed ingested (g) = $pellet\ intake\ (count) \cdot pellet\ mass\ (g)$

Growth efficiency index: Since the appetite data is observational and actual feed intake is unknown, traditional growth efficiency calculations like FCR can't be done. Therefore, the appetite and growth data was used to create a growth efficiency index that can compare the diets in this project, both for the entirety of the project and for segments between each weighing. This calculation uses weight change and a collective normalized appetite index from video recordings of feed intake and appetite score. For the first period there was not done appetite score assessment, resulting in a GEI only based on feed intake.

Total normalized appetite index:

$$\frac{Average\ appetite\ score\ \boldsymbol{per\ tank}}{Max\ average\ appetite\ score\ for\ selected\ period} \cdot \frac{Feed\ intake\ \boldsymbol{per\ tank}}{Max\ average\ feed\ intake\ for\ selected\ period}$$

Weight change: $Weight_{final} - Weight_{initial}$

Growth efficiency index (GEI): $\frac{Weight\ change}{Total\ appetite\ index}$

2.8 Statistics

Data was collected and organized in Microsoft Excel (Version 16.69). Further data management, visualization and statistical analysis was done in RStudio (Version 1.4.1717) using the programming language R (Version 4.1.1) (R Core Team, 2021). Packages used were "tidyr" (Hadley Wickham, 2021), "stringr" (Wickham, 2019), "ggplot2" (Wickham, 2016), "cowplot" (Wilke, 2020), "hms" (Müller, 2021) and "lubridate" (Grolemund and Wickham, 2011), as well as the base packages in R.

The trial setup is based on a three component mixed gradient design (Cornell, 1990). This method uses systematically varied gradients of the three macronutrients continuously and evenly varying within set limits. Using this setup, calculations can be done to estimate the ideal macronutrient ranges. The statistical software Design-Expert was used to visualize models based on the mixed design experiment. This software fits the input data into the best fitting model, of which represented in this thesis are linear, quadratic and cubic models. Variation in data was in this thesis explored and tested using the Kruskal-Wallis test and one-way ANOVAs. The Kruskal-Wallis test was used to test variance for non-parametric data. Dunn's test was used to further present the interactions and differences. ANOVA was chosen for normal distributed data, and because of its ability to handle multiple groups and assessing the interaction and relative differences in the results. Pearson's correlation test was used to test the significance of linear correlation between continuous variables, while Spearman's correlation test was used for non-parametric data that didn't follow linear relationships.

3 Results

3.1 Mortality

Mortality for each of the tanks was monitored throughout the trial and are presented in **Table 3** and **Figure 1** in the Appendix. For most tanks the mortality was in the range of two to seven fish per tank. Exceptions were tank 4, fed Diet 4 (P50L2010), with a mortality of 10, and tank 1, fed Diet 12 (P60L05C20), with a mortality of 17. A major part of mortality was fish that were euthanized, and taken out of the trial, due to health-related issues, with the two most frequent being loss of eyes and severe damages to the gill covers. Additionally, almost all mortality was related to the smaller in-tank "losers", with dead fish averaging at a body weight of only 267 g.

3.2 Fish health statement

There were some problems surrounding the health of the fish, particularly showing towards the end of the trial. This mainly seemed to impact the gill health, resulting in malformation and open sores, that developed for a lot of the fish. Sterling White Halibut AS, the supplier of fish for the trial issued a statement regarding this condition based on tests they did on the fish:

It was not found any virus explaining the gill changes found on the fish in the trial. Costia and chlamydia is proven, which may contribute to changes in the gill structure. It remains unclear whether the changes seen on the gills originate from an agent or if the gill changes might have come as a consequence of handling, like for example vacuum pumping. Gill status might have impacted the single individual's ability to perform throughout the trial (Kjetil Solheim, pers. comm. 2023).

3.3 Environmental data

Mean temperature was 8.12 ± 0.25°C, and was incrementally lower in the later stages of the trial (**Figure 8**). In the periods where oxygen measurements were saved, data showed that shortly after trial start the oxygen saturation decreased and stabilized. The oxygen saturation (mean ± SD) was 87.00 ± 3.28%. Measurements are presented in **Figure 9.** Pearson's

correlation analyses show a small positive correlation between the oxygen saturation and temperature ($r = 0.200$, $p<0.001$).

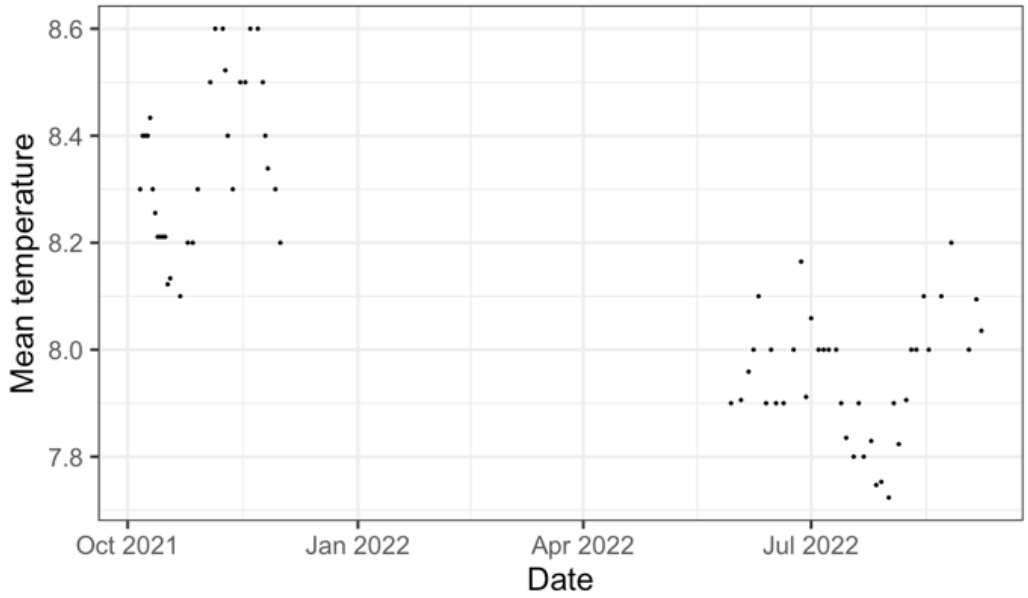


Figure 8 Mean water temperature for all experimental tanks in the facility through the trial period. Due to a technical error, data from December 1st to the 30th of May were lost.

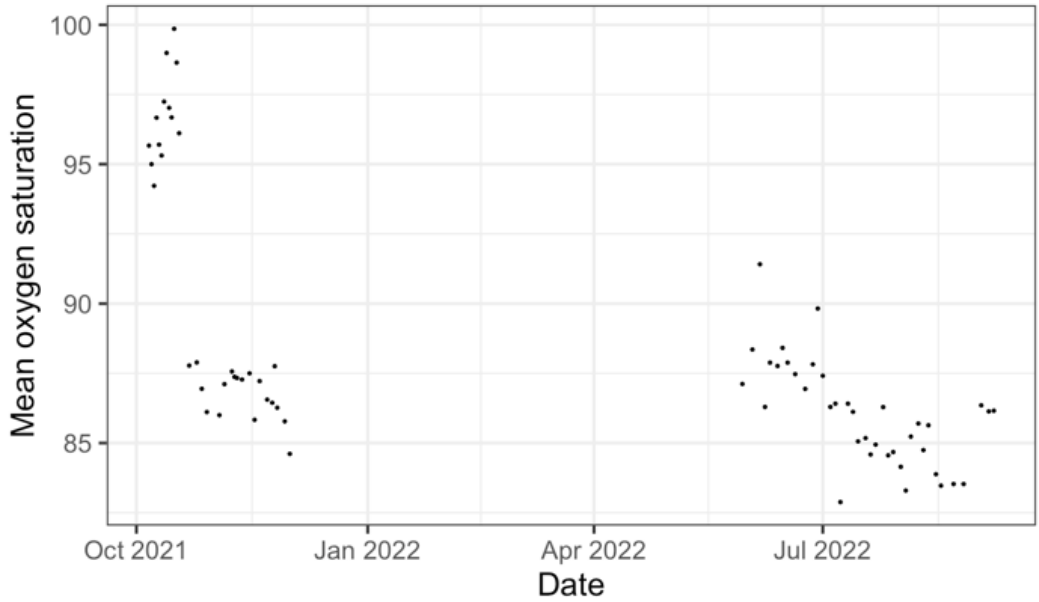


Figure 9: Mean oxygen saturation (%) for all experimental tanks in the facility through the trial period. Due to a technical error, data from December 1st to the 30th of May were lost.

3.4 Growth

The mean (± SD) starting weight was 316.0 ± 79.4 g, with no significant differences between the tanks at the trial start. At the mid-trial, there were some significant weight differences (**Table 6**) between the fish groups: Diet 6 (P60L30C5) was at this point the biggest fish, being the only group of fish that grew to a mean weight over 600 g. On the other end of the scale, Diet 7 (P50L30C5) had less than 150 g mean weight increase and, thus, a significant negative effect on the fish growth in relation to diets 2 (P60L20C20), 4 (P50L20C10), 5 (P50L20C20), 6 (P60L30C05) and 10 (P50L20C30) in the mid-trial weight, (Kruskal-Wallis, $p<0.01$) (**Table 6**). This lack of growth of Diet 7 (P50L30C05) was so pronounced that this group had to be euthanized due to emaciation.

On week 46, Diet 6 (P60L30C5) still had a relatively high mean weight, significantly higher than diets 9 (P80L05P05) and 3 (P70L10C10) (Kruskal-Wallis, $p<0.05$) (**Table 6**). Additionally, growth for fish fed Diet 3 (P70L10C10) deviated from the others, with a significant negative effect on weight in relation to diets 2 (P60L20C20), 4 (P50L20C10), 5 (P50L20C20), 6 (P60L30C05), 10 (P50L20C30) and 11 (P50L10C30) (Kruskal-Wallis, $p<0.5$) (**Table 6**).

At the end of the trial, these two diets, 6 (P60L30C5) and 3 (P70L10C10), deviated most from the mean weight. Diet 3 (P70L10C10) deviated negatively in relation to the four highest weight diets; 2 (P60L20C20), 6 (P60L30C05), 10 (P50L20C30) and 11 (P50L10C30) (Kruskal-Wallis, $p<0.01$) reaching a weight of only 676 g at end of trial. Diet 6 (P60L30C5) gave highest weight, although without significant deviance, in total going from 322 to 948 g over the whole trial. **Figure 10** shows final weight presented in a cubic model. However, the statistical model output is not significant ($p>0.05$). These data however don't include the fish fed with the high lipid Diet 7 (P50L30C5), because as previously mentioned, these fish had to be slaughtered at mid-trial.

Table 6: Mean fish weight with standard deviation (SD) at the start, mid (week 31) and end of trial (week 60) weighing for the different diets.

	Body weight (g)			
Diet	Start	Mid	Final	Composition name
1	311.0 ± 91.7	529.1 ± 223.7	835.2 ± 492.4	P60L20C05
2	320.4 ± 77.2	560.6 ± 218.2	864.0 ± 411.0	P60L20C20
3	313.1 ± 80.4	524.9 ± 205.3	675.5 ± 327.7	P70L10C10
4	314.4 ± 78.5	557.0 ± 240.5	798.9 ± 385.9	P50L20C10
5	318.7 ± 79.4	537.6 ± 217.5	782.4 ± 374.3	P50L20C20
6	322.0 ± 83.5	608.4 ± 235.3	947.8 ± 455.9	P60L30C05
7	311.4 ± 80.9	451.8 ± 170.0	-*	P50L30C05
8	304.1 ± 77.4	522.2 ± 216.6	805.7 ± 424.6	P70L05C20
9	313.3 ± 69.1	564.5 ± 343.3	765.3 ± 362.0	P80L05C05
10	310.6 ± 77.8	553.3 ± 202.7	879.0 ± 390.6	P50L20C30
11	299.7 ± 73.7	530.9 ± 204.7	888.1 ± 382.1	P50L10C30
12	330.3 ± 83.2	517.1 ± 230.7	774.3 ± 343.0	P60L05C20

*: This fish was euthanized mid-trial because of bad fish welfare.

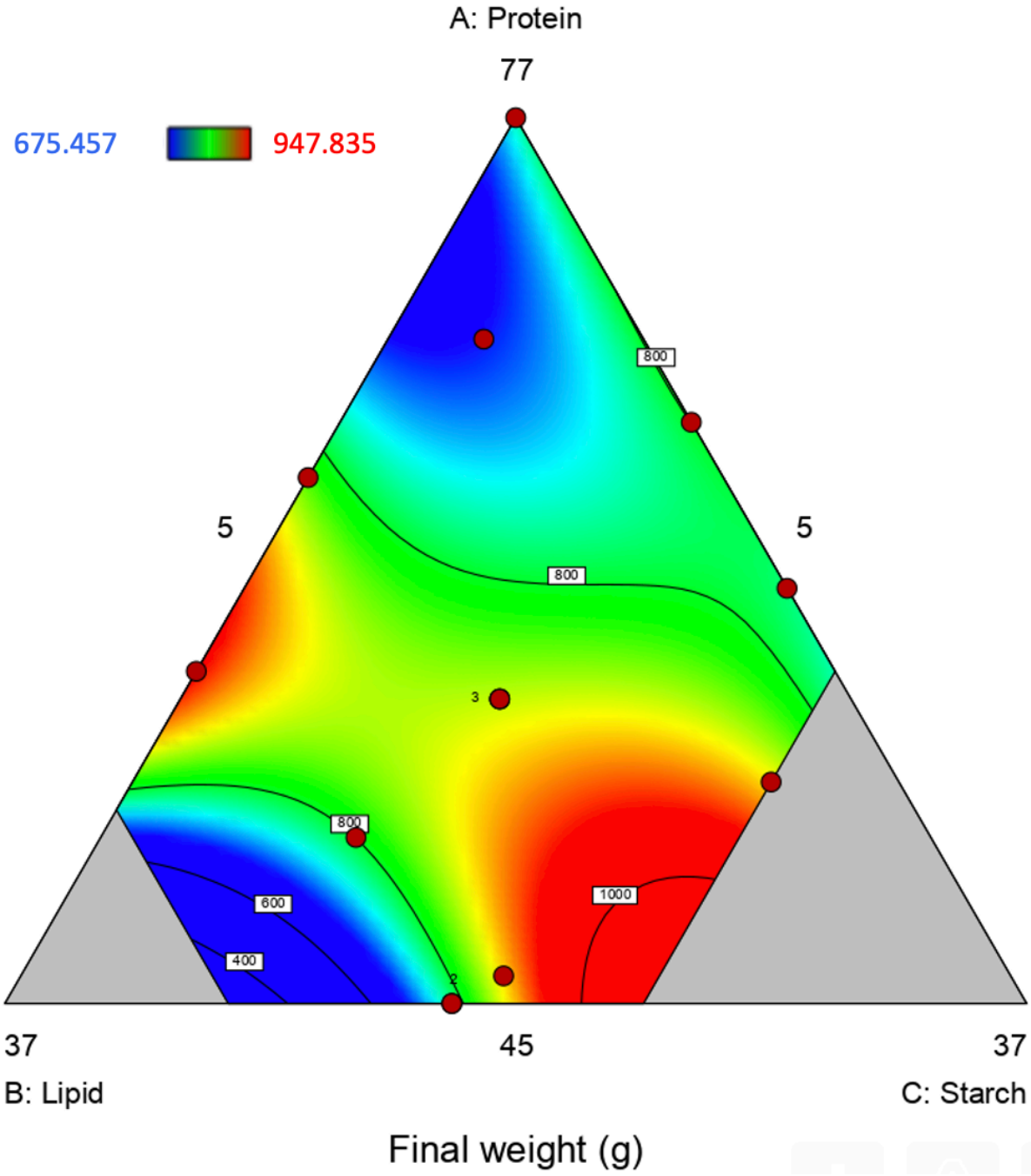


Figure 10: Cubic model for fish weight (g) at the end of trial related to gradients of the macronutrients presented in a contour plot over the triangular mixed design. Red dots show the distribution of the experimental diets. The color scale on the contour plot goes from lowest fish weight values at dark blue areas to highest values for red areas, values shown in the top left.

The weight change of fish related to the protein, lipid and carbohydrate content groups are shown in **Figures 11, 12 and 13**, respectively. Very few differences were seen in the low/high/mid groups of the three macronutrients at the first weighing, 20 weeks into the trial and at the mid-trial weighing in week 31.

In week 46 of the trial the low- and mid-protein groups both had notably better growth (87.9 and 114.1 g, respectively) than the high-protein group ($p<0.0001$ and $p<0001$, respectively). This trend was still present at final weighing ($p<0.01$). On the other hand, the low-lipid group weight was significantly lower, approximately 80 g, than the mid lipid diets both in week 46 and in week 60 (Kruskal-Wallis, respectively $p<0.0001$ and $p<0.05$), and the low lipid group was significantly lower than the high lipid group in week 46 (Kruskal-Wallis, $p<0.001$). For carbohydrate groups, significant decrease in growth was found for low carbohydrate groups in relation to the mid groups ($p<0.5$) in week 46, while no significant differences were found for weight in week 60.

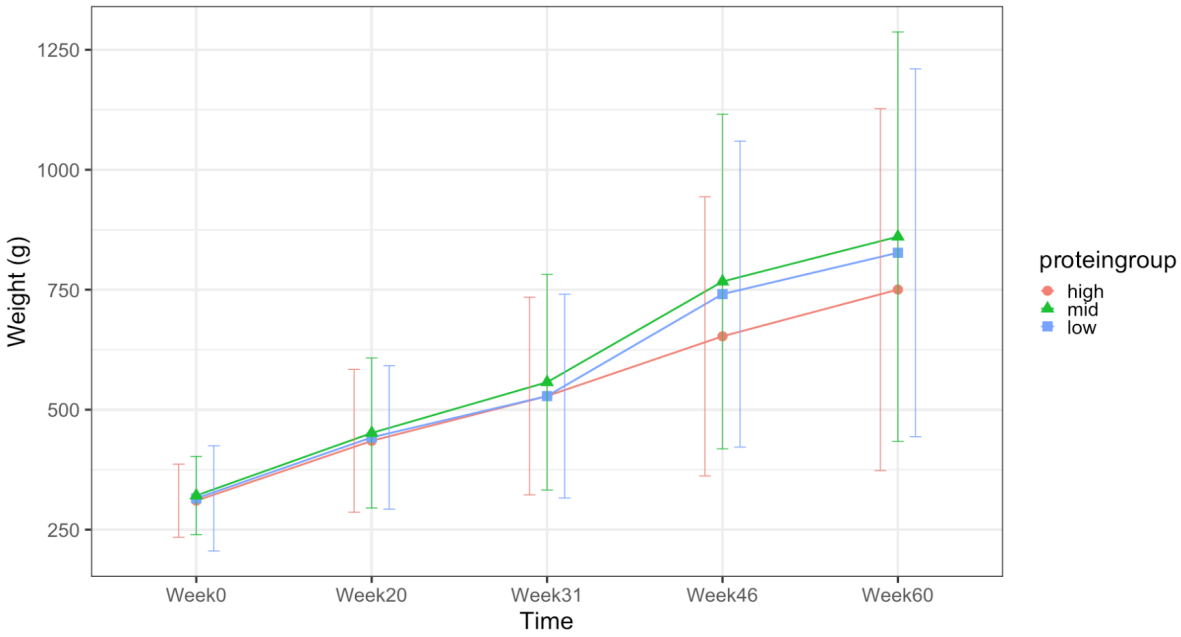


Figure 11: The change in mean weight (± SD) for the three protein groups through the trial period.

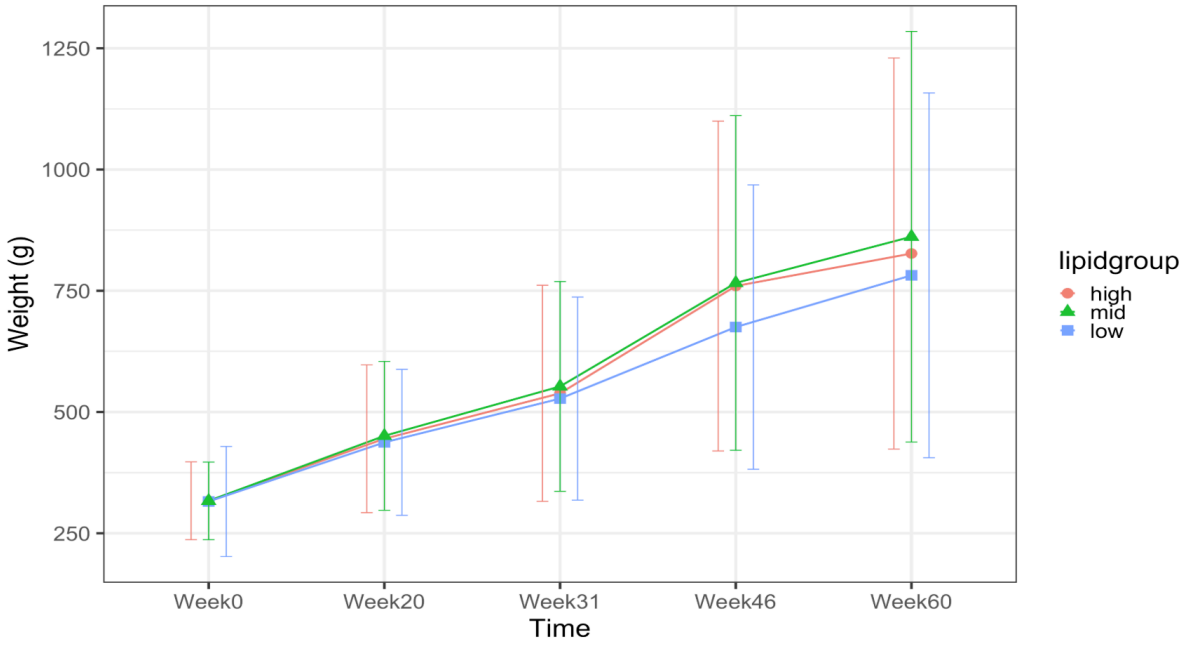


Figure 12: The change in mean weight (± SD) for the three lipid groups through the trial period

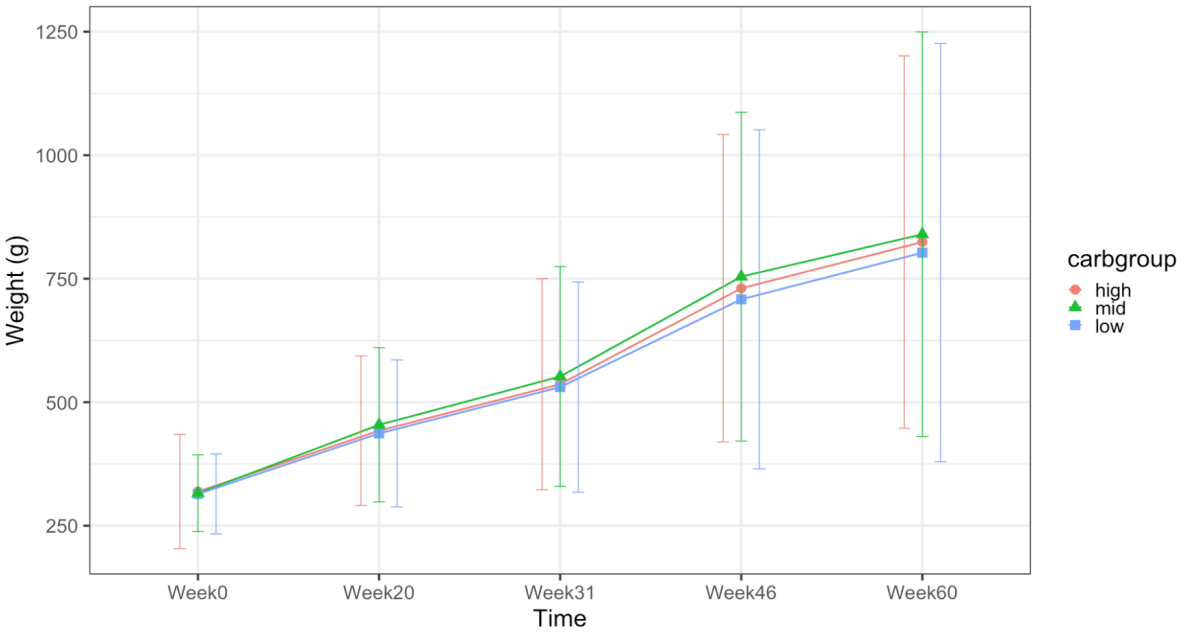


Figure 13: The change in mean weight (± SD) for the three carbohydrate groups through the trial period.

3.4.1 Hepatosomatic index (HSI)

Fish fed Diet 12 (P60L05C20) had significantly lower HSI in relation to the six diets with highest HSI (2, 3, 4, 5, 10 and 11) ($p<0.05$) (**Table 7**). Fish fed Diet 11 also had a significantly higher HSI than fish fed Diet 9 ($p<0.05$). Pearson´s correlation test showed a strong negative relation between protein content and HSI ($r = -0.298$, $p<0.001$), while both dietary lipid and

carbohydrates positively correlated with HSI ($r = 0.190$, $p<0.0001$, and $r = 0.198$, $p<0.0001$) HSI and weight also showed to weakly but significantly correlate ($r = 0.190$, $p<0.0001$). No significant relationship was found between HSI and feed intake nor appetite score.

Table 7: Mean hepatosomatic index (HSI ± SD) of fish fed the twelve diets. HSI data was collected at the mid and final sampling.

Diet	HSI (%)	Composition name
1	1.29 ± 0.42	P60L20C05
2	1.50 ± 0.41	P60L20C20
3	1.57 ± 0.39	P70L10C10
4	1.57 ± 0.43	P50L20C10
5	1.76 ± 0.57	P50L20C20
6	1.48 ± 0.27	P60L30C05
7	1.40 ± 0.17	P50L30C05
8	1.45 ± 0.35	P70L05C20
9	1.15 ± 0.25	P80L05C05
10	1.85 ± 1.12	P50L20C30
11	1.73 ± 0.62	P50L10C30
12	1.08 ± 0.30	P60L05C20

3.5 Appetite analysis data

Video analysis revealed that Diet 11 (P50L10C30) induced the highest feed intake in halibut (**Figure 14**). There was a significantly higher feed intake (20-30 g difference) for fish fed with Diet 11 in relation to three of the lowest feed intake diets (4 (P50L20C10), 5 (P50L20C20) and 6 P60L30C05)) ($p<0.05$). For the rest of the diets no significant differences were found.

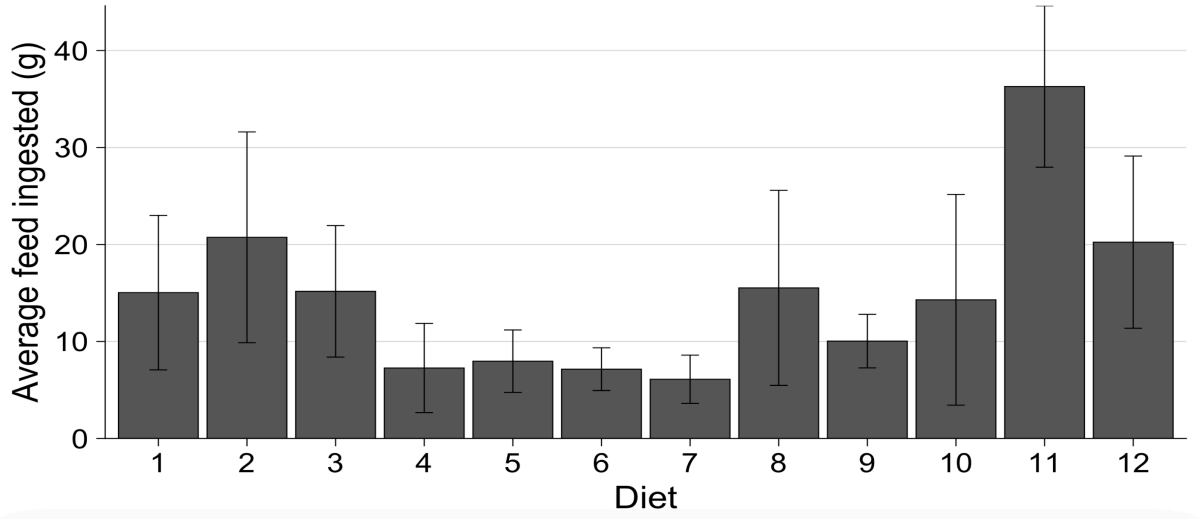


Figure 14: Mean feed intake (± SD) observed from the video recordings over the whole trial period for the twelve experimental diets.

The **Figure 15** shows a quadratic model for the mean feed intake in function of the macronutrient gradients. The peak in feed intake is observed at the highest carbohydrate content level. The low points are located towards highest lipid levels and highest protein levels. The model is significant ($p<0.01$).

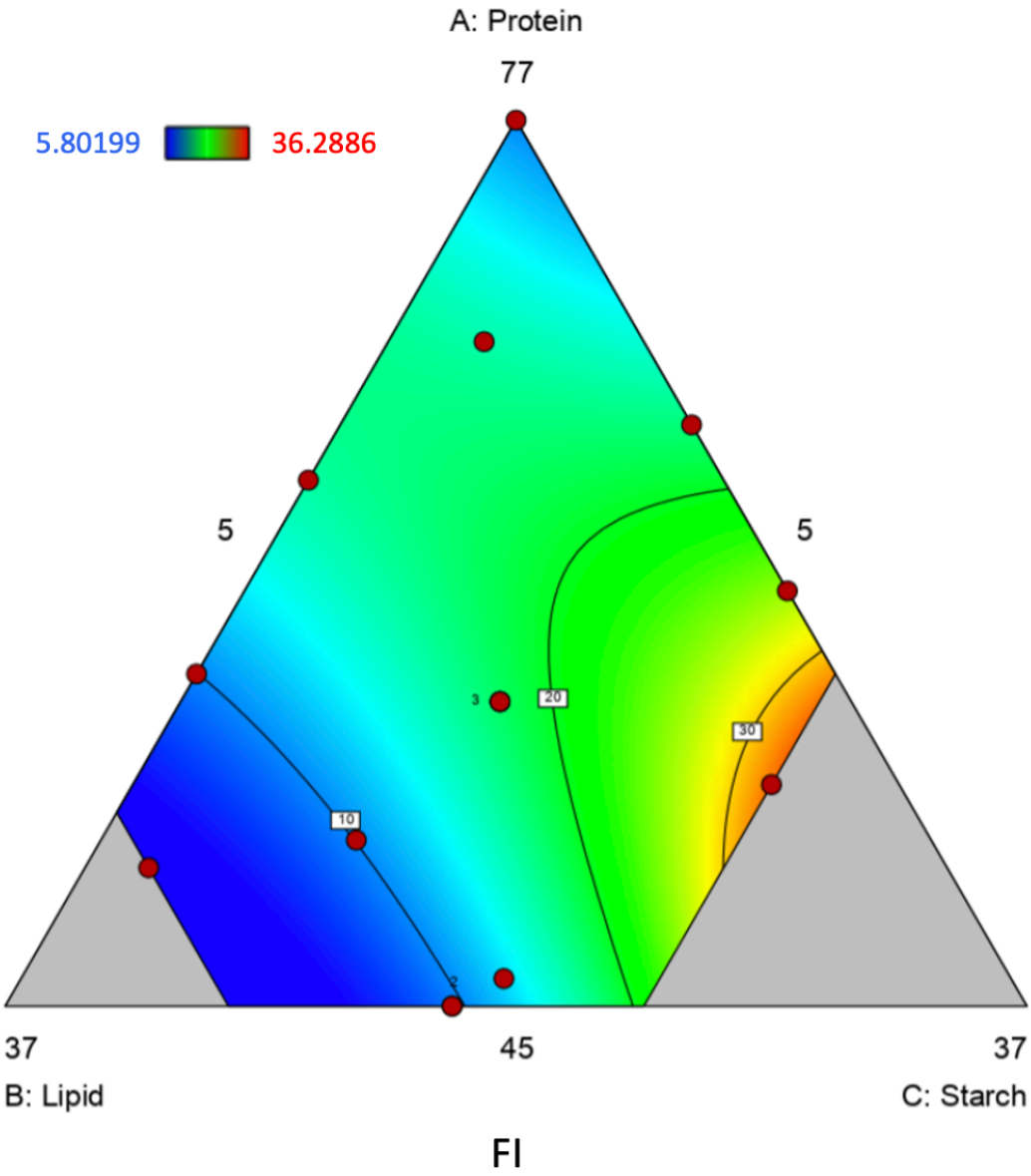


Figure 15: Quadratic modelling of feed intake (g) for gradients of macronutrients presented in a contour plot over the triangular mixed design. Red dots show the distribution of the experimental diets. The color scale on the contour plot goes from lowest feed intake values at dark blue areas to highest values for red areas, values shown in the top left.

Feed intake as a function of time since trial start did have a varying effect for the different tanks and diets (**Figure 16)**. In total over all diets, there was no significant increase in feed intake over the trial period, with even the difference from week 20 to week 60 being non-significant. The mean increase in feed intake was at only 7 g.

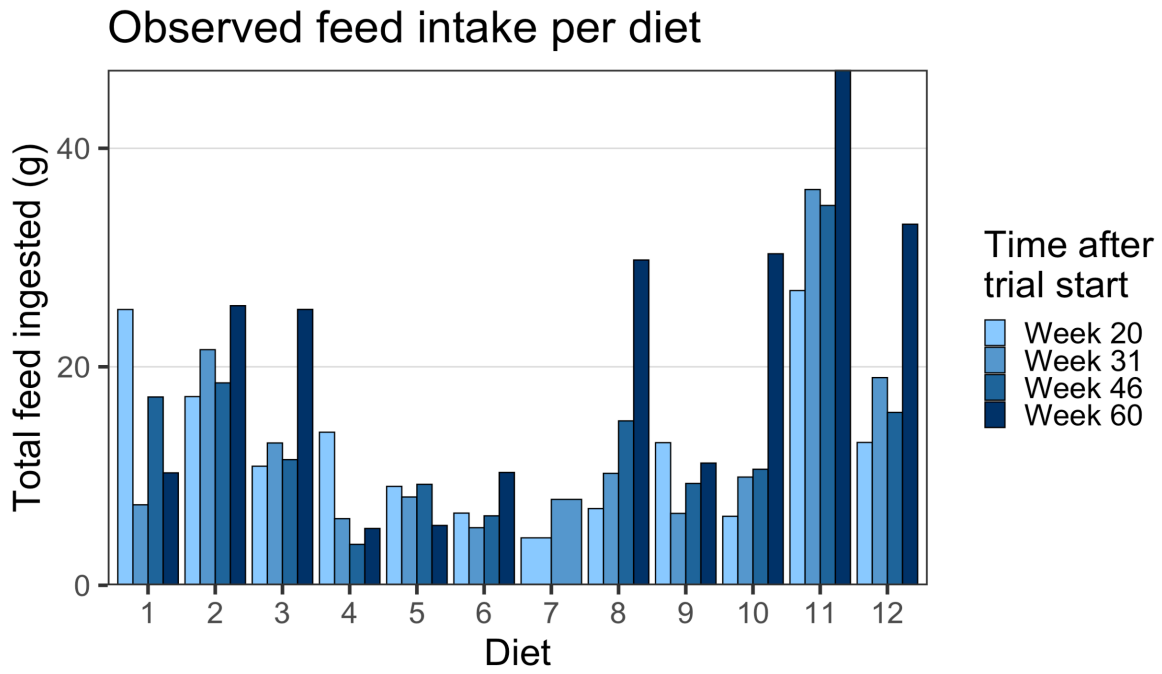


Figure 16: Feed intake per diet for each of the video recordings through the trial period.

The feed intake for the macronutrient groups is shown in **Figures 17, 18 and 19**. The diets with high protein levels perform worst for the first three periods (week 20, 31 and 40), before drastically improving in the last video recording (week 60). The tanks with low and mid protein content are quite even throughout the whole trial. No statistically significant results are seen.

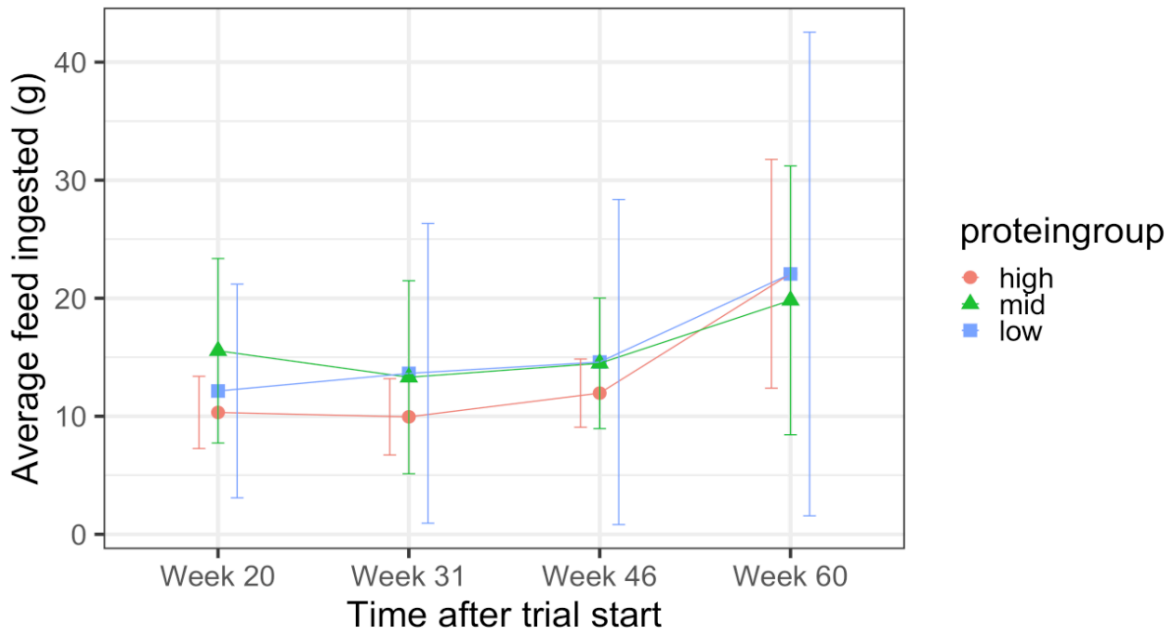


Figure 17: Mean feed intake (± SD) for the protein groups through the trial period.

The lipid level gives higher variations in feed intake, with the high lipid group showing a significantly lower appetite than the mid- and low-lipid diets (Kruskal-Wallis, $p<0.001$), resulting in a feed intake that is almost halved in relation to the other groups throughout the whole trial. Low lipid diets seem to be performing best, stimulating a higher intake.

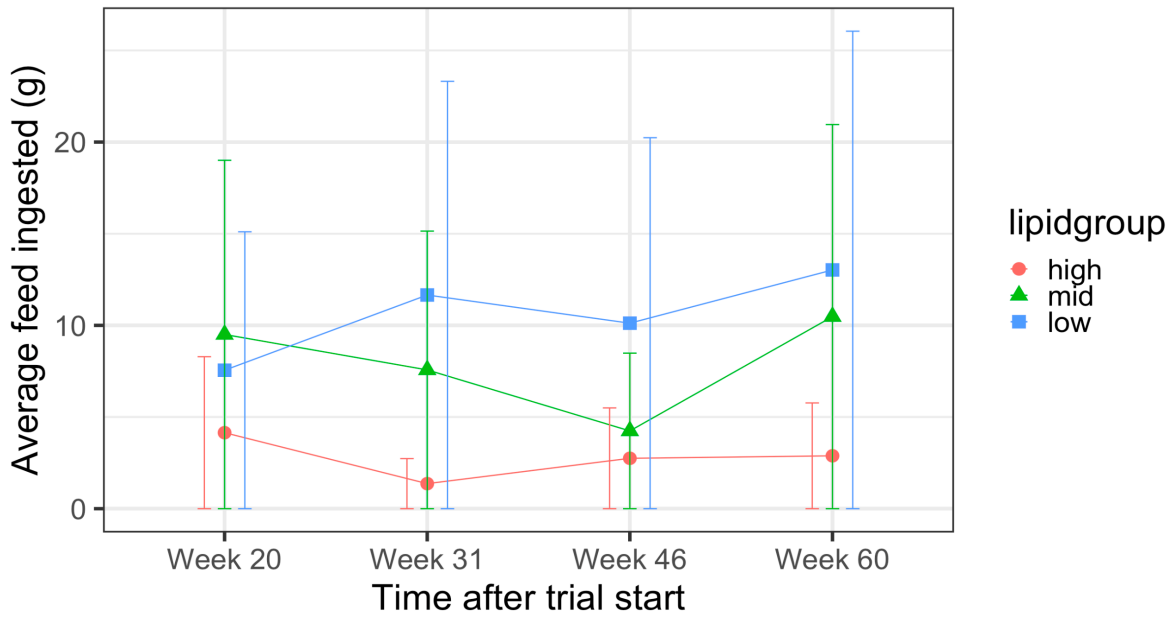


Figure 18: Mean feed intake (± SD) for the lipid groups through the trial period.

For increased carbohydrate content level in the feed, it was observed an increased feed intake. Difference between low and high carbohydrate diets was of statistical significance (Kruskal-Wallis, $p<0.05$), shown in **Figure 19**. At mid-trial and final recording, the high carbohydrate feed intake was double that of low carbohydrate diets.

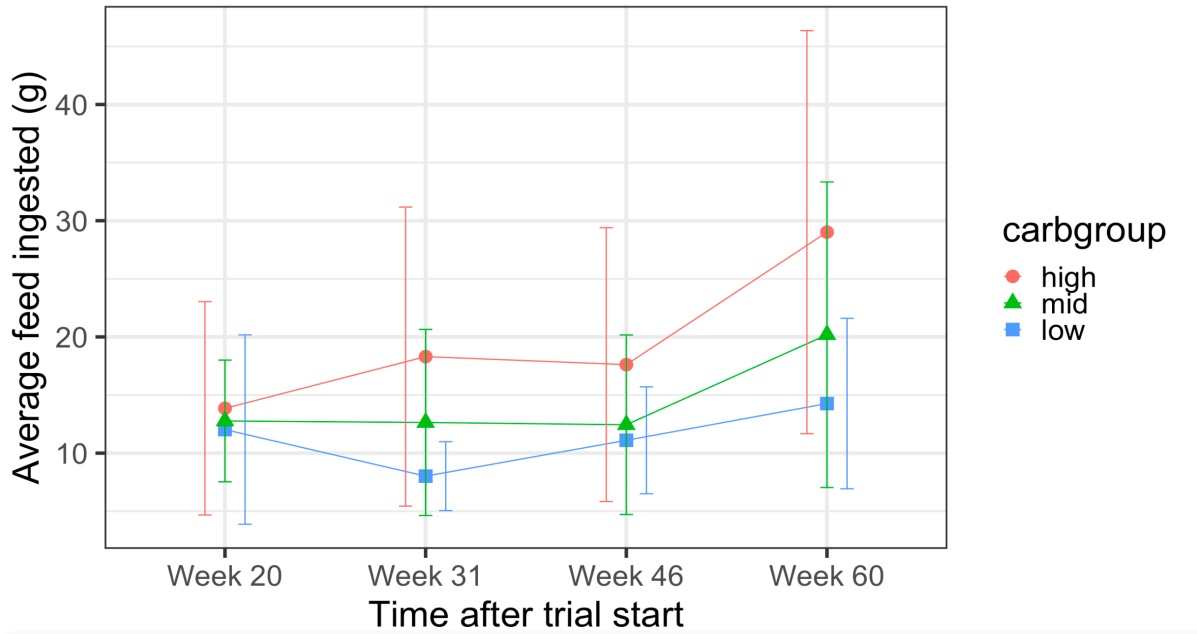


Figure 19: Mean feed intake (± SD) for the protein groups through the trial period.

3.6 Appetite score

The operator's daily appetite assessments throughout the trial resulted in the appetite scoring data presented in **Figure 20**, sorted by the higher to the lowest mean value. The different diets had a significant effect on appetite score (Kruskal-Wallis, $p<0.0001$). The two diets scoring highest are 3 (P60L20C10) and 11 (P50L10C30), averaging scores of 2.88 and 2.54 over the trial. On the other end, the diets 6, 5, 4 and 7 scored the lowest (P60L30C5, P50L20C20, P50L20C10 and P50L30C5), with appetite scores ranging from 0.48 to 0.32.

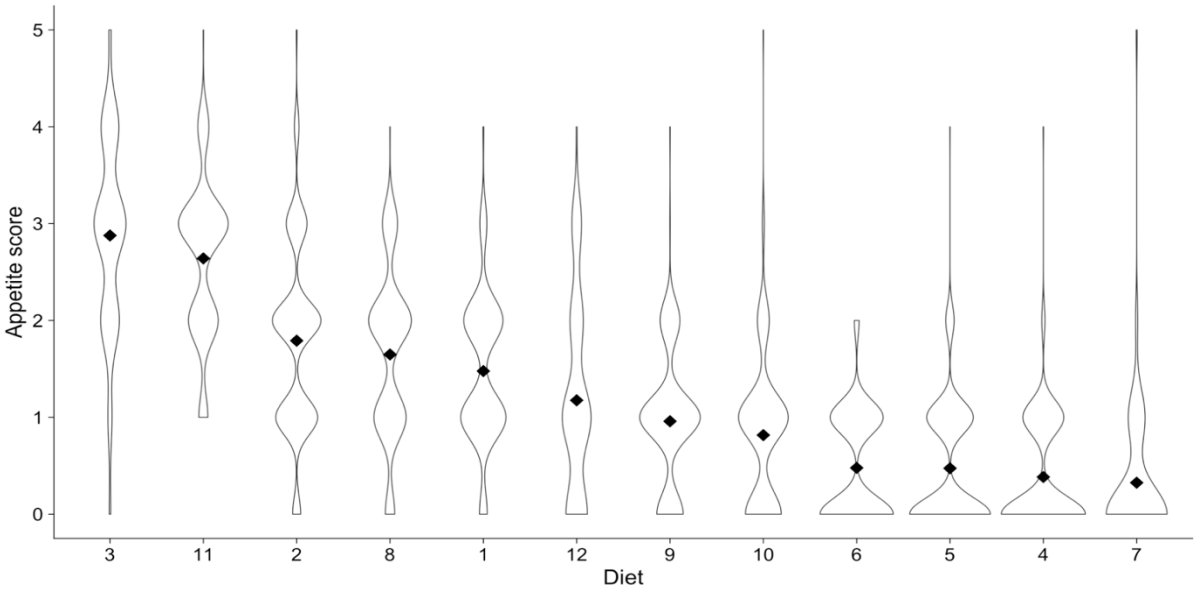


Figure 20: Appetite scoring plot for the entire scoring period. Sorted by mean values (marked by the squares).

Figure 21 shows a modelling of the appetite score data for the macronutrient gradients using a quadratic model ($p<0.01$, significant). This model shows that high lipid diets score vastly lower in appetite than diets with mid to low lipid-levels. Separating into the three lipid content groups an increase of over 80% can be seen when going from high to low lipid levels ($p<0.0001$). The difference from high lipid group to the mid lipid group is more than threefold ($p<0.0001$).

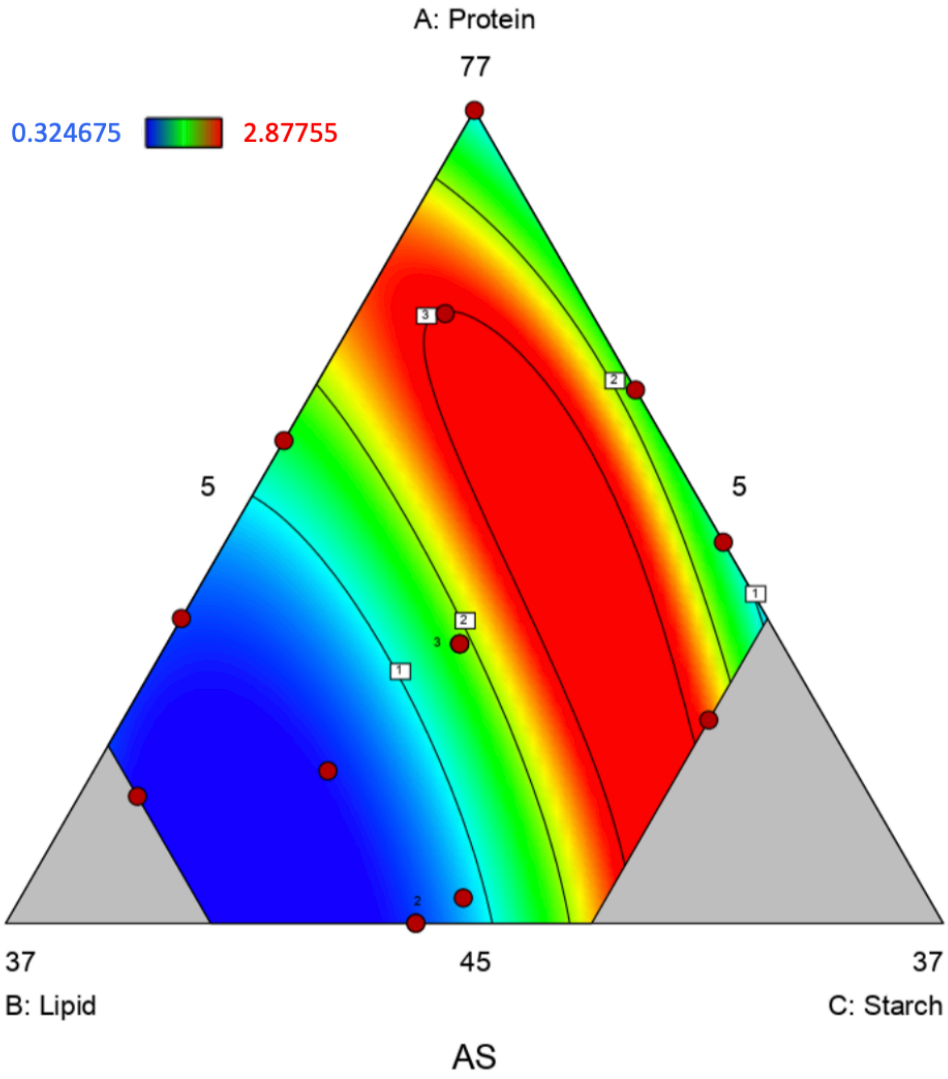


Figure 21: Quadratic model for mean appetite score for gradients of macronutrients over the whole trial. Red dots show the distribution of the experimental diets. The color scale on the contour plot goes from lowest mean appetite score values at dark blue areas to highest values for red areas, values shown in the top left.

Over the period of appetite scoring, fluctuations in scoring were noticeable (**Figure 22**). Different trends were seen over the experimental groups, with some diets showing an increase in appetite as time went by, while others decreased. Relation of appetite score with time was found using Pearson's correlation test, showing a significant decrease for diets 1, 2, 4, 5**,** 6, 7, 9 and 10 (r values and significance level in **Table 8**). Appetite score for diets 3 and 12 increased significantly ($p<0.001$) with time, while no significant correlation was found for diets 9 and 11.

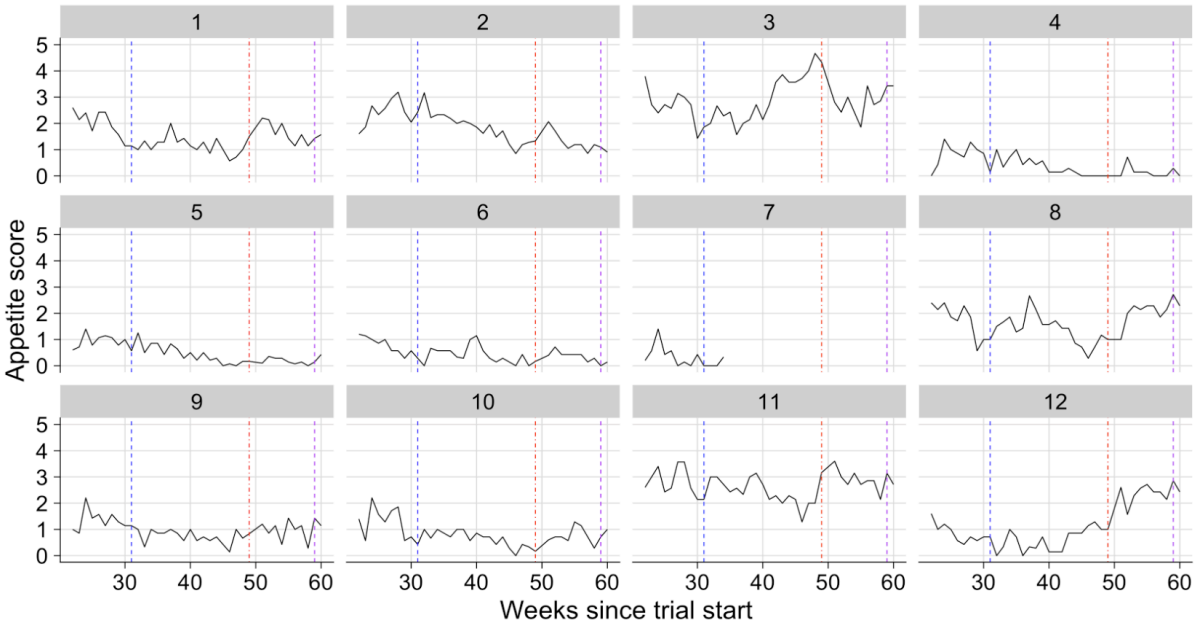


Figure 22: The weekly mean appetite score through the trial period. Dotted lines indicate the weeks of the recordings.

Table 8: Appetite score and its relationship with time since trial start. ([***] indicate p<0.001, [**] indicate p<0.01, no star indicates a non-significant correlation.

Diet	Mean appetite score	Correlation between AS and time (r value)	Composition name
1	1.48 ± 0.77	- 0.21 (***)	P60L20C05
2	1.79 ± 0.96	- 0.53 (***)	P60L20C20
3	2.88 ± 0.99	0.22 (***)	P70L10C10
4	0.38 ± 0.58	- 0.47 (***)	P50L20C10
5	0.47 ± 0.63	- 0.47 (***)	P50L20C20
6	0.48 ± 0.57	- 0.34 (***)	P60L30C05
7	0.32 ± 0.73	- 0.24 (***)	P50L30C05
8	1.65 ± 0.86	0.08	P70L05C20
9	0.96 ± 0.69	- 0.18 (**)	P80L05C05
10	0.82 ± 0.77	- 0.26 (***)	P50L20C30
11	2.54 ± 0.81	-0.04	P50L10C30
12	1.18 ± 1.02	0.58 (***)	P60L05C20

The appetite score change over time for the different macronutrient groups is shown in **Figures 23, 24 and 25**. On average, high protein diets gave the highest appetite score (mean score = 1.83 ± 1.00) (Kruskal-Wallis, p<0.0001). Thereafter the mid protein diets followed (mean score = 1.43 ± 1.79) (Kruskal-Wallis, p<0.0001) with the low protein diets giving lowest appetite score (mean score = 0.93 ± 0.94) (Kruskal-Wallis, p<0.0001). The high protein groups score slightly increased with time (Pearson's correlation test, r = 0.002, p = 0.9518, not significant), while low and mid protein groups decreased significantly (Pearson´s, r = -0.214, p<0.001, and r = -0.153, p<0.001).

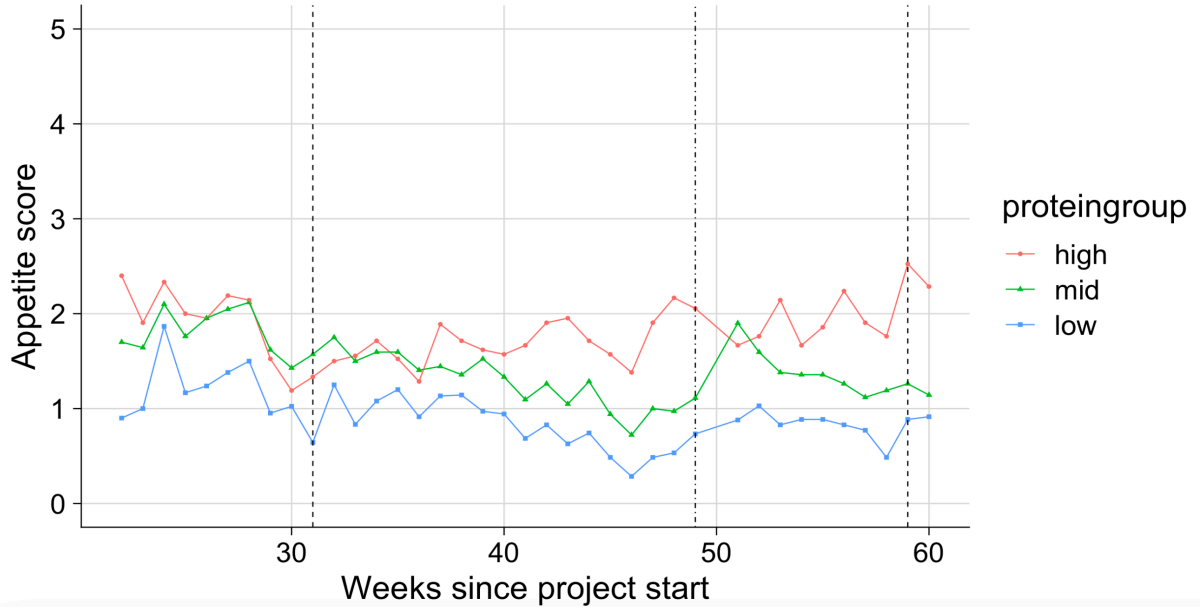


Figure 23: The weekly mean appetite score development through the trial period for the protein groups. Dotted lines indicating the weeks where video recordings took place.

The mid lipid group notably gave the highest appetite score (mean score = 1.86 ± 1.01) (Kruskal-Wallis, p<0.0001), followed by the mid lipid diets, differing significantly from the high and low group (mean score = 1.56 ± 0.70) (Kruskal-Wallis, p<0.0001). Recording the lowest mean score was high lipid diets, averaging at a score of only 0.44 ± 0.38, significantly lower than the low and mid lipid groups (Kruskal-Wallis, p>0.0001). For low lipid diets, the appetite score increased over time as the fish grew bigger (Pearson´s, r = 0.198, p<0.001), while mid and high lipid diets significantly decreased (Pearson´s, r = -0.221, p<0.001, and r = -0.383, p<0.001).

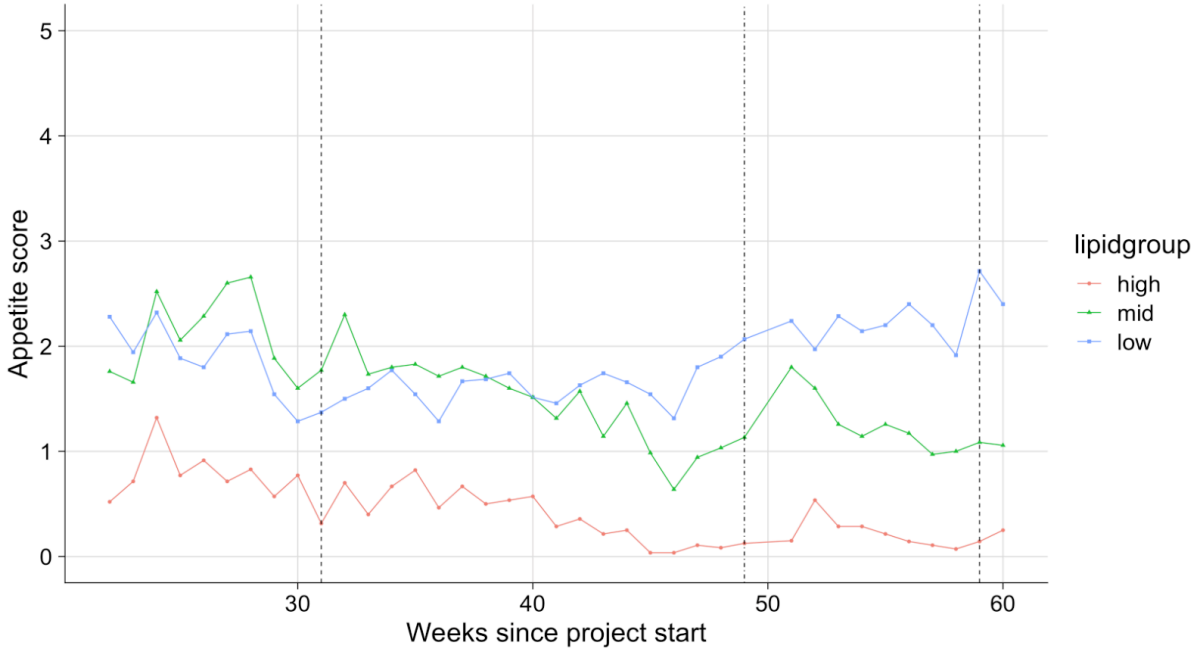


Figure 24: The weekly mean appetite score development through the trial period for the lipid groups. Dotted lines indicating the weeks where video recordings took place.

High carbohydrate diets on average gave the lowest appetite score (mean score = 1.12±0.97) (Kruskal-Wallis, $p<0.001$), followed by low carbohydrate diets (mean score = 1.36±1.05) ($p<0.001$) and best, the mid carbohydrate diets (mean score = 1.50±0.81) ($p<0.001$). Low carbohydrate diets had a very slight, non-significant increase in AS over time ($r = 0.022$, $p = 0.474$), while mid carbohydrate diets notably decreased ($r = -0.332$, $p<0.001$). High carbohydrate diets had a slight non-significant decrease ($r = 0.043$, $p = 0.134$).

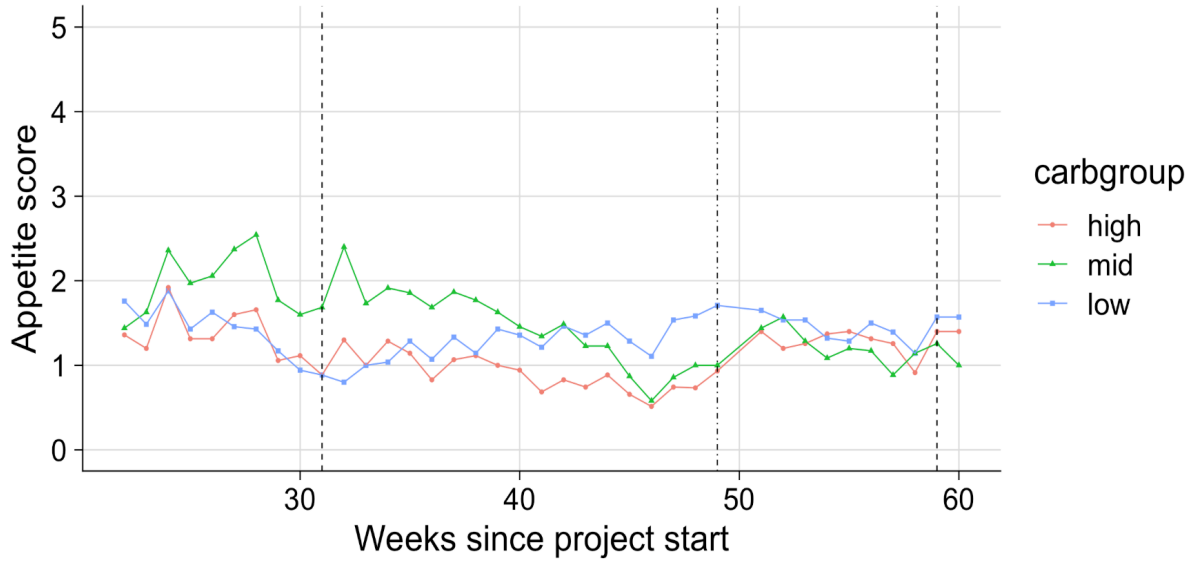
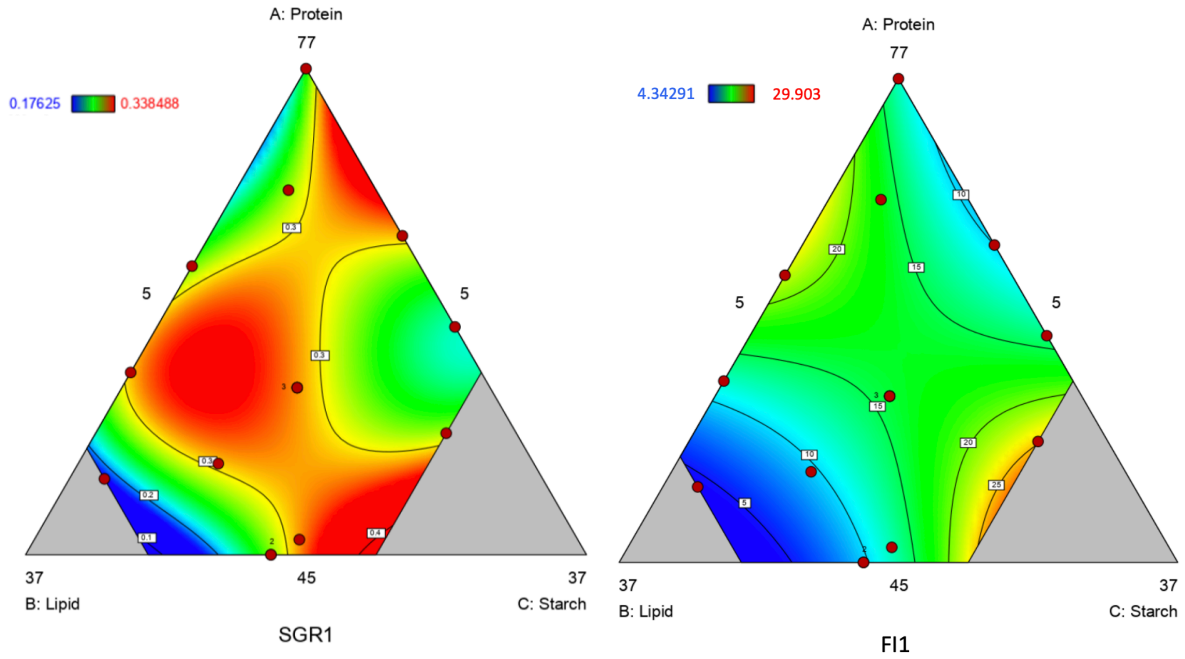


Figure 25: The weekly mean appetite score development through the trial period for the carbohydrate groups. Dotted lines indicating the weeks where video recordings took place.

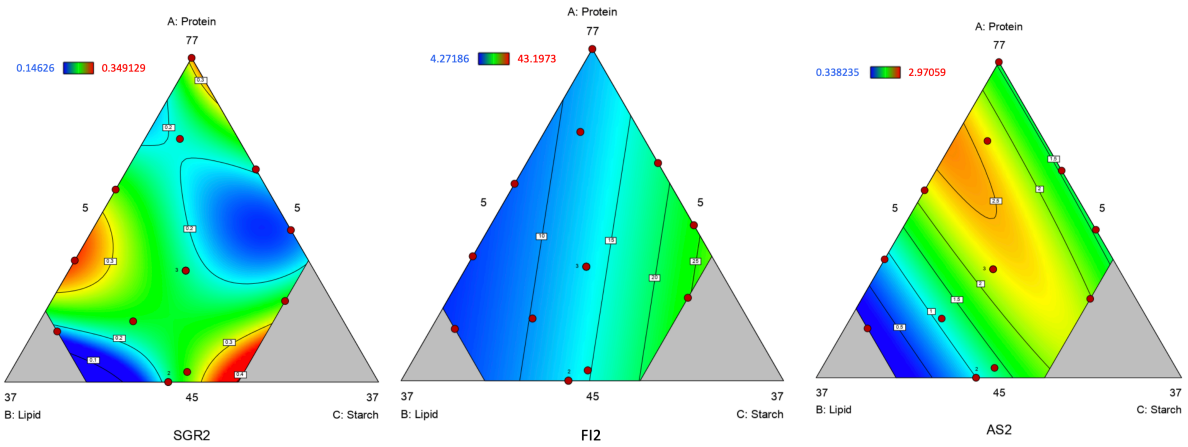
3.7 Relationship between appetite and growth through the trial period

The specific growth rates (SGR) in between each weighing was compared with the appetite data (video analysis and appetite scoring) from the same time periods. For each period these data are presented and compared below. SGR, Feed intake (FI), Appetite score (AS) and Growth efficiency index (GEI) are categorized after which interval they represent (1-4). In **Figure 26,** SGR data from the first in-tank period is presented in a cubic model ($p<0.05$). The model points at three peaks, with the lowest SGR is seen towards the highest lipid content. Data for feed ingested (FI1) which is shown in **Figure 27** as a linear model ($p>0.05$) shows its feed intake peaks at a middle lipid content, with absolute peaks in both ends of the carbohydrate gradient. Lowest point also at the highest lipid content. The calculated Growth efficiency index (GEI1) didn't show any significant differences in growth efficiency related to feed intake data for any of the diets or macronutrient groups (**Table 9**). There was a positive relation between SGR1 and FI1, however this correlation was non-significant ($r = 0.423$, $p = 0.116$).



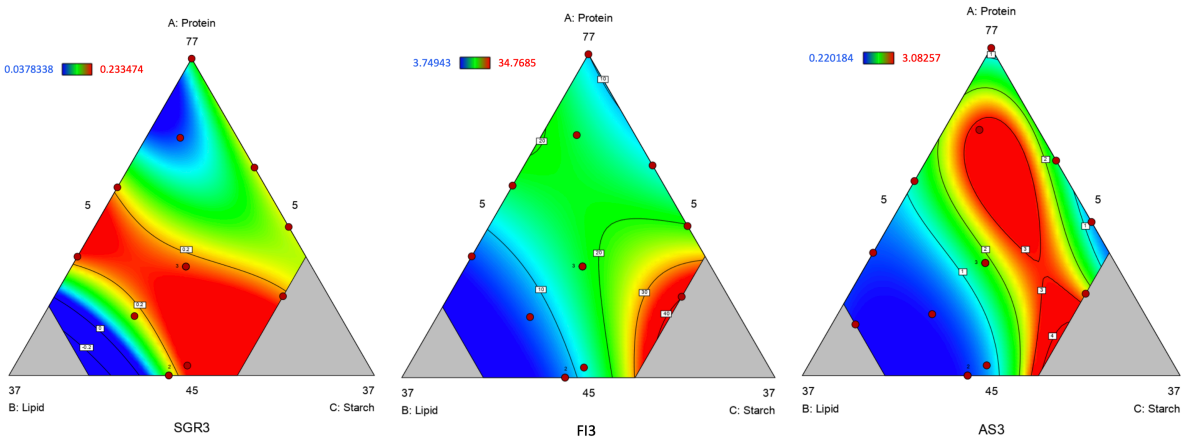
Figures 26 and 27: Models for specific growth rate and feed intake (g) (FI1) for the first inter-weighing interval. Red dots show the distribution of the experimental diets. The color scale on the contour plot goes from lowest values at dark blue areas to highest values for red areas, values shown in the top left.

For the second inter-weighing interval appetite-scoring, along with feed intake, were compared with the specific growth rate. The SGR2 cubic model seen in **Figure 28** is not significant ($p = 0.458$), neither is the FI2 linear model in **Figure 29 (**$p = 0.091$). Appetite score is modeled using a quadratic model in **Figure 30**, which is statistically significant ($p<0.01$). Pearson's correlation test showed no significant correlation between neither SGR2 and FI2 ($r = -0.028$, $p = 0.923$), nor SGR2 and AS2 ($r = 0.099$, $p = 0.716$). Fish fed the high lipid group diets shows a significant positive effect in GEI for this period (One-way ANOVA, $p<0.05$). However, a very strong, significant correlation is seen from FI2 from video recordings and AS2 from day-to-day scoring ($r = 0.610$, $p = 0.015$).



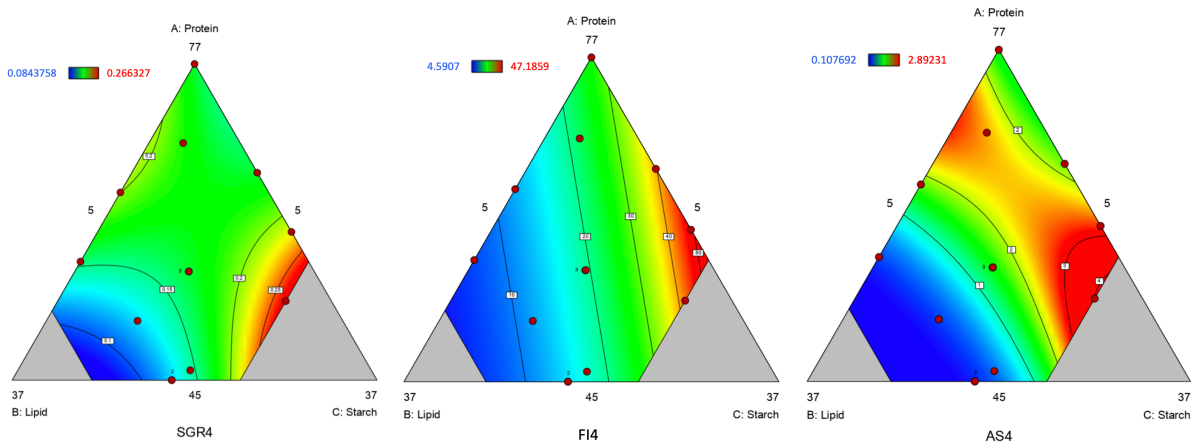
Figures 28, 29 and 30: Models for specific growth rate, feed intake (g) (FI2) and appetite scoring (AS2) for the second inter-weighing interval. Red dots show the distribution of the experimental diets. The color scale on the contour plot goes from lowest values at dark blue areas to highest values for red areas, values shown in the top left.

For the third inter-weighing interval, Diet 7 (P50L30C5) was excluded from the trial, thus, removing the outer point in lipid content. The cubic model for SGR3 in **Figure 31** has a p value of 0.071, thus the model is non-significant. Nevertheless, fish fed with low lipid content seems to be the slowest growing, along with low values towards high protein content. FI3 quadratic model (**Figure 32**) ($p<0.05$) shows a peak for feed intake for high carbohydrate diets, and low points towards high lipid. AS3 cubic model (**Figure 33**) ($p<0.01$, significant) shows peak appetite score at a mid to low lipid level, with the same low points towards high lipid. Correlation tests shows no significant correlation between SGR3 and FI3 ($r = -0.303$, $p = 0.292$) or SGR3 and AS3 ($r = -0.335$, $p = 0.189$). For this period, high lipid still have significantly higher GEI than the rest of the lipid groups (one-way ANOVA, $p<0.05$, Appendix, **Table 5**). FI3 and AS3 are here strongly correlated ($r = 0.636$, $p = 0.015$).



Figures 31, 32 and 33: Models for specific growth rate, feed intake (g) (FI3) and appetite scoring (AS3) for the third inter-weighing interval. Red dots show the distribution of the experimental diets. The color scale on the contour plot goes from lowest values at dark blue areas to highest values for red areas, values shown in the top left.

For the fourth inter-weighing interval SGR4 gives a linear model (**Figure 34**) with statistical significance ($p<0.05$) where specific growth rate followed the gradient for lipid levels, lowest at low lipid, highest at high lipid. FI4 is also distributed linearly, with a significant linear model ($p<0.05$) in **Figure 35** where feed intake increases with increasing lipid and carbohydrate gradients. A quadratic model ($p<0.05$, significant) for AS4 is seen in **Figure 36**, following a similar trend towards mid to high lipid, and high carbohydrate. Pearson's correlation test shows a strong significant correlation between SGR4 and FI4 ($r = 0.698$, $p = 0.005$) and a very strong correlation between SGR4 and AS4 ($r = 0.789$, $p = 0.0008$). Still, fish fed high lipid diets have significantly higher GEI than the fish fed low and mid lipid group diets (one-way ANOVA, $p<0.05$, Appendix, **Figure 5**). Correlation between the two types of appetite data also remain very strong ($r = 0.774$, $p = 0.001$).



Figures 34, 35 and 36: Models for specific growth rate, feed intake (g) (FI4) and appetite scoring (AS4) for the fourth inter-weighing interval. Red dots show the distribution of the experimental diets. The color scale on the contour plot goes from lowest values at dark blue areas to highst values for red areas, values shown in the top left.

Table 9: Calculated growth efficiency index for each of the tanks over the four inter-weighing intervals and in total for the full trial. Fish fed the high lipid Diet 7 (P50L30C05) were euthanized midway, the diet is therefore omitted from the data for GEI3, GEI4 and GEI.

Tank	Diet	GEI1	GEI2	GEI3	GEI4	GEI	Composition name
1	12	288.6804	236.2495	218.078	168.9115	1074.284	P60L05C20
2	2	161.8685	141.3416	434.6334	127.9731	739.5091	P60L20C20
3	9	237.9631	602.8284	424.8507	358.9222	1206.316	P80L05C05
4	4	309.5606	872.6578	2342.248	402.0318	1811.627	P50L20C10
5	2	252.419	202.1529	411.1132	223.5774	971.2702	P60L20C20
6	5	470.1004	821.9667	2468.026	321.4863	2476.407	P50L20C20
7	2	533.3802	270.7229	570.5752	122.1083	1099.451	P60L20C20
8	6	613.6229	1151.513	1711.562	457.3884	3329.728	P60L30C05
10	7	844.6152	243.0605	-	-	-	P50L30C05
11	8	570.2302	237.7114	296.3448	194.1623	1145.652	P70L05C20
12	3	376.7609	103.358	66.62906	220.7077	485.8747	P70L10C10
13	10	683.3721	468.8816	773.2764	218.56	2231.646	P50L20C30
14	11	126.4456	124.1061	217.3234	139.8611	626.3931	P50L10C30
15	1	135.8699	354.8741	375.2567	315.4048	811.7924	P60L20C05
18	5	334.7174	615.2675	876.4455	311.1015	1863.637	P50L20C20

3.8 Activity data

Figure 37 shows activity measurements analyzed from the video recordings. The overall trend is an increase in activity leading up to feeding, with an elevated activity response of varying length in the time after feeding. Diets 2 (P60L20C20), 4 (P50L20C10), 9 (P80L05C05) and 10 (P50L20C30) had significantly higher total activity compared to the other experimental diets (one-way ANOVA, $p<0.05$). These diets also had a higher activity in the time post feeding (one-way ANOVA, $p<0.05$, $p<0.05$, $p<0.05$ and $p<0.01$, respectively) The anticipatory index, a relation between activity two minutes and eight minutes before feeding, did not differ significantly for any of the diet groups.

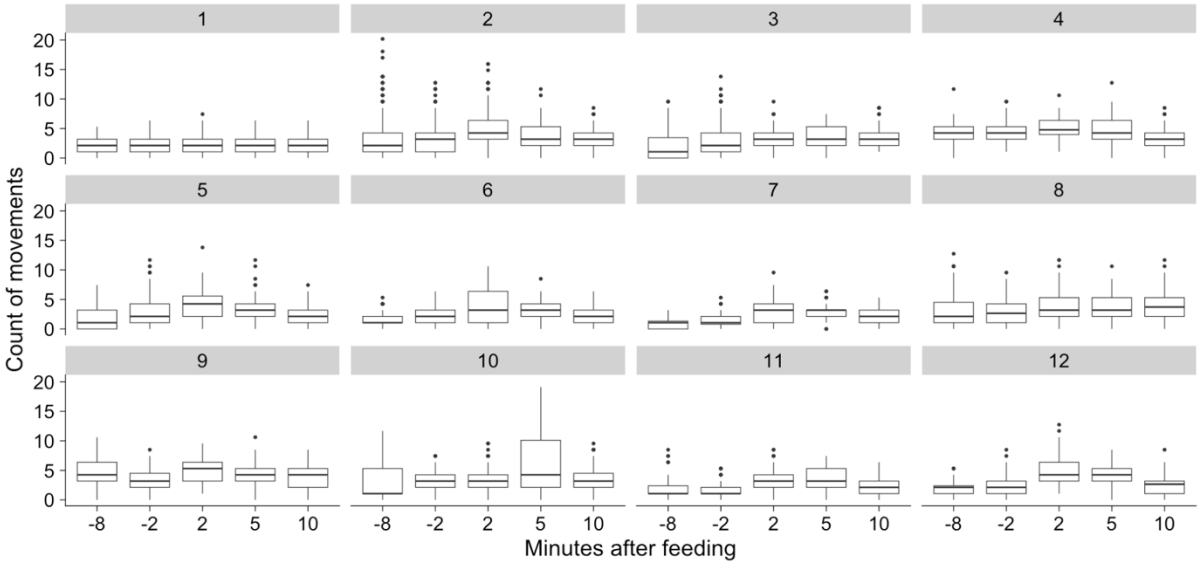


Figure 37: Boxplot showing the quantification of tank activity before and during feeding. Quantification is done for average number of movements longer than one body length in each quartile of the tank per 10 seconds. Data is from different time segments surrounding feeding and presented for the twelve diets.

The relation between the different activity data and the feed ingested was investigated to understand their impact on appetite. Spearman's correlation test was used between total activity and amount of feed ingested, finding a significant negative correlation ($r = -0.316$, $p = 0.018$) in **Figure 38**. A similar result was found between feed intake and total post-feeding activity, with a borderline significant negative correlation ($r = -0.260$, $p = 0.053$) illustrated in **Figure 39**. The anticipatory index also showed a negative correlation with feed intake ($r = -0.291$, $p = 0.029$) as seen in **Figure 40**.

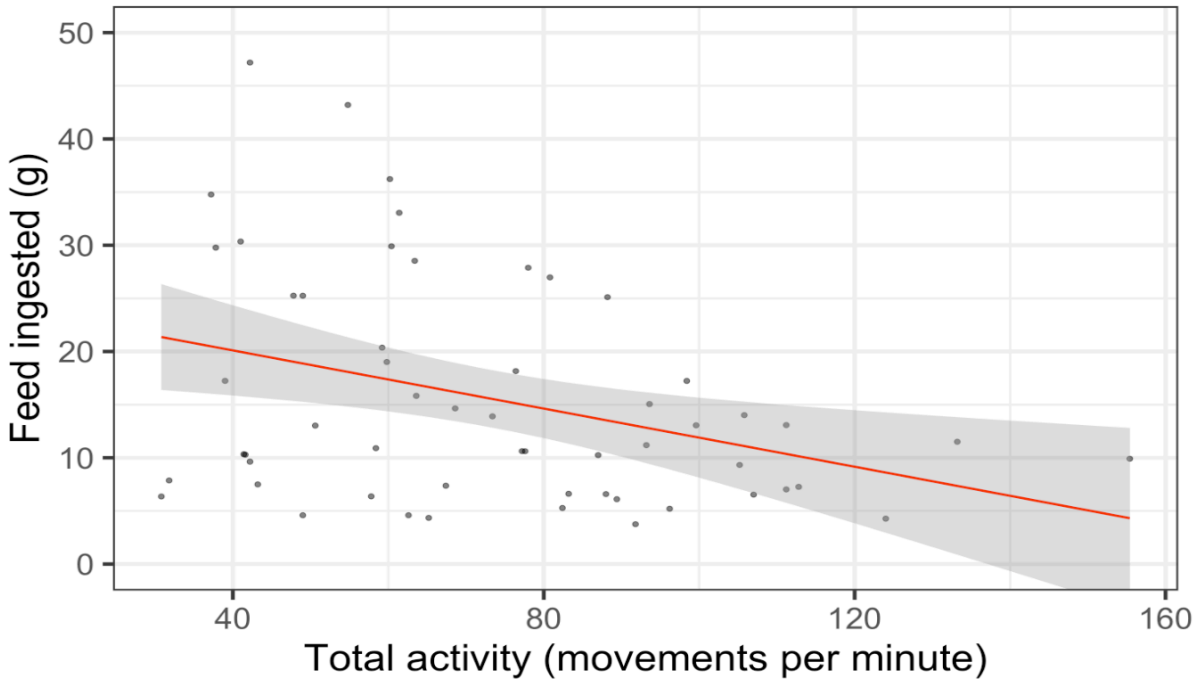


Figure 38: Correlation plot between feed intake and total observed activity with a trend line and confidence area based on a linear regression model of the data

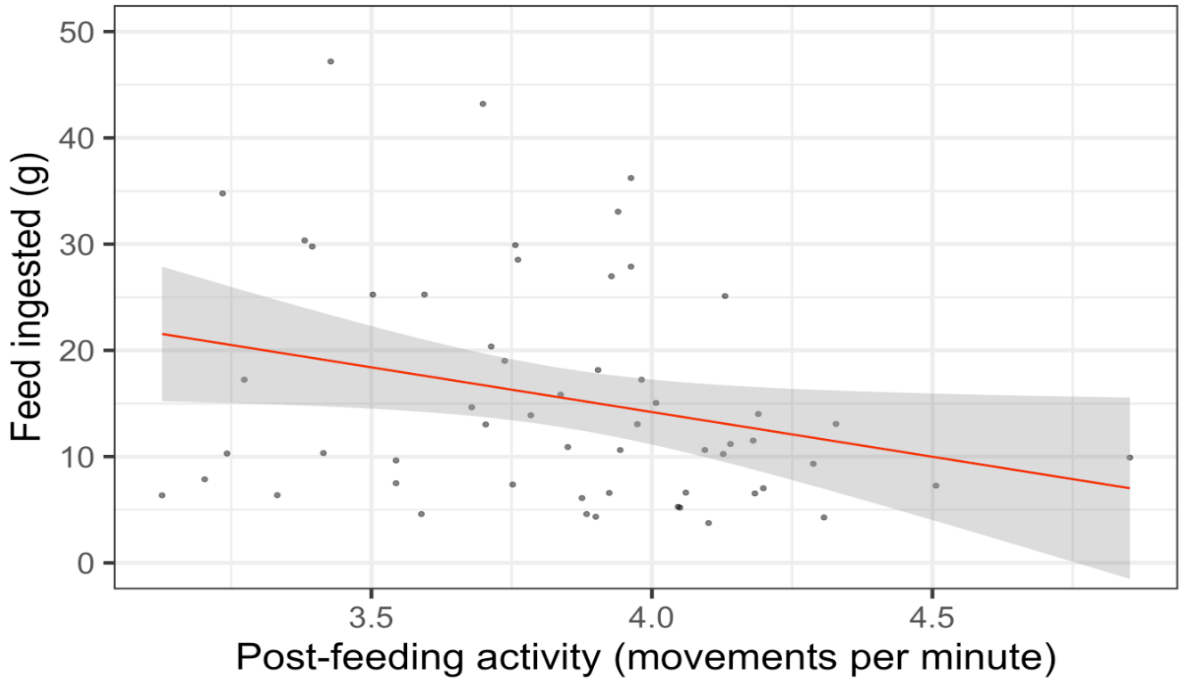


Figure 39: Correlation plot between feed intake and post-feeding activity with a trend line and confidence area based on a linear regression model of the data.

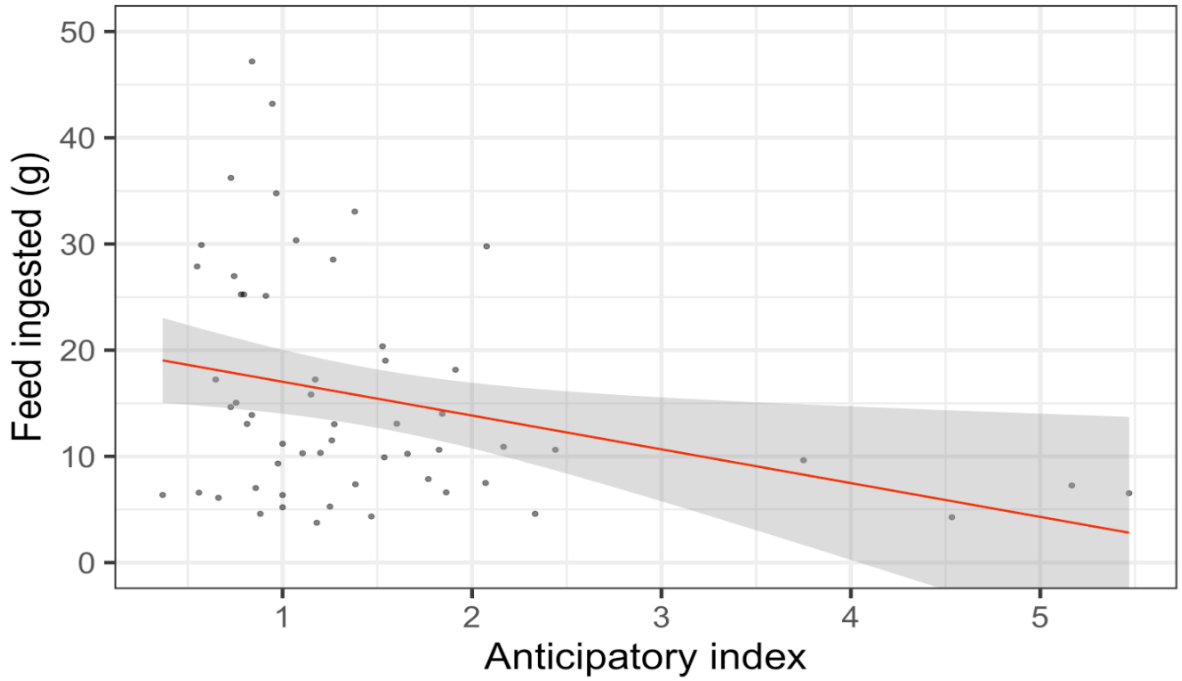


Figure 40: Correlation plot between feed intake and the calculated anticipatory index, with a trend line and confidence area based on a linear regression model of the data.

3.9 Ingestion behavior

Table 10 and **Figure 41** presents the relative ingestion behavior frequency of the halibut for the experimental diets. IB2 (Moderate movement ingestion) is the most frequent ingestion behavior for all the diets, with IB3 (Swimming ingestion) being second most frequent altogether. For IB2 and IB3 no significant effects are found for the 12 different diets, however there is a significantly higher frequency of IB1 (Stand-still ingestion) for Diet 4 (P50L20C10) (ANOVA, $p<0.01$). No significant effects are found for the macronutrient groups.

There was a significant negative correlation between the frequency of IB1 and feed intake (Pearson´s, $r = -0.356$, $p<0.01$). For the other ingestion behaviors, no such significant impact is found. There is a non-significant low negative correlation between IB2 and total activity (Pearson´s, $r = -0.247$, $p = 0.060$).

Table 10: Relative frequency of the different ingestion behaviors.

Diet	IB1 frequency (%)	IB2 frequency (%)	IB3 frequency (%)	Composition name
1	15.33	63.58	21.09	P60L20C05
2	12.43	52.70	34.87	P60L20C20
3	12.72	51.38	35.91	P70L10C10
4	34.14	43.52	22.34	P50L20C10
5	14.39	57.97	27.65	P50L20C20
6	13.00	59.03	27.97	P60L30C05
7	27.68	42.86	29.46	P50L30C05
8	19.77	56.11	24.12	P70L05C20
9	19.53	59.54	20.93	P80L05C05
10	16.31	55.36	28.32	P50L20C30
11	7.57	56.37	36.06	P50L10C30
12	15.16	68.00	16.84	P60L05C20

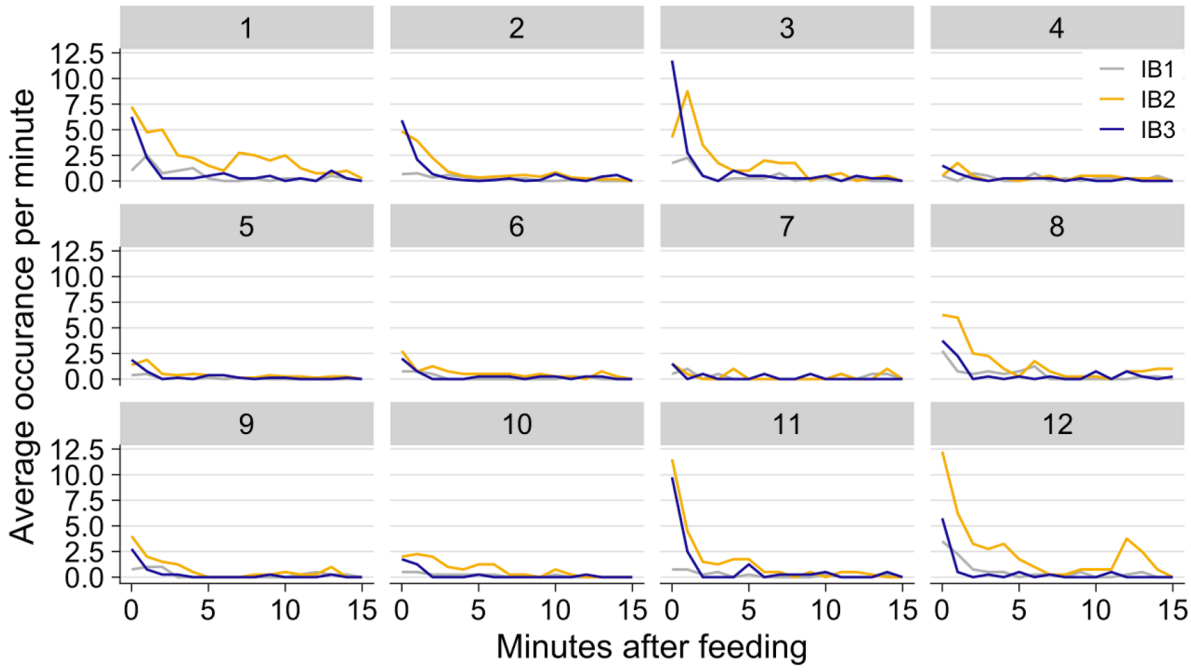


Figure 41: *Average occurrence of the three ingestion behaviors (IB1, IB2 and IB3) per diet identified in the video recorded feeding period.*

3.10 GI-tract content

In the sampling the GI-tract was dissected and gut content from different GI-tract segments were analyzed. This content was taken at different time after the start of feeding. There was a tendency for a slight, but non-significant correlation between time of sampling after feeding and stomach content (**Figure 42**) (Spearman´s, r = 0.097, p = 0.188). After filtering the fish with no stomach or midgut content, there was no correlation between stomach and midgut content (r = 0.010, p = 0.914).

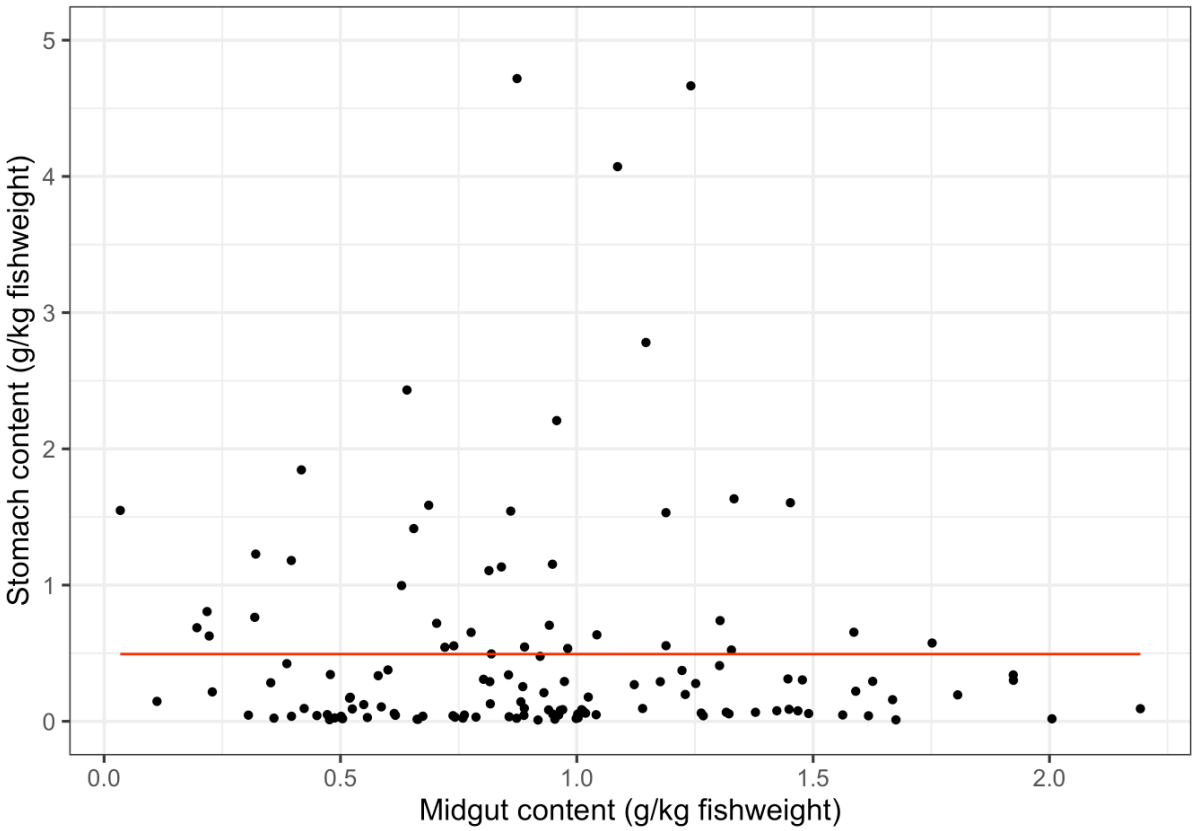


Figure 42: Correlation plot between the stomach and midgut content. Fish with no content in both stomach and midgut (<0.3 g) were removed from the analyses. A trend line based on a Generalized Additive Model is fitted to show the trend of the data.

4 Discussion

4.1 Methodological considerations

The three-component mixture design used in this trial was generated for easy analysis and modelling. Upper and lower limits for each of the macronutrients components were based on previous research (Berge and Storebakken, 1991; Hamre *et al.*, 2003, 2005; Hatlen *et al.*, 2005; Martins *et al.*, 2007; Árnason *et al.*, 2009). This was considered to maximize and optimize the investigated dietary inclusion ranges of protein, lipid, and carbohydrates.

The methods used for video analysis of feed intake were based on previous experience from similar appetite analysis of grow-out halibut above 1 kg (Endre Lygre, 2022, Pers. comm.). This process was quite time-consuming. Even though it was not possible to follow individuals, since the fish were not externally tagged, video analysis was effective in detecting instances of feed intake in these relatively small tanks because of the halibut's distinct ingestion movements. However, for the video feed intake analysis, differences in the automatic feeder's setup (Appendix, **Table 1**) can be seen as a source of error, offering to a small extent varying amounts of feed through the 15-minute video period analyzed. The operator's assessment of appetite, i.e., the appetite scoring, gave us a broad overview of the appetite on a day-to-day basis, as well as the development of appetite and feed interest over time. This assessment was done in the morning, around 09:00-10:00 (Yvonne Rong, 2023, Pers. comm.). This makes it so that both video appetite data and appetite scoring show the feeding response during morning feeding. Even though there is expected a general appetite to be at its highest at the start of feeding, it is a limiting factor that only the feeding interest early in the day is captured. This can result in potential gaps of data, particularly for the tanks where fish have a more slow and steady feed intake throughout the whole feeding period. The procedure for activity analysis worked well in capturing the activity dynamic around feeding time. Regarding the collection of the gut content during sampling, the method applied was suboptimal. During sampling days, the tanks were fed at usual feeding hours, i.e., from 08:00 to 18:00 for the mid-trial sampling and 09:00 to 17:30 for the final sampling. The tanks were sampled in a consecutive order, i.e., one after another, resulting in a varying amount of time between feeding start to the time of sampling, ranging from 0 to 7 hours. Thus, the sampled fish did not get a full meal before being sampled, but instead were given a varying share of their daily meal, based on time of sampling

related to time of feeding start. Even though it would be assumed that this varying feed amount would heavily affect stomach content, correlation test disproved this, showing no significant correlation between time of feeding and stomach content. Nevertheless, in future trials, to guarantee efficient sampling of the gut content, this will be taken into consideration and the feeding schedule will be adjusted so that the fish will be sampled at the same time after the meal has ended.

4.2 Dietary effect on growth

This trial maps the performance of halibut fed a range of varying macronutrient diets. As with all forms of aquaculture, a major measurement of success is growth, as well as feed efficiency. Thus, based on this parameter, in this trial, the clearly best performing diet from start to final weighing was Diet 6 (P60L30C05), containing 57% protein, 25% lipid and 5% carbs. Atlantic halibut fed this diet reached a mean weight of 948 g (total mean growth of 626 g). Following, diets 2 (P50L10C30), 10 (P50L20C30), and 11 (P60L20C20), resulted in a mean final weight of 888, 879, and 864 g (total mean growth of 588, 568 and 544 g), respectively. These top four growth-performing diets have a protein content ranging from 46 to 57%, a lipid range of 9 to 25% and a carbohydrate content from 5 to 25%. This supports that there is good performance of medium-to-low protein diets, while a wide range of lipid and carbohydrate levels are tolerated, while giving satisfying growth results.

The diet with the lowest growth performance was Diet 3 (P70L10C10) followed by Diet 12 (P60L05C20) and Diet 9 (P80L05C05), resulting in a mean final weight of 675, 765 and 774 g (total growth of 362, 452 and 444 g), respectively. Diets 3 and 9 are high protein (dietary content of 69 and 77%, respectively), low lipid (10 and 5%) and low carbohydrate (8 and 5%) diets. These show that elevated protein content doesn't necessarily result in high growth. Diet 12, however, has a lower protein content at 60% and higher carbohydrate content at 16%. In common with diets 3 and 9 is the low lipid levels (at 5%). Diet 7 (P50L30C05) was all in all the worst performing diet. Diet 7 had the highest lipid content (at 30% dietary lipid, 50% protein and 7% carbohydrates), and the fish fed with this diet had to be slaughtered due to emaciation. This matches the findings of Hamre *et al.* (2003), where lipid levels at 30% also resulted in growth depression.

When categorizing the diets based on levels of protein, lipid and carbohydrates, the major differences are observed. Surprisingly, and contradictory to previous findings (Aksnes *et al.*, 1996; Hamre *et al.*, 2003, 2005), the fish given the low carbohydrate diets (5-8% dietary carbs) showed the lowest growth, marginally but non-significantly lower than the mid and high carbohydrate diets (13-16% and 19-25% dietary carbohydrates). Literature on carbohydrate inclusion in fish feed suggested a maximum of 5% dietary carbohydrates for small, juvenile halibut (Hamre *et al.*, 2003, 2005), and that for larger halibut (400-600g) it was shown that growth increased when dietary carbohydrates were lowered towards 3%, a trend not observed in this trial.

For fish fed different lipid levels, there were no major differences in the first half of the trial. Subsequently, the fish fed with the low lipid group (5-10% dietary lipid) had significantly worse growth than the mid and high lipid groups (16-18% and 23-30% dietary lipid, respectively) in the second half of the trial, at 46 and 60 weeks. Here, however, it is important to note that the worst performing diet, i.e., Diet 7 which had the highest lipid content, was removed from the trial mid-way, with the fish fed this diet euthanized. Previous literature has shown that smaller, juvenile halibut (0.5-100 g) growth is unaffected by varying lipid levels, ranging from 12 to 21% (Berge and Storebakken, 1991), 5 to 25% (Hamre *et al.*, 2003) and 14 to 25% (Martins *et al.*, 2007). For larger fish, at 600 to 1500 g, the same trend has been described for diets ranging from 8 to 20%, meaning no growth variations were observed (Berge and Storebakken, 1991). Thus, the results support and match these previous findings, although it may seem like lowering the dietary lipid level to 5% can result in a decreased growth to the fish size used in this thesis.

In terms of protein content, the worst performing diets are the high protein group (66-77% dietary protein). Fish fed these diets had significantly lower growth in latter stages of the trial (weeks 46 and 60) than the mid and low protein groups (at 56-60% and 45-53% dietary protein). Previous work on dietary protein content has focused mainly on its lower requirement, and the recommendations seem to vary dependent on life stages, with relatively high protein requirements for small fish, and lower requirements as the fish size increases (Hatlen *et al.*, 2005; Árnason *et al.*, 2009). Requirements for fish sized 6-556, 58-126, 140-266, 559-877 and 980-1493 g are reported at 62, 56, 51, 41, and 35% respectively (Aksnes *et al.*, 1996; Helland and Grisdale-Helland, 1998; Hamre *et al.*, 2003, 2005; Hatlen *et al.*, 2005; Árnason *et al.*,

2009). The results showed that diets ranging from 45 to 60% performed satisfactorily, however, increasing the dietary protein to and beyond 66% decreased the growth significantly. With protein being the most expensive food ingredient, minimizing its content is favorable, since lowered protein content can in the end lead to lower feed cost, higher growth and net economic gain for halibut farmers. However, it is interesting to observe that there seems to be an upper limit in protein content, which can have negative impacts on fish growth.

HSI, measured at mid-trial and end-of trial sampling correlated negatively with dietary protein content, while correlating positively with both dietary lipid and carbohydrate content. These trends are similar to the relations seen between macronutrients and growth. Previous research on halibut has showed no such correlation for higher lipid levels (Berge and Storebakken, 1991; Helland and Grisdale-Helland, 1998). Hamre *et al.*, (2005) described an increased HSI due to accumulation of carbohydrate in the liver, along with decreased growth when increasing dietary carbohydrates over 5%. In this trial HSI also positively correlated with the weight of the sampled fish, further indicating a relation between good growth and high HSI.

4.3 Dietary impacts on appetite

As stated previously, a large problem in Atlantic halibut farming is to induce appetite and interest to ingest commercial formulated feed (Yacoob and Browman, 2007). This lack of interest in the formulated feed was present throughout the trial and were observed in both the video recordings and for the appetite scorings given.

Regarding feed intake, the best performing diet, taking into consideration only the video recordings, was Diet 11 (P50L10C30) which is a diet with one of the highest carbohydrate contents (25% dietary carbohydrate). Diet 2 (P60L20C20) and 12 (P60L05C20) followed, which also had high carbohydrate levels (15 and 16%). In the other end, Diets 4, 5, 6 and 7, all high on lipid, gave the lowest observable feed intake during morning feeding. In addition, if looking at appetite for the macronutrient groups, the clearest trends was still related to lipid and carbohydrate levels. The fish fed with diets grouped within the high lipid content seemed to have a markedly lower feed intake, while fish fed mid and low lipid diets groups were quite even in terms of feed intake, with low lipid performing slightly better. This lower observed appetite for high lipid diets might be related to varying energy density for the feed, that

increased with elevated levels of lipid, and thereby reduced the food intake needs related to energy requirements. As for carbohydrate groups, the trend is the opposite, with fish fed diets in the low, mid and high carbohydrate groups having the lowest, middle and highest feed intake, respectively.

The daily appetite scoring showed Diet 3 (P70L10C10) as the highest mean score, followed by Diet 11 (P50L10C30) and Diet 2 (P60L20C20). Like observed for video recorded feed intake, Diets 4, 5, 6 and 7 scored the lowest also in appetite scoring, indicating that fish had a very low appetite when fed diets at the highest lipid levels. This same trend is was registered when looking at the macronutrient groups. For protein the high dietary content groups scored best, contradicting feed intake data from videos, while carbohydrate groups had no clear pattern throughout the trial period, but with mid carbohydrate levels averaging higher appetite. Olfaction, and gustation is expected to be important for inducing feed intake. One of the most common groups of chemicals (attractants) stimulating these senses is believed to be FAA and smaller peptides (Yacoob and Browman, 2007). This indicates the role of the protein ingredient as an enhancer of feed intake motivation. However, whether the high protein diets actually contained higher amount of FAA/peptides in this study is not known, since it was not analyzed. Thus, based on the available information it cannot be concluded if this high appetite scoring for the high protein group is related to FAAs as attractants. Another aspect that could be affecting appetite is under- or malnutrition. Hereunder, the lowered energy densities for some of these high protein diets might come into effect. The fish fed with Diet 3 showed an extreme, "feeding-frenzy"-like response to feed that could be seen in the last two video recordings (week 46 and 60), but, at the same time, had a very low growth. This "feeding-frenzy"-like response included a mass vertical swimming as a response to vibrations from the automatic feeders, even before pellets hit the water surface, and combined with the low growth efficiency, is an indicator that all instances of elevated feed interest aren't necessarily favorable.

The feed intake analyzed from video recordings showed no significant increase in feed intake from start to end of the trial. At the same time, the appetite scoring data over the trial timeline showed that appetite score declined for a vast majority of the diets in relation to time. Only Diet 3 (P70L10C10) and 12 (P60L05C20) had significant appetite score increase, while Diet 8 (P70L05C20) and 11 (P50L10C30) showed no significant changes. All the other diets had an appetite scoring decrease. This might be, to some degree, caused by loss of interest in

commercial feed, a common problem in halibut farming described by Yacoob and Browman, (2007). The appetite decline observed in some tanks might also be linked to the implications surrounding the fish health that developed throughout of the trial, where increasing health problems towards the end of the trial were observed. The scoring procedure was also based on human assessments; thus, expectation and habits of the operator's scoring appetite are plausible factors that can have impacted the scores to some extent.

4.4 Appetite and growth

Feed conversion and growth efficiency are, as previously stated, affected by a wide range of environmental and biological factors (Rønnestad et al., 2017). However, this thesis has focused on the effects of dietary macronutrient levels on feed intake and, consequently, growth. Under commercial fish farming conditions, with an ideal commercial diet, a clear relationship is expected to be found between feed intake and growth. Deviations from this relationship can be, therefore, used to identify diets with low performance regarding utilization of the feed. In the present study, a high variability in the relationship between appetite and growth between the diets was observed. Indeed, the only period where the specific growth rate was significantly correlated with the appetite measurements was from 46 weeks into the trial until the final weighing in week 60. This growth-appetite relation, or rather; deviation, is an interesting result. For protein, the fish fed with the high protein content group performed worse than the rest in regards of growth. It also showed low feed intake on video recordings, but a high appetite score. It is hard to pinpoint why the difference between video feed intake and daily assessment of appetite scoring is so large. One reason might be due to randomized scheduled videos, which might result in videotaping and analyzing the fish's feed intake on days with lower appetite. This possible high day-to-day appetite variation is described to be normal for Atlantic halibut (Tuene and Nortvedt, 1995). The fish fed with high lipid diets (23-30% dietary lipid) clearly showed the lowest appetite response. However, with exception of the terminated group Diet 7, they managed to perform well in regards of growth, showing higher growth rates than the low lipid diets throughout the trial timeline. With these three diets with 23-25% dietary lipid levels performing well and the 30% lipid diet as mentioned resulting in emaciation, a lipid tolerance limit seems to be reached with the 25-30% range.

The growth efficiency index describes the relation of the collected appetite data with growth. The major finding related to the GEI was that fish fed the high lipid diets had much higher

apparent growth efficiency than the fish fed with mid and low lipid level diets. This can either be explained by high lipid levels having a potential in giving a very good feed conversion rate growth boost. This however, has not been seen in previous findings, where increasing dietary lipid over 14% has not shown any beneficial effect (Martins *et al.*, 2007), and since our project did not have the total feed intake data by the use waste feed collectors, this is a rough claim. Like previously mentioned, and presented in **Table 3**, the high energy density in the high lipid diets might affect the data here, since the feeding automat doses, were calibrated to feed set amounts of feed, determined in grams. Another possible explanation is that the high lipid levels alter the halibut´s feeding behavior away from a high morning feeding activity, and that their actual feed intake is higher than our measurements show. Shifting towards a slower, steady intake over the whole period would make it so that our early morning-feeding measurements of both feed intake and appetite score will fail to capture and estimate the total feed intake, especially when comparing to fish desperate for food. Like previously stated, Diet 3 showed an especially high response and interest at start of feeding for the latter half of the trial. This high protein and relatively low energy diet scored high in feed intake, and in appetite score, while resulting in the lowest fish mean weight at end of trial, indicating an especially low feed efficiency. Calculated GEI for these fish showed to be the lowest of all experimental groups, indicating a very suboptimal feed conversion in regard to this diet.

4.5 Feeding behavior

The quantified activity in the tanks around morning feeding was examined to assess its impact and effect on appetite. It was found that some of the experimental diets (2, 4, 9 and 10) had a significantly increased total activity both through the whole video period, and after feeding started. These diets, however, didn't have any remarkable similarities in macronutrient compositions, making it difficult to link increased activity to any dietary factor. The anticipatory index, i.e., the change in fish activity leading up to start of feeding, did not present any significant differences for any of the diets. A negative correlation between elevated activity and appetite was found, which might be a pattern linked to stress. Increases in activity has been previously described to affect the halibut´s performance through decreased growth rates (Kristiansen *et al.*, 2004). Reasons for this seemed to be the energy cost of swimming, and like we describe; a lowered feeding motivation. Further, increased anticipatory index was negatively correlated with appetite, i.e., the highest appetite was observed at an anticipatory

index slightly under one. This suggests that a slight decrease in activity ahead of feeding start results in higher pellet intake. This supports the findings of Nilsson *et al*., (2010), the halibut´s conditioning response ahead of feeding showed to be much more cautious than what´s seen for other species, possibly reflecting the halibut´s "sit-and-wait" foraging strategy. The three different ingestion behaviors; IB1 (Stand-still ingestion), IB2 (Moderate movement ingestion) and IB3 (Swimming ingestion) were quantified, mapping the characteristics of the typical halibut feed intake. This analysis resulted in some interesting findings. Not too surprisingly, IB1 frequency showed to negatively correlate with the total feed intake. When low feed interest made it so pellets had time to sink, and thereby rest on the tank floor, there was more occurrence of the more passive IB1. This was most frequent in diets 4 and 7, both high lipid diets, and both among the lowest in feed interest in the video recordings. This supplements the argument that the fish fed high lipid diets showed less interest in food during morning feeding, seeming "lazier" so to say. Further, there was a significant negative correlation between IB2 and total activity in the tank during feeding. This ingestion behavior, with a feeding movement involving a shorter lunge towards the pellet is described as a typical halibut ingestion behavior (Gibson, 2007; Nilsson *et al.*, 2010). In video analysis this was seen as a very characteristic movement. Reduced frequency of this natural behavior potentially caused by a decreased feeding motivation, along with the correlating high activity thus might be a stress responses, like described in (Berge and Storebakken, 1991).

4.6 Gut transit and return of appetite

It has been suggested that stomach-emptying is linked with the return of appetite in multiple teleost species (Grove *et al.*, 1978, 1985; Gwyther and Grove, 1981; Sims *et al.*, 1996; Álvarez and Ramírez, 2001). This is particularly well described for rainbow trout (Grove *et al.*, 1978). However, this thesis failed to show a clear correlation between the GI-tract compartments content and appetite for Atlantic halibut. Although, no conclusion should be drawn from these results, since the sampling procedures shown to be suboptimal. These non-ideal feeding regimes for the gut content analysis, mentioned in methodological considerations, combined with a low appetite in the sampling period made it hard to get enough content in the different GI-tract segments to do the analyses. The role of stomach emptying in appetite control should be investigated further.

4.7 Conclusion

This thesis investigated and mapped the appetite response, feeding behavior and growth performance of grow-out Atlantic halibut. The following conclusions were drawn based on the initial hypothesis and results obtained:

- Appetite, based on recordings of feed intake and appetite score assessments, was affected by the macronutrient level of the diets. Fish fed high lipid diets (23-25% dietary inclusion) clearly showed the lowest appetite, while increasing carbohydrate levels up to 25% positively affected appetite.
- Feeding behavior, measured in in-tank activity and different behaviors during feed ingestion did not show any significant differences between the macronutrient levels.
- The relationship between feed intake and growth, assessed by the growth efficiency index (GEI), showed great differences for the varying dietary macronutrient levels. GEI was mostly correlated with dietary lipid levels, where the elevated lipid level resulted in increased GEI.
- There were clear differences in growth performance between the fish fed different macronutrient diets, with highest growth found for fish fed diets with dietary protein levels from 46 to 56%, lipid from 9 to 25% and carbohydrates from 5 to 25%.
- The role of GI-tract content and its effect on appetite control remains unclear due to inconclusive results and technical issues.

Additionally, signs of tolerance limits for macronutrient levels were found. High lipid levels (at 30%) resulted in marked reduced growth. Elevated protein levels at 69 and 77% also negatively affected the fish growth, while the full range of carbohydrate content (5 to 25% dietary inclusion) did not affect the fish growth.

The findings indicate the possibility to formulate lower protein feed compositions, with elevated dietary inclusion of lipid and carbohydrates: However, more studies are needed to explore this further.

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Appendix

Table 1: The set pause and pulse times determining pauses between each feed pulse and the length of each pulse.

Tank	Pause time (s)	Pulse time (s)
1	859.5	2.0
2	801.9	2.0
3	729.9	2.0
4	182.4	0.5
5	188.5	0.5
6	197.4	0.5
7	419	1.0
8	436.9	1.0
9	400.9	1.0
10	373.4	1.0
11	354.1	1.0
12	374.5	1.0
13	361.3	1.0
14	404.5	1.0
15	380.5	1.0
16	372.1	1.0
17	319.3	1.0
18	366.1	1.0

Table 2: Ingredient list for the trial diets.

Diet	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)
Fish meal	59.10	50.50	63.30	46.00	40.20	52.90	45.70	60.90	71.60	41.70	48.70	55.50

Wheat gluten	15.00	12.82	16.07	11.68	10.20	13.43	11.60	15.46	18.17	10.58	12.36	14.09
Wheat	9.49	14.09	9.32	15.06	15.61	9.30	13.24	3.83	2.82	9.04	4.37	1.44
Tapioca starch	0.00	8.40	3.30	5.40	12.10	0.00	0.00	16.25	4.00	23.40	26.50	24.90
Fish oil	8.00	7.15	2.95	11.50	11.70	12.80	15.60	0.20	0.00	7.50	3.10	0.46
Marine lecithin	4.60	3.95	1.80	6.50	6.60	6.90	9.30	0.20	0.00	4.20	1.75	0.35
NaH2PO4	1.40	1.28	1.20	1.50	1.40	1.91	1.70	1.30	1.30	1.50	1.50	1.45
CaCO3	0.45	0.00	0.00	0.50	0.50	0.90	1.00	0.00	0.00	0.40	0.00	0.00
Stay-C	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Vitamin mix	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.5	0.50	0.50	0.50
Mineral mix	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.5	0.50	0.50	0.50
Lys	0.50	0.45	0.55	0.45	0.35	0.45	0.45	0.50	0.6	0.35	0.40	0.45
Thr	0.25	0.20	0.25	0.20	0.18	0.20	0.20	0.20	0.25	0.17	0.16	0.20
Met	0.05	0.00	0.05	0.05	0.00	0.05	0.05	0.00	0.05	0.00	0.00	0.00
His	0.10	0.10	0.15	0.10	0.10	0.10	0.10	0.10	0.15	0.10	0.10	0.10
Yttrium oxide	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01

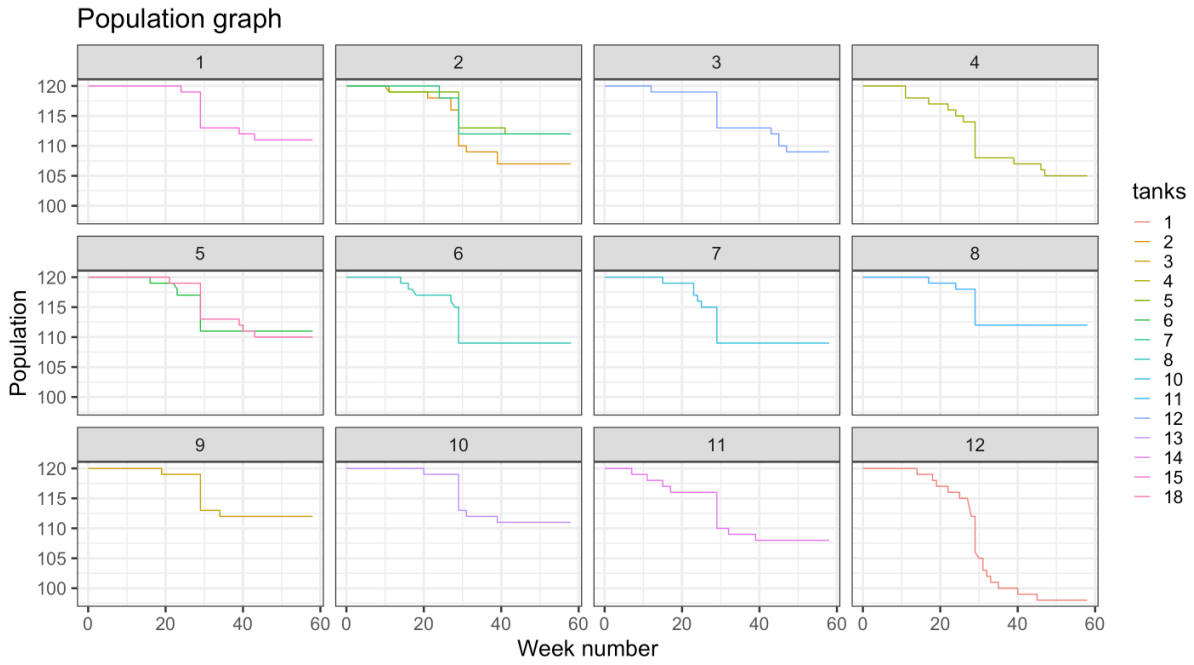

Figure 1: Population graph for the 15 experimental tanks over the trial period.

Table 3: Reported mortality in the facility over the trial period, with weight, length and PIT tag of the dead fish.

Date	PIT TAG	Length (cm)	Weight (g)	Tank
1/10/2022	2484	31	392	1
2/2/2022	.0464	30	256	1
2/11/2022	no tag	26	171	1
3/7/2022	33A1	28	155	1
3/28/2022	3996	28	185*	1
4/12/2022	0F1A	29	270	1
4/12/2022	5873	29	211	1
4/12/2022	5709	29	228	1
4/26/2022	51BE	27	170	1
5/6/2022	143B	29	242	1
5/6/2022	1E.19	30	191	1
5/16/2022	5A1E	28	226	1
5/20/2022	0F0D	29.5	203	1

6/6/2022	556B	34	277	1
7/8/2022	1787	32	197	1
8/15/2022	3B70	27.5	187	1
10/26/2022	21F8	33	246	1
12/14/2021	28EB	29	202	2
2/23/2022	467E	29	230	2
4/6/2022	0BAA	30	323	2
4/8/2022	2DEB	30	262	2
5/4/2022	5017	32	295	2
7/1/2022	578A	29	227	2
7/4/2022	3B74	27.5	213	2
2/14/2022	1D65	26	188	3
5/25/2022	805	28	180	3
10/25/2022	3554	31	263	3
10/31/2022	2322	29	235	3
12/17/2021	2E.42	29.5	250	4
12/17/2021	2F98	29	239	4
1/26/2022	3F0A	27	121	4
3/3/2022	1CC2	28	180	4
3/18/2022	0F11	25	164	4
3/31/2022	366C	27.5	174	4
7/4/2022	1134	31	282	4
8/22/2022	4414	31	294	4
8/24/2022	09F5	30	265	4
9/28/2022	13F9	35	496	4
12/20/2021	0B1C	27.8	210	5
7/18/2022	3A0C	32	224	5
1/20/2022	07B4	31.5	313	6
3/8/2022	46B5	27	192	6
3/14/2022	2077	27	285	6
3/18/2022	377E	31	409	7
8/26/2022	5437	28	247	7

1/5/2022	CC18	26	178	8
1/19/2022	CFE7	28.5	214	8
2/2/2022	A950	28.5	225	8
4/7/2022	4AE1	26	186	8
4/12/2022	B744	28	194	8
5/9/2022	277A	33.5	426	9
6/8/2022	4F88	30	294	9
6/25/2022	20F7	28.5	220	9
7/11/2022	.0666	33.5	414	9
9/20/2022	1D71	31.5	881	9
10/25/2022	54A5	34	347	9
10/25/2022	26A9	32	312	9
1/17/2022	2F5B	29	227	10
3/14/2022	21A1	26	136	10
3/14/2022	3950	29	239	10
3/21/2022	58CD	27.5	155	10
3/23/2022	07C4	30	204	10
1/31/2022	14A1	26	150	11
3/21/2022	5344	30	251	11
10/11/2022	4E6C	34	379	11
12/23/2021	2539	17.5	191	12
7/28/2022	3F03	33.5	373	12
8/10/2022	5016	33.5	356	12
8/12/2022	2E9A	31.4	271	12
8/26/2022	06C9	29.5	243	12
2/21/2022	2A60	28.5	196	13
5/7/2022	1AF0	29	269	13
7/4/2022	130A	28	243	13
10/3/2022	0DDE	31	260	13
10/28/2022	15ED	32	288	13
11/22/2021	B52E	25	193	14
12/20/2021	B5D9	29	207	14

1/12/2022	A89F	26	142	14
1/26/2022	AFA2	27.5	162	14
5/13/2022	CCD3	29	201	14
7/1/2022	4F91	32	373	14
3/21/2022	04D2	28.5	197	15
7/1/2022	47B5	29	262	15
7/28/2022	2546	31.5	325	15
9/27/2022	2257	40	709	15
9/28/2022	4500	29	254	15
11/2/2021	22A5	24.5	135	16
12/21/2021	37ED	33	372	16
1/13/2022	1593	28	205	16
3/1/2022	2361	28.5	223	16
4/6/2022	3E.92	31	293	16
6/8/2022	4520	29	290	16
3/2/2022	1ED8	27.5	209	17
3/29/2022	0A96	32.4	355	17
5/9/2022	0D58	36.5	610	17
10/4/2022	542E	30	291	17
2/27/2022	26F3	28.5	220	18
7/4/2022	3FBE	30	317	18
7/8/2022	54B2	34.5	510	18
7/27/2022	041A	35.5	444	18
10/16/2022	26AD	32	302	18
11/1/2022	31C5	34	305	18

Table 4: Growth efficiency index for each dietary protein level over the four inter-weighing intervals

Proteingroup	**Mean GE1**	**Mean GE2**	**Mean GE3**	**Mean GE4**	**Mean GE**

high	394.9847 ± 167	314.633 ± 258	262.608 ± 181	257.932 ± 88	945.948 ± 400
mid	384.839 ± 207	580.576 ± 302	1335.464 ± 1009	278.608 ± 101	1501.618 ± 973
low	330.9735 ± 198	392.809 ± 378	620.2031 ± 547	235.894 ± 130	1337.672 ± 986

Table 5: Growth efficiency index for each dietary lipid level over the four inter-weighing intervals

Lipidgroup	Mean GE1	Mean GE2	Mean GE3	Mean GE4	Mean GE
high	514.523 ± 221	740.893 ± 338	1849.571 ± 729	373.002 ± 69	514.523 ± 1224
mid	320.016 ± 167	260.851 ± 201	244.645 ± 131	216.513 ± 85	320.016 ± 328
low	353.382 ± 243	287.59 ± 129	512.971 ± 163	201.525 ± 79	353.382 ± 609

Table 6: Growth efficiency index for each dietary carbohydrate level over the four inter-weighing intervals

Carbgroup	Mean GE1	Mean GE2	Mean GE3	Mean GE4	Mean GE
high	380.663 ± 209	453.294 ± 282	910.630 ± 923	231.984 ± 82	1654.473 ± 782
mid	341.054 ± 288	491.127 ± 412	644.5747 ± 729	338.106 ± 98	1166.742 ± 1288
low	365.492 ± 179	344.917 ± 299	810.9829 ± 862	213.971 ± 114	1153.502 ± 400