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Evaluation of plastic responses to nutritional programming by various feed sources in brown and rainbow trout fry

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Stéphanie Céline Michl

(Master of Science der Biologie)

geboren in Fontainebleau, Frankreich
aus Erding, Bayern

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Dekan:	Prof. Dr. Joachim Krieter
Erster Berichterstatter:	Prof. Dr. Carsten Schulz
Zweiter Berichterstatter:	Prof. Dr. Jeffrey A. Hutchings
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This is for all the people who devoted their lives to build a better world.

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General introduction

Current constraints and future prospects in global aquaculture

Aquaculture, the farming of aquatic animals, has always been highly dependent upon capture fisheries for the production of fishmeal and fish oil which are used in aquafeeds [1]. 16.3 million tonnes of the global marine fisheries have been processed to fishmeal and fish oil in 2012, although total landings in capture fisheries decreased by 2.4% since 2000 [2]. Thus, feed manufacturers were increasingly taking steps towards finding promising fishmeal and fish oil substitutes for aquafeeds. In salmonid aquaculture for example, plant-derived feed ingredients are of high importance. In Norway, one of the largest salmon producing countries, the average use of plant ingredients increased from 10% to 70% from 1990 until 2013 [3]. Likewise, the use of fishmeal rapidly decreased from 65% to an average use of 18% in salmon feeds in 2013 [3]. However, aquaculture is one of the fastest growing food production sectors in the world and thus from a broader perspective, the growing demand for fish products will eventually result in a continuously growing demand for fishmeal and fish oil [4]. Therefore, the potential and impact of fishmeal and fish oil substitutes on growth performance and health of fish have also been a central question in aquaculture research. Several studies in the last two decades have focused on plant-based raw materials. Products from soybeans, peas and lupines, canola, wheat and corn, amongst others, have been investigated for their suitability in aquafeeds [5–14]. However, despite all this research effort, quite a few challenges remain that impair a successful utilisation of plant-derived proteins at high dietary inclusion rates [15]: The nutritional value of plant-based raw materials compared to fishmeal is often limited. For example, the crude protein content of corn gluten meal is only about 60% and not only contains high levels of indigestible carbohydrates, but is also deficient in lysine which requires the supplementation of artificial amino acids [15]. Soybean meal, especially soya saponins have been associated with enteritis in salmonids [6, 8]. Hence, quite a lot of effort needs to be taken to produce purified protein concentrates which are also more suitable for efficient feed formulations. Soy protein concentrates can be used in much higher percentages without causing enteritis [15]. However, glucosinolates, phytic acid and other anti-nutritive factors that can be co-purified in protein concentrates, e.g. from canola and rapeseed, can cause additional severe physiological problems. Other challenges in replacing fishmeal with plant meals are, for example, the necessary supplementation of trace minerals that are insufficient with reduced dietary fishmeal levels, or the unknown function of so called “unidentified bioactive growth factors”, possibly amines or steroids found in fishmeal that are beneficial for fish growth [15]. Nevertheless, the percentage of fishmeal that is used in early feeding diets is still about 46-68% [16] and trout fry should be fed

intensively with a daily ratio of 3-5% of body weight [17]. The production time of rainbow trout varies between nine months and three years until harvesting, depending on region (e.g. Mediterranean or North Atlantic) and rearing conditions (e.g. sea cages or recirculating aquaculture systems, RAS). In salmonid farming the rearing from eggs to juveniles is the most labour and cost intensive phase of the whole production cycle. A reduction of the fish meal content in diets for first feeding salmonids would thus result in a reduction of costs for hatcheries during the first year rearing phase. It was demonstrated [18, 19] that rainbow trout can be fed from first feeding on with plant proteins and plant oils, but the growth of these fish was significantly reduced. Moreover, the microarray based study [19] revealed an upregulation of the immune system in fish fed a completely plant-based diet, indicating a generation of physiological stress through this type of feeding.

Essential components of the gastro-intestinal system

First feeding is one of the crucial time points in all fish larvae. After hatching, the yolk sac is the only nutrient source until fish larvae are able to ingest external feed. Depending on the species, this time period varies between some days and several weeks. In marine fish larvae the digestive tract at first feeding is only a tube without stomach, however with functional pancreas, liver and gall bladder [20]. Salmonids in contrast already have a functional stomach with fully developed gastric digestion before the onset of first feeding, which leads to higher protein assimilation efficiencies compared to stomach-less fish larvae [21]. The reason for this is the large yolk sac of salmonids which results in a relatively longer developmental period of salmonid embryos before they need to start exogenous feeding [22]. The gastro-intestinal (GI) tract of fully developed juveniles and adults consists of several compartments with different functionalities with regard to the digestion of feed (please see [22] and [23] for the detailed description of the following summary). After ingestion, feed particles pass the oesophagus to enter the stomach – an acidic environment generated by the secretion of HCl from the gastric glands, which provides ideal working conditions (pH 1.5-2.5) for digestive enzymes like the protease pepsin. Pepsin is breaking down large proteins into smaller, soluble peptides and the initial cleavage efficiency of pepsin is crucial for subsequent proteolytic reactions like the tryptic digestion of peptides in the intestine [24]. Pepsin is an endopeptidase and its main cleavage sites are between aromatic amino acids, such as phenylalanine and tyrosine. The stomach not only provides the optimal environment for digestive enzymes, it also mixes the ingesta by peristaltic movements and its gastric mucous cells are releasing hormones that regulate digestion and feeding behaviour. The stomach is followed by the intestine, which is divided into several functional regions. The pyloric sphincter opens the stomach towards the intestine and hence controls the time that feed remains in the stomach, but also the amount of acidic digesta that enters the rather

neutral (pH 7-8) intestine and the pyloric caeca. Pyloric caeca accompany the intestine and are only present in fish that possess a stomach [23]. The number of pyloric caeca is highly variable between species and so are their functions: as storage for digestive enzymes, the secretion of hormones and an inflation of the absorptive surface for already soluble nutrients. The digestive enzymes present in pyloric caeca and the intestine are released by the pancreas and the gall bladder, accompanied by sodium bicarbonate to neutralize the acid gastric fluids and to promote an optimal environment for intestinal activities of several other digestive enzymes, e.g. trypsin, chymotrypsin, elastase, collagenase, lipases or amylase. The breakdown of proteins continues in the intestine, where the endoprotease trypsin digests the smaller polypeptides released from the stomach. The resulting amino acids can then be absorbed. At the same time, small proteins or polypeptides can also be assimilated via pinocytosis and intracellular digestion [22]. Dietary carbohydrate breakdown is maintained in the intestine by various enzymes, but amylase, a member of the glycosyl hydrolases, is one of the main ones involved. The membrane linked enzymes are synthesised in enterocytes and hydrolyse the α -1,4-glycoside bonds in starch and glycogen. α -amylase is only produced in the exocrine pancreas [25] and specialised on the cleavage of starch and is highly conserved between organisms. Furthermore, it is a fundamental component of the enzymes present in the early development of piscivorous fish [26], although prey fish does not contain starch. It has also been demonstrated that amylase is associated with the gut microflora because of carbohydrase-producing bacteria [27]. The release of digestive enzymes by the intestinal microbiome is a phenomenon that has been actively investigated in recent years [28]. Not only amylase has been detected, but also proteases, chitinases or lipases of *Bacillus* species have been isolated from the digestive tract of Atlantic salmon [29]. Hence the intestinal microbiome can have a tremendous additional effect on the digestive capacity of a fish.

Effects of plant-based diets on the gastro-intestinal tract

In fish nutrition, knowledge about the digestion of dietary protein is of high importance due to its key role for fish growth [30]. Digestibility studies have been successfully used to determine the potential of plant proteins in feeds for salmonids [5, 7, 31]. However, due to size limitations these methods are hardly transferrable to small fry and *in vitro* methods seem to be still not suitable for a direct correlation to *in vivo* digestibility of feed ingredients [32]. Plant derived feedstuffs need to fulfil certain criteria to be an appropriate substitute for fishmeal [33]: low levels of fibre, starch and especially non-soluble carbohydrates and anti-nutritive factors, a relatively high protein content, a favourable amino acid profile, and high nutrient digestibility and a reasonable palatability. The ability to digest different types of feeds like plant meals or fishmeal is primarily based on the individual digestive capacity of the fish,

which includes all the components of the GI tract. Digestive enzymes for example, can be strongly affected by the diet-type. A comparison of rainbow trout fed a fishmeal diet and diets partially or totally replaced by plant materials [34] showed that three hours post feeding of the fishmeal diet, a peak in total protease activity occurred, whereas no such an effect was seen for fish fed the plant meal diet. Another study [35] additionally suggests that proteins of plant origin are processed differently by digestive enzymes compared to animal derived proteins, which might result in an altered amino acid bioavailability. Plant meals furthermore contain high amounts of long-chain carbohydrates which are difficult to digest [25] and can potentially reduce protein [36] or whole diet [37] digestibility. Furthermore it was shown in sea bass that amylase activity increased in direct relation to dietary carbohydrate levels [38]. However, not only digestive enzymes are affected by plant based diets, but also the intestinal microbiome. [39] found significant differences in the gut microbial community of rainbow trout fed with peas, soybean and canola products compared to fish fed with fishmeal. However, these differences were smaller if plant protein concentrates were used and bigger if plant meals were used. Moreover, a quantitative reduction of cultivable bacteria was observed when soybean meal was fed to juvenile rainbow trout [40] with a fishmeal-replacement of 45%. The replacement of fish oil by rapeseed oil and the simultaneous substitution of fishmeal by pea meal also induced a significantly different development of the gut microbiota in first feeding trout fry [41].

Adaptation, imprinting and nutritional programming

All of the introduced studies so far clearly demonstrate that the feeding of plant based feedstuff to salmonids – especially juveniles – still implies physiological consequences that have not yet been overcome. This dissertation focuses on a novel approach that addresses those challenges by evaluating the concept of nutritional programming (a specific form of *metabolic programming*) with regard to the impact of plant based diets not only on growth or digestive enzyme activities, but also on the intestinal microbiome of trout. An organism can adjust to external stimuli in several ways. One possibility is *adaptation*, defined as “[...] the modification of the characteristics of organisms that facilitates an enhanced ability to survive and reproduce in a particular environment” [42]. In other words: an organism modifies itself to fit the surrounding parameters and to be able to survive or reproduce. Such modifications can occur at several levels – morphological, behavioural or cellular. Adaptation can be found in the skeletal muscle metabolism of athletes [43] or in the body temperature regulation of mammals [44], in the freeze tolerance of amphibians [45] and on a cellular level as biochemical adaptation of enzymes [46]. Adaptation usually leads to two possible outcomes: homeostasis, the preservation of a current status, and enantiostasis, the maintenance of a given physiological function [42]. Enhancements of adaptation are *metabolic imprinting* and

metabolic programming. Metabolic programming is defined as “[...] a dynamic process whose effects are dependent upon a critical window [...]” and metabolic imprinting in contrast is “[...] more strictly associated with imprinting at the genomic level” [47], which means that the effects of imprinting are directly associated with modifications of DNA, such as methylation or histone modifications [48]. Important for both concepts is a stimulus that occurs in an early life stage and provokes a permanent or long-term physiological effect in later life stages. From large epidemiological studies in humans it is known that early nutrition can have severe effects on the later health status of children. The Avon Longitudinal Study of Parents and Children (ALSPAC), for example, demonstrated that obesity, neurodevelopment, asthma, allergies or diabetes are linked to an early dietary exposure – either as embryos with stimuli that are coming from the mother’s diet or as infants [47]. In aquaculture research, the idea of nutritional programming is becoming of higher importance considering the challenges aquaculture is currently facing [48]. It was demonstrated that an early exposure of Siberian sturgeon to high dietary glucose levels disrupts gluconeogenesis later in life [49]. In rainbow trout however, the early feeding with a hyperglucidic diet significantly enhanced the activity of digestive enzymes involved in the carbohydrate metabolism when fed with a high carbohydrate diet as later juveniles [50]. Moreover, a second study with rainbow trout [51] revealed that feed intake, growth rate and feed efficiency of fish fed with a plant-based diet were significantly enhanced after early feeding of plant derived proteins during the larval stage. In contrast, during a third study [52] no beneficial and long-term effects of an early stimulus on the carbohydrate utilisation could be detected, even though a lasting influence on the glucose metabolism has been shown. As known from humans, metabolic programming can also occur via parental feeding history: in juvenile gilthead sea bream it was observed that fish, fed with low fishmeal and low fish oil diets, demonstrated better growth performance if the corresponding broodstock had been fed with plant oils instead of fish oil [53].

The aim of this thesis: nutritional programming of carnivorous fish

Nutritional programming can be a fundamental tool to address the problems of plant-based diets in carnivorous fish. Several attempts have proven that nutritional programming in fish is possible; however it remains unclear whether the outcome is permanent, and beneficial. The approach used in this thesis is built upon the hypothesis that digestive enzymes and the intestinal microbiome are amongst the most critical parameters that influence an efficient utilisation of plant material by trout. Two independent experiments will be presented in this thesis and several parameters investigated will contribute to a better understanding of the underlying mechanisms, necessary for a successful application of nutritional programming in aquaculture. The first experiment was conducted with all-female rainbow trout

(*Oncorhynchus mykiss*), originating from a well-established trout strain in North America. The second experiment was conducted with the offspring of wildly caught brown trout (*Salmo trutta trutta*) from Germany. The experimental animals were reared in a recirculating aquaculture system and fed for several weeks with different levels of fishmeal substitution by plant-derived proteins. Most of previous research in metabolic programming of fish has focused on a short-term stimulus of several days to promote an improved and long-lasting utilisation of plant based feedstuff in carnivorous fish. This project follows a novel approach by applying a long-term 'stimulus' at the onset of first feeding for a period of, at least, eight weeks, immediately followed by a diet change in a complete cross-over design. The advantage of this procedure is the unrequired intermediate feeding with another diet-type that could again influence the activity of digestive enzymes and the gut microbiome. For example, [51] were feeding a fishmeal diet in-between the stimulus and a final challenge with a plant-based diet, which lead to a drastic drop of voluntary feed intake at the start of the challenge, however this drop was even stronger if fish had never been fed with plant ingredients before. Part of the concept in this thesis was therefore the integration of an intermediate diet-type that contained at least 50% fishmeal, but might not induce the same repelling feeding behaviour. Previous research mostly focused on the nutritional programming effects on components of the carbohydrate metabolism. However, there is already evidence that the digestion of plant proteins is not comparably efficient as the digestion of animal protein, and pepsin is the first digestive enzyme involved in the cleavage of proteins. Nevertheless, plant-based feedstuff is almost always accompanied by unwanted carbohydrates and hence amylase and pepsin were the two digestive enzymes selected to illustrate ongoing metabolic responses to the dietary treatment. As pointed out in previous sections, the metabolic contribution of the intestinal microbiome is of high importance in fish nutrition. The bacterial gut community has been shown to be severely affected by diet-type and subsequent community-shifts could tremendously contribute to an altered digestive capacity. During the early development of fish, the bacterial community evolves and thus it was hypothesised in this thesis, that the early nutritional stimulus applied will induce a permanent effect on the intestinal microbiome of fish. The strength of this influence can be modulated by nutritional stimuli, but it could also have already been affected by the parental life history of experimental fish. During the raise of salmonid aquaculture in the last decades, rainbow trout has been facing several attempts of selective breeding and optimisation processes with regard to the use of feed optimised for tank feeding and rapid growth. The ability to utilise diets other than those usually used in trout production could have already been diminished by imprinting mechanisms of trout strains selected for production since a long time. Wild brown trout, compared to rainbow trout, has not faced years of selective breeding for certain diets and could therefore maintain another genetic potential for the

utilisation of plant meals. Thus, this dissertation will provide new insights into the metabolic adaptation and programming mechanisms of carnivorous fish with regard to plant-based feed ingredients. It will explicitly investigate whether a long-term stimulus with plant-derived proteins can permanently enhance the utilisation of this type of diet in juvenile trout.

The contribution of this thesis to current knowledge

Chapter 1 discusses the following research questions:

- How does the inclusion of plant-derived proteins into first feeding diets affect the growth performance of wild brown trout fry?
- Do dietary plant-derived proteins affect the amylase and pepsin activities of first feeding fry or of juvenile fish, and how do digestive enzyme activities contribute to growth performance?
- Can early feeding of wild brown trout fry with plant-derived proteins induce a permanent improvement in utilising plant-based diets later in life due to the concept of nutritional programming?
- Does the parental feeding history of strictly carnivorous wild brown trout broodstock affect the potential of first feeding fry in utilising plant-derived proteins?

Chapter 2 examines the following scientific issues:

- Do plant-based diets impact the microbial gut community of wild brown trout fry?
- How are plant-derived dietary proteins affecting the formation of the intestinal tract microbiome of first feeding fry?
- What impact do different plant-derived dietary protein inclusion levels have on the intestinal microbial diversity of brown trout?
- Are specific microbes of the gut microbiome of wild brown trout similar to the gut microbiome of other salmonids?
- Do plant-based feed ingredients promote specific bacterial taxa compared to fishmeal, and if yes what are the physiological consequences?

Chapter 3 addresses the following research aims:

- Can rainbow trout be reared from first feeding on with diets exclusively derived from plant proteins?
- If yes, what effects can be expected for growth performance in comparison to fishmeal or to 50% dietary plant proteins?
- How do antinutritive factors affect growth and digestive enzyme activities of trout fry?

- Is it possible to mediate a positive long-term response of rainbow trout to the early feeding of plant-based diets?
- What levels of plasticity are involved in physiological responses of juvenile trout towards the feeding of plant-derived proteins?

Chapter 4 highlights the following scientific topics:

- Do plant-based diets influence the intestinal microbiome of first feeding rainbow trout fry?
- If yes, what are the specific effects for the bacterial community structure?
- Is the microbial diversity in the gastro-intestinal tract of first feeding rainbow trout fry affected by plant-derived dietary proteins?
- Does the bodymass of fish have an impact on the bacterial gut community?

The **General Discussion** will focus on the following superordinate subjects:

- A comparison of the effects of plant-based diets on the growth performance of rainbow trout and wild brown trout
- The activities of amylase and pepsin prior to first feeding in both species
- The phenotypic plasticity of wild and farmed trout towards plant-based diets with regard to digestive enzyme activities and to the intestinal microbiome
- The potential of nutritional programming in carnivorous fish species for long-term improvement of plant-derived feedstuff
- The concept of a core microbiome for salmonids

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Chapter 1

Plastic responses by wild brown trout (*Salmo trutta*) to plant-based diets

Stéphanie Céline Michl^{a,b,c}, Benjamin Weis^a, Jeffrey A. Hutchings^{d,e}, Carsten Schulz^{a,b}

^aGesellschaft für Marine Aquakultur mbH (GMA) Büsum, Hafentörn 3, 25761 Büsum, Germany

^bInstitute of Animal Breeding and Husbandry, Department for Marine Aquaculture, Christian-Albrechts-Universität zu Kiel, Hermann-Rodewald-Str. 6, 24118 Kiel, Germany

^cGEOMAR Helmholtz Centre for Ocean Research, Wischhofstr. 1-3, 24149 Kiel, Germany

^dDepartment of Biology, Dalhousie University, 1355 Oxford Street, Halifax, Nova Scotia B3H 4R2, Canada

^eCentre for Ecological and Evolutionary Synthesis (CEES), Department of Biosciences, University of Oslo, PO Box 1066 Blindern, NO-0316 Oslo, Norway

Abstract

Decreasing fishmeal availability and increasing prices promote the usage of plant-derived feedstuff as a substitution for fishmeal in commercial salmonid diets. However, little is known about the impact of plant-derived feedstuff on juvenile brown trout (*Salmo trutta*), a species that exhibits strong phenotypic plasticity with various genetic sub-structures and high overall genetic diversity. Thus, the production of brown trout for restocking purposes preferentially uses wild fish as broodstock to avoid loss of genetic variability. Because of nutritional programming, the strictly carnivorous feeding habit of wild brown trout broodfish could nevertheless have a negative impact on the digestive physiology of fry and fingerlings that are fed with commercial trout diets. The present study, therefore, investigated whether the feeding of plant-based diets from first feeding onwards induced a permanent improvement in the utilisation of plant-derived protein sources in wild brown trout juveniles. Any plastic responses to the experimental diets resulting in a long-term physiological effect were hypothesised to be not only observed in growth performance, but also in altered amylase and pepsin activities. We demonstrated that (i) the feeding of wild brown trout fry with dietary plant proteins is beneficial during the first weeks of life and (ii) continuous feeding of at least 50% plant-derived dietary protein resulted in the same rate of growth when compared to the growth resulting from fishmeal as the exclusive dietary protein source. Pepsin and amylase activities were only partly affected by diet-type and it can be concluded that intestinal amylase and pepsin activities in juvenile brown trout are primarily regulated by intrinsic mechanisms. In the present experiment, we were not able to induce a permanent nutritional programming effect of the first feeding diet; instead, a cross-over diet change applied 89 days post first feeding demonstrated that wild brown trout fry exhibit highly plastic responses to different feeding strategies during the first months of life.

Key words: Amylase; Brown trout; Digestion; Digestive enzymes; Nutritional programming; Pepsin; Plant proteins; Proteases

1 Introduction

The production of brown trout (*Salmo trutta*) in aquaculture mainly focuses on juveniles for restocking purposes and increasing angling opportunities for recreational fisheries [1]. The production of adult fish for human consumption is of minor interest: growth rates of brown trout are not comparable to rainbow trout (*Oncorhynchus mykiss*) or Atlantic salmon (*Salmo salar*) and, thus, production is economically less competitive [2–4]. Juvenile brown trout are usually fed diets commercially manufactured for rainbow trout [1], which implies an increasing percentage of plant-derived feedstuff as a substitution for fishmeal [5] because of decreasing fishmeal availability and increasing prices [6].

The effects of various dietary plant-based resources on growth and health of salmonids have been intensely studied in the last two decades [7–11]. It has also been demonstrated that the digestive physiology of fish can be heavily affected by plant-based diets, especially due to digestive enzyme inhibitors or indigestible carbohydrates [12]. Nevertheless, most studies have focused on Atlantic salmon and rainbow trout, and little is known about the impact of plant-derived feedstuff on brown trout. Furthermore, no family-based breeding program has been established for brown trout aquaculture, in contrast to Atlantic salmon or rainbow trout [13]. Instead, wild fish are preferentially used as broodstock for restocking purposes to avoid loss of genetic variability [13].

However, it is known from studies on gilthead sea bream (*Sparus aurata*) that the composition of diets for broodstock directly influences the utilisation efficiency of diets for juveniles because of *nutritional programming* [14], a concept well known from studies on humans [15] and becoming of increasing interest in aquaculture [16]. *Nutritional programming* is dependent on an early-life stimulus that provokes a permanent or long-term physiological effect on later life stages [15]. Thus, the strictly carnivorous feeding habit of wild brown trout broodfish could have a negative impact on the digestive physiology of fry and fingerlings that are fed with commercial trout diets containing about 50% plant-derived proteins. On the other hand, *nutritional programming* is not only effective via reproduction: early feeding with plant-based diets has been shown to significantly improve growth and feed intake of rainbow trout later in life when fed again with plant-based diets [17]. Moreover, it was demonstrated that α -amylase gene expression in rainbow trout responded significantly to a hyperglucidic diet fed at first feeding and was significantly upregulated when this diet was fed again later in life [18].

Amylase is highly conserved between organisms and is a fundamental component of the enzymes present in the early development of piscivorous fish [19], although prey fish does

not contain starch. Moreover, it has been demonstrated that amylase is associated with the gut microflora of fish because of carbohydrase-producing bacteria [20]. Fish amylases are susceptible to anti-nutritive factors present in plant products. Proteinaceous amylase inhibitors are present in a variety of agricultural plants, such as wheat and beans [21, 22], and the processing of plant meals for use in aquafeeds not necessarily eliminates proteinaceous enzyme inhibitors. For example, α -amylase inhibitory activities were even increased after dehulling of dry beans [23] and α -amylase inhibitors were still present in a protein concentrate from winged bean [24]. Thus, an impact of plant-based feed ingredients on amylase activities, even from highly purified protein sources, could still be expected.

Pepsin is the key enzyme for gastric digestion. In contrast to marine fish larvae, salmonids possess a fully developed gastric digestion already at the onset of first feeding, which significantly improves protein assimilation efficiencies [25]. Although the intestinal enzyme trypsin functions as the main enzyme involved in protein digestion, it has been shown in a gastro-intestinal model that gastric digestion significantly influences subsequent bio-accessibility and bio-availability of nitrogen and phosphorous, in contrast to an exclusive alkaline digestion [26]. Moreover, another *in vitro* study demonstrated a significantly reduced nitrogen release from the protein hydrolysis of soybean meal compared to that of fishmeal by digestive enzyme extracts (acid and alkaline) of seabream (*Sparus aurata*) [27]. Furthermore, in humans it has been observed that acid secretion and gastrin release were significantly reduced by isolated soy protein compared to beef protein [28] and gastrin, a neuroendocrine hormone, stimulates the release of pepsinogen, the precursor enzyme of pepsin. Those observations support a continuous monitoring of pepsin activities in rainbow trout fry when plant-based proteins are applied from first feeding onwards.

A primary means of adjusting to external stimuli is through phenotypic plasticity: the non-genetic modification of physiological or morphological characteristics of an organism in response to environmental change. Brown trout exhibit strong phenotypic plasticity with various genetic sub-structures and high overall genetic diversity, which is hypothesised to allow wild trout to adapt to a variety of feeding habitats [13]. The present study, therefore, aimed to induce *nutritional programming* in wild brown trout by feeding plant-based diets from first feeding onwards. It was hypothesised that the early feeding of plant-derived protein sources would act as a nutritional stimulus provoking improved growth performance when the same diets were applied later in life. Therefore, the potential of wild brown trout fry to exhibit plastic responses to different plant-based feeding strategies was explored and investigate whether the feeding of plant-based diets from first feeding onwards induced a permanent improvement in the utilisation of plant-derived protein sources. Additionally, the

digestive enzymes pepsin and amylase were monitored from hatching until the end of the experiment for their response to the different feeding regimes and evaluated for their involvement in nutrient utilisation.

2 Material & Methods

2.1 Experimental Animals

The experiment was conducted at the “Gesellschaft für Marine Aquakultur mbH” (Büsum, Germany). Eyed brown trout eggs (*Salmo trutta*) were reproduced at the Fischbrutanstalt Altmühlendorf (Germany) from wild brown trout caught in Schleswig-Holstein. All animal handling procedures were approved by the animal welfare officer of the “Gesellschaft für Marine Aquakultur mbH” and the local authority of Schleswig-Holstein, according to the German animal welfare law (TierSchG).

2.2 Experimental Diets

Three isoenergetic diets (diet X, diet Y and diet Z; see Table 1-1) were formulated and produced in cooperation with Skretting ARC (Stavanger, Norway). All diets were isonitrogenous in order to evaluate the effects of different protein sources on digestive enzyme activities without masking these effects by varying dietary protein levels. Fishmeal served as the exclusive protein source for diet X. In contrast, fishmeal contributed only 10% to the protein supply of diet Z. The remaining percentage was covered by various plant-protein sources. Diet Y is intermediate between diet X and diet Z with 50% plant-derived proteins and 50% fishmeal. All diets were extruded and crumbled in five different particle sizes from 0.5 to 3.0 mm to adjust feed particles according to the size of fry. The amino acid composition of each diet was formulated according to the NRC (2011) digestible amino acid requirements for small Atlantic salmon fry (0.2 - 20.0 g) and likewise was the composition of the vitamin and mineral premixtures in accordance with the NRC (2011) guidelines.

2.3 Chemical analysis of dietary nutrients and whole-fish body homogenates

The chemical nutrient analysis of all experimental diets and of whole-fish body homogenates was performed according to the European Commission Regulation (EC) No 152/2009 (European Union 2009). Dry matter content of whole-fish body homogenates was determined by freeze-drying whole fish for 72 hours before homogenisation. Dry matter content of feed samples was determined by drying samples directly at 103°C for 4 hours (ED 53, Binder GmbH, Germany). Crude ash content of feed and body homogenates was determined in a

combustion oven at 550°C for 4 hours (P300, Nabertherm, Germany). Methods after Kjeldahl (InKjel 1225 M, WD 30, Behr, Germany) and Soxhlet (Soxtherm, C. Gerhardt GmbH & Co. KG, Germany) were applied to determine the crude protein and crude lipid content of all samples. Gross energy was determined in a bomb calorimeter (C200; IKA, Germany). Fatty acid analysis and amino acid analysis of diets were performed by Skretting ARC.

2.4 Experimental Design

6000 eyed trout eggs were randomly distributed among three commercial hatching troughs (2000 eggs each) that were integrated into a recirculating waterbody. Average water temperature was $11.2 \pm 0.3^\circ\text{C}$ until hatching day and $12.4 \pm 1.3^\circ\text{C}$ for the remainder of the experiment. Average pH was 8.1 throughout the entire feeding trial. Feed was provided for the first time on day 20 post hatch (dph), but active first feeding of trout fry was observed on dph 28. The fish in each hatching trough were fed with one of the experimental diets, resulting in the 1st Feeding Diet groups X, Y and Z (see Fig 1-1). Each group was fed *ad libitum* by automatic feeders. Experimental animals were fed once per hour until dph 71, which was then gradually reduced until fish were fed four times per day until dph 89. The light regime was 15h light / 9h dark, starting at 06:00 am. On day 89 post hatch, the second feeding period started. 1440 trout fry of each 1st Feeding Diet group were transferred into 50 L aquaria that were integrated in the same water body. All diets were changed in a cross-over design for the second feeding period, resulting in 9 final treatment groups that were fed in triplicates, i.e. 27 aquaria with 160 fish each (Fig 1-1). Until day 137 post hatch, each of the experimental 2nd Feeding Diets was applied four times per day - in total 2.2% of the total biomass per day.

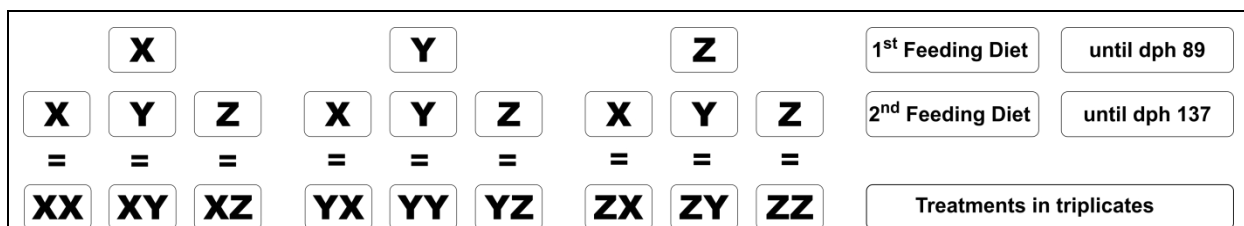


Fig 1-1. Experimental design of the feeding trial. The 1st Feeding Diets have been fed from first feeding until dph 89 in three separate hatching troughs without replication. After the diet-change, the 2nd Feeding Diets have been fed from dph 89 until dph 137 in aquaria. The cross-wise combination of 1st and 2nd Feeding Diets resulted in nine different dietary treatments that were fed in triplicates.

Table 1-1. Composition of experimental diets.

Ingredients (in % of dry matter)	Diet X	Diet Y	Diet Z
Fishmeal	77.56	33.50	11.00
Corn gluten		10.00	16.66
Sunflower meal		4.46	3.12
Soy protein concentrate		15.00	20.00
Wheat gluten		14.19	25.00
Faba bean meal		4.50	2.00
Wheat starch	12.97	5.00	5.00
Vitamin & Mineral Premixtures	0.72	1.04	3.11
Fish oil	8.72	12.32	14.11
Proximate composition (in % of dry matter)			
Dry matter (in % of diet)	93.43	94.69	92.93
Crude protein	57.31	58.34	57.63
Crude fat	18.04	18.74	18.84
Crude ash	9.49	6.23	4.57
Gross energy (MJ kg ⁻¹)	23.09	23.96	24.30
Total starch content (calculated)	11.40	8.43	8.71
Amino acid composition (in % of diet)			
Arginine	2.41	3.21	2.94
Histidine	1.03	1.16	1.16
Isoleucine	1.95	2.12	2.09
Leucine	4.43	3.86	4.33
Lysine	2.49	3.63	2.66
Methionine	0.92	1.43	1.12
Cystine	0.77	0.53	0.68
Phenylalanine	2.48	2.03	2.42
Tyrosine	1.02	1.26	1.40
Threonine	1.61	2.15	1.90
Valine	2.09	2.49	2.35
Alanine	2.38	3.02	2.70
Aspartic acid	3.48	4.50	4.15
Glutamic acid	12.15	7.94	10.60
Glycine	1.87	2.96	2.38
Proline	3.96	2.51	3.38
Serine	2.40	2.33	2.47
Fatty acid composition (in % of total fatty acids)			
n-6 / n-3 <i>ratio</i>	0.52	0.17	0.35
Total n-6	10.10	3.99	7.55
Total n-3	19.46	23.68	21.61
ALA / LA <i>ratio</i>	0.17	0.35	0.18
Total C18:2n-6 (LA)	9.09	2.80	6.40
Total C18:3n-3 (ALA)	1.58	0.99	1.18
EPA / DHA <i>ratio</i>	0.88	0.69	0.79
Total C20:5n-3 (EPA)	6.46	7.63	7.18
Total C22:6n-3 (DHA)	7.32	11.06	9.05

2.5 Sampling

2.5.1 Growth data

Ten fish from one hatching trough were sampled for determining dry body weights on hatching day and on four additional dates during the yolk sac stage. Ten fish per hatching trough were further sampled for dry body weights on dph 20 (feed provision), 23, 25 and 28 (first feeding). After first feeding, dry body weights of ten fish per hatching trough were measured on seven additional dates until dph 89 (start of second feeding period). Furthermore, on dph 89 and 137 individual body masses were measured and samples for total body composition analysis were taken (in total ~100g fish per treatment). Specific growth rates (SGR) for the first feeding period and for three intermediate periods were calculated according to [29] with mean dry body weights of ten individual fish obtained separately for every sampling day based on the formula

$$\text{SGR} = [\ln(m_f) - \ln(m_i)] / t * 100,$$

with m_f = dry body weight at dph f , m_i = dry body weight at dph i , $t = f - i$, $f > i$.

2.5.2 Digestive enzyme analysis

Ten eggs from one hatching trough were sampled for amylase and pepsin activity assays (five eggs each) five days before hatching and on hatching day. Five fish from one hatching trough were sampled for digestive enzyme analysis on hatching day and on three additional dates during the yolk sac stage. After feed provision on dph 20, five fish per hatching trough were sampled on seven dates until dph 35. Additional samples for digestive enzyme analysis were taken on dph 89 (five fish per hatching trough) and dph 137 (five fish per aquarium, three aquaria per treatment).

2.6 Sample preparation

Prior to sampling, experimental animals were narcotized with MS222 (Tricaine methanesulfonate, E10521, Sigma-Aldrich Co. LLC.) and immediately killed by cutting the gill vein. Smaller fry were narcotised and killed with an overdose of MS222. Samples for measuring dry body weight were immediately frozen at -20°C and freeze-dried for 72 hours prior to weighing. The assessment of dry body weight is more appropriate than wet body weight, particularly for the comparatively small yolk-sac fry, because of increased measurement error caused by remaining water droplets. Due to size limitations, whole egg homogenates were used for digestive enzyme analysis before hatching and whole body homogenates were used after hatching, before first feeding and for the first seven days after first feeding (until dph 35). All samples were frozen at -80°C until analysis. On dph 89 and

dph 137, the whole gastro-intestinal tract was dissected on ice and instantly frozen at -80°C for digestive enzyme analysis. Fish had been fasting for 12 hours before sampling on days 89 and 137 post hatch to avoid acute feeding-induced peaks of enzyme activities and to rather access enzymatic baselines.

2.7 Amylase and Pepsin activity assays

All samples for amylase and pepsin activity assays were thawed at 4°C . Amylase activity was measured with the fluorescence based EnzChek® Ultra Amylase Assay Kit (E33651, Molecular Probes, Inc.) according to manufacturer's specifications. For homogenisation and further reactions, a buffer solution of 20 mM $\text{H}_2\text{PO}_4\text{Na}$, 6.7 mM NaCl and 0.5 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (pH 6.9) was prepared. Pepsin activity was measured with the EnzChek® Protease Assay Kit *green fluorescence* (E6638, Molecular Probes, Inc.) according to manufacturer's specifications. For homogenisation and further reactions, a buffer solution of 10 mM HCl (pH 2.0) was prepared. Respective buffer solutions were added with a final volume to weight ratio of 3:1 (buffer:tissue). The samples were homogenised (KT Micra D9 homogeniser, ART Prozess- & Labortechnik GmbH & Co. KG, Germany) on ice for 30 seconds with a speed of 25,000 RPM and centrifuged (Universal 320R centrifuge, Hettich Zentrifugen, Germany), at 4°C for 10 minutes with 10,000 RPM (whole body homogenates) and for 20 minutes with 5,000 RPM (whole digestive tract homogenates). The clear supernatant was pipetted into a new reaction tube and glycerine was added to a final volume of 10% of the sample solution. The samples were stored frozen at -20°C until performance of activity assays; α -amylase from *Bacillus spec.* (A-6380, Sigma-Aldrich Co. LLC.) was used as the standard for amylase activity assays. The reaction temperature was constantly set to 25°C . The fluorescence intensity was measured after 20 min at 485 nm (excitation) and 520 nm (emission). Pepsin from porcine gastric mucosa (P-6887, Sigma-Aldrich Co. LLC.) was used as a standard for pepsin activity assays. The reaction temperature was constantly set to 37°C . The fluorescence intensity was measured after 30 min at 490 nm (excitation) and 525 nm (emission). The readout of the activity assay of each sample was Fluorescence Units (FU) performed on a spectrophotometric microplate reader (TECAN infinite M200, Tecan Trading AG, Switzerland). The standard curves with pepsin and amylase solutions of known activity were used to convert FU into activity units. Activity data are, thus, expressed as mU standardised per mg of used tissue (i.e. egg homogenate, whole body homogenate or whole digestive tract homogenate). Gastro-intestinal tract weights of individual fish were significantly correlated to corresponding wet body weights ($\rho=0.915$, $p<0.001$; Pearson's product-moment correlation). Samples that had activity units below zero after the conversion from FU to mU were excluded from further calculations and statistics.

2.8 Statistical analysis

The statistical software R (2014) was used to evaluate the data. Data collected during the first feeding period, when fish were reared in the hatching troughs, were described by a statistical model based on generalised least squares [30]. The data were assumed to be approximately normally distributed and to be heteroscedastic. These assumptions were based on a graphical residual analysis. The statistical model included diet as a fixed factor. Based on this model, an analysis of variance (ANOVA) was conducted, followed by multiple contrast tests according to [31], using the R package SimComp [32], in order to compare individual treatments. For data collected in the aquaria during the second feeding period, an appropriate statistical mixed model ([33]; [34]) was established. The data were assumed to be approximately normally distributed and to be heteroscedastic. The statistical model included the different diets as a fixed factor and the aquaria were regarded as a random factor. Based on this model, an ANOVA was conducted, followed by multiple contrast tests (e.g. [35]), using the R package multcomp [36], in order to compare the several levels of the influence factor. Significant interactions between the first and the second feeding diet were interpreted as being reflective of a nutritional programming effect. In case of a non-significant interaction, data were pooled for the first feeding diet and multiple contrast tests were rerun to compare only the three second feeding diets (X, Y and Z). Statistical differences of growth data and enzymatic activities between the end of the first feeding period and the end of the second feeding period were evaluated for continuously fed fish (treatments X, XX, Y, YY, Z and ZZ). A statistical mixed model was established with sampling day as a fixed factor and the hatching troughs and aquaria as a random factor. An ANOVA was conducted, followed by multiple contrast tests to compare the two sampling days as described before [35, 36].

3 Results

3.1 Growth performance and body composition

Average dry body weights of individual fish were used for polynomial curve fitting of growth curves for the first feeding period (Fig 1-2). Fish fed with the two diets X (0% derived from plant proteins) and Z (10% derived from plant proteins) exhibited similar dry body weights at the end of the first feeding period, but dry body weights of fish fed with diet Y (50% derived from plant proteins) were significantly higher. Specific growth rates (Table 1-2), determined for the entire first feeding period and for three intermediate periods, supported these findings with higher growth rates for fish fed with diet Y, compared to diets X and Z. Separation of the three growth curves (Fig 1-2) became evident approximately between dph 45 and dph 55. In contrast, wet body weights obtained on day 89 post hatch were statistically not significantly

different between the dietary groups X, Y and Z (Fig 1-3), although wet body weights of fish fed with diet Y were increased ($0.76 \text{ g} \pm 0.13$) compared to fish fed with diet X ($0.69 \text{ g} \pm 0.16$) or diet Z ($0.66 \text{ g} \pm 0.17$).

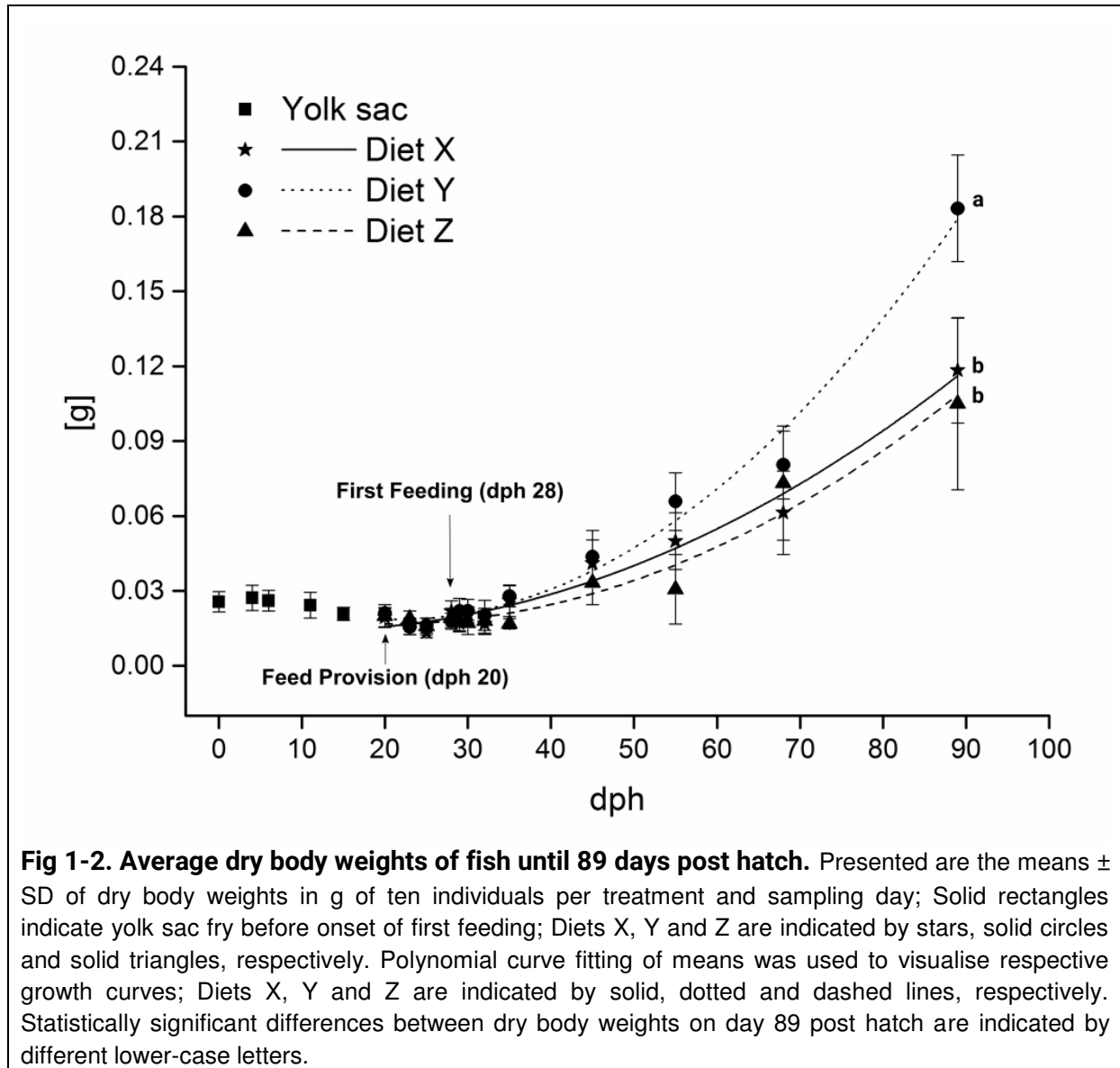


Table 1-2. Specific growth rates (SGR) of fish until 89 days post hatch.

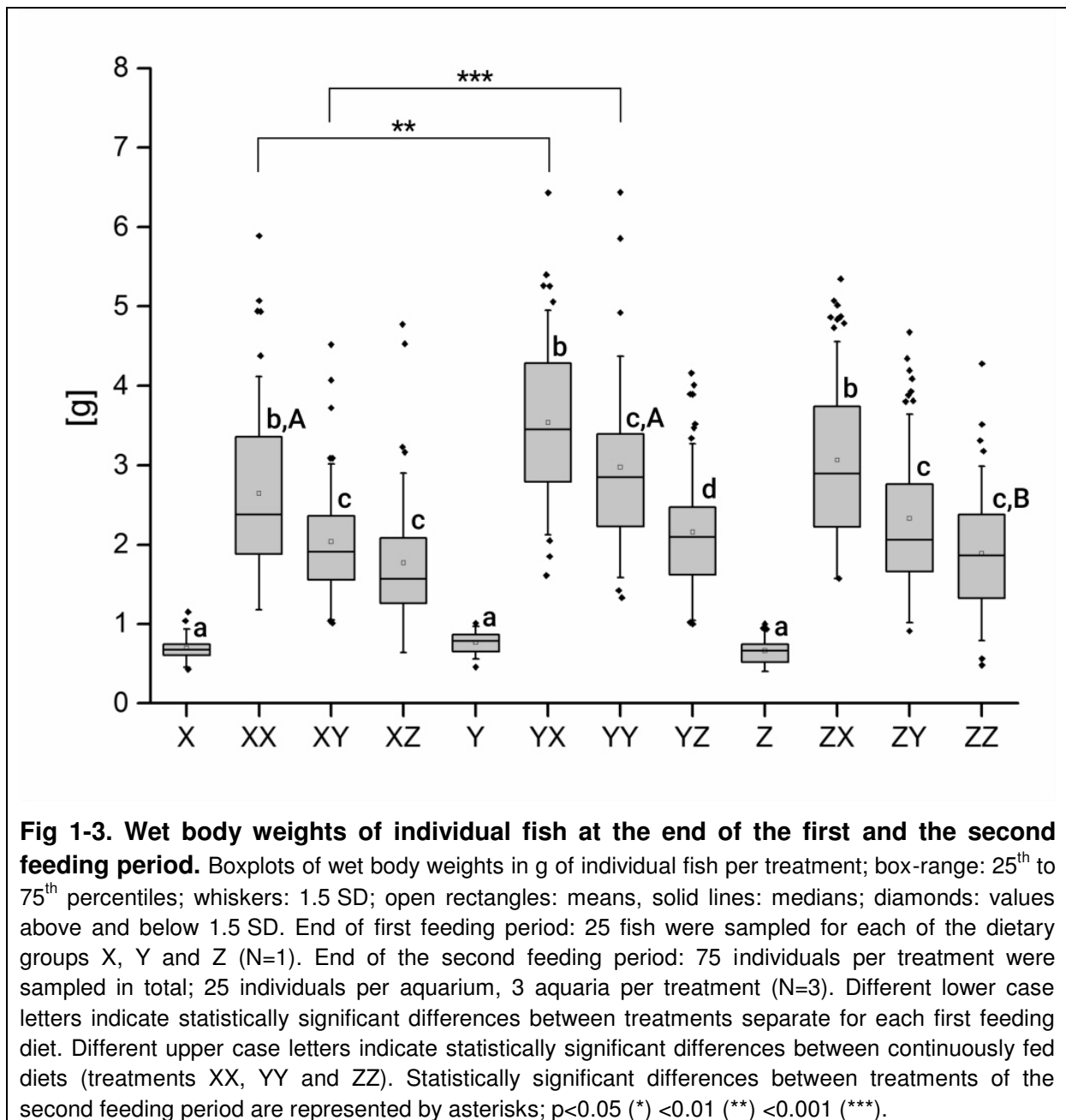
1 st Feeding Diet	dph 28 – 89	dph 28 – 45	dph 45 – 68	dph 68 – 89
X	2.77	3.71	1.74	3.13
Y	3.82	5.26	2.66	3.92
Z	2.80	3.27	3.43	1.72

SGRs were calculated from mean dry weights of ten individual fish per 1st Feeding Diet, obtained separately for every sampling day.

$SGR = [\ln(m_f) - \ln(m_i)] / t * 100$; m_f = dry body weight at dph f , m_i = dry body weight at dph i , $t = f - i$, $f > i$.

On day 137 post hatch, however, all fish had significantly higher wet body weights when compared to those measured on dph 89. Moreover, fish fed with the second feeding diet X

after the diet change had significantly higher wet body weights when compared to the second feeding diets Y and Z ($p < 0.05$). The statistical model revealed no significant interaction between the first and the second feeding diet. Nevertheless, there was a significant influence of the first feeding diet on wet body weight of fish on day 137 post hatch ($p < 0.01$): the first feeding diet Y promoted significantly higher wet body weights when compared to those achieved on diet X when fish were fed with the second feeding diets X and Y. Highest overall wet body weights were observed for fish in treatments YX and ZX, followed by XX and YY. Fish that were continuously fed with diet Z (treatment ZZ) had significantly reduced wet body weights compared to treatments XX and YY.



The chemical analysis of nutrients of whole-fish body homogenates indicated slightly decreased water contents in fish homogenates of all treatments at the end of the second feeding period, compared to the end of the first feeding period (Table 1-3), except for fish of treatments Z and ZZ. In contrast, crude fat contents increased from dph 89 to dph 137 in fish of all treatments. The diet change at the end of the first feeding period, however, had no significant effect on crude protein or crude fat of whole-fish homogenates. The ash content was nevertheless significantly reduced in fish of treatments XY and YY compared to fish of treatments XX and YX, respectively.

Table 1-3. Proximate body composition of pooled fish.

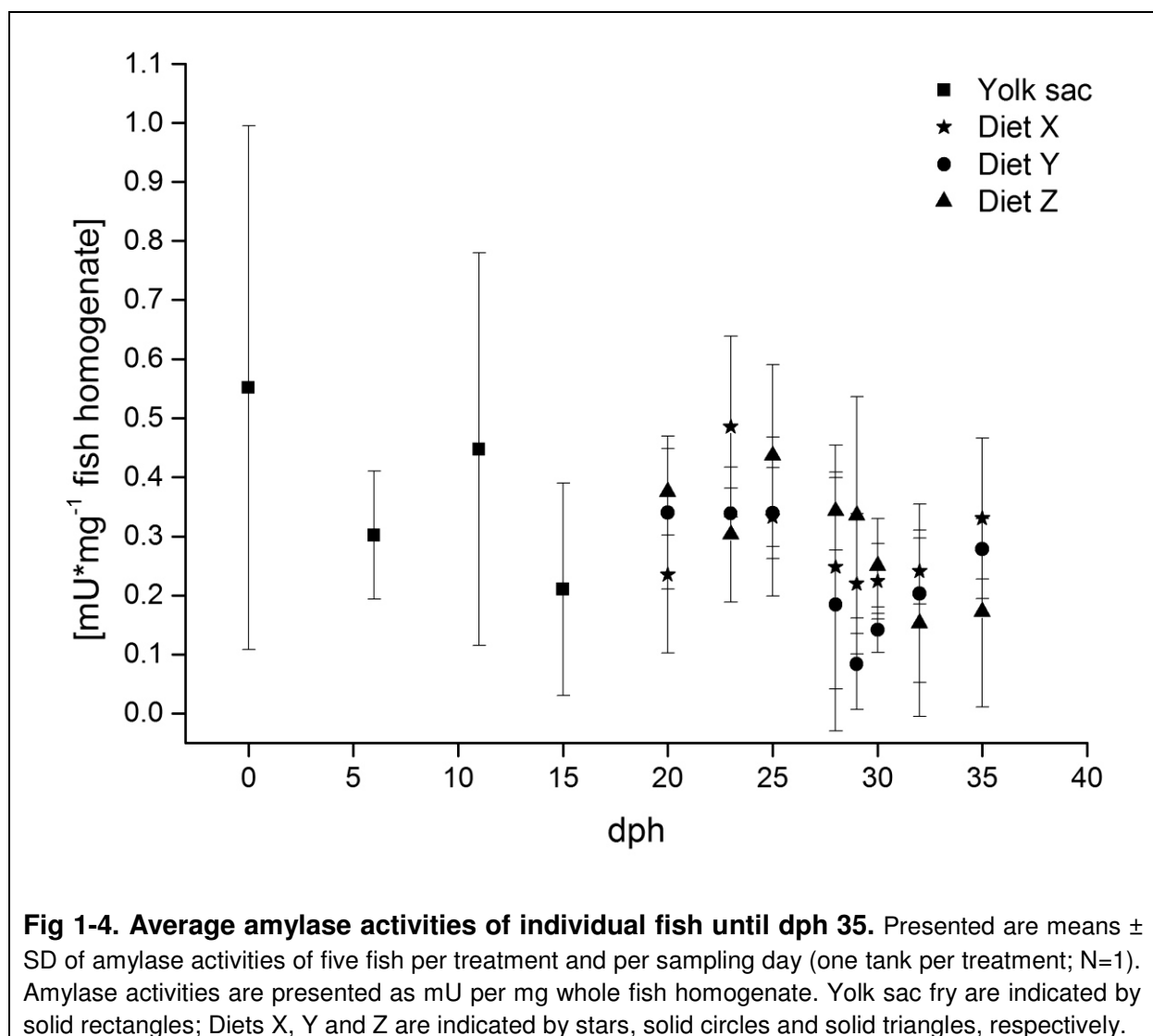
	in [%]	Moisture	Crude Protein	Crude Fat	Ash
1 st Feeding Period	X	79.5	14.4	3.8	2.3
	Y	78.3	14.6	5.0	2.1
	Z	78.2	14.8	4.7	2.3
2 nd Feeding Period	XX	76.5 ± 0.4	15.2 ± 0.4	5.2 ± 0.0	3.1 ± 0.1
	XY	76.9 ± 0.5	14.8 ± 0.3	5.8 ± 0.2	2.6 ± 0.1
	XZ	77.4 ± 1.0	14.9 ± 0.6	5.4 ± 0.2	2.3 ± 0.2
	YX	75.7 ± 0.4	15.4 ± 0.0	5.9 ± 0.3	2.9 ± 0.1
	YY	76.6 ± 1.2	14.9 ± 0.9	6.2 ± 0.2	2.3 ± 0.1
	YZ	76.7 ± 0.2	15.0 ± 0.2	5.8 ± 0.3	2.5 ± 0.3
	ZX	76.4 ± 0.4	15.2 ± 0.2	5.7 ± 0.2	2.8 ± 0.3
	ZY	76.1 ± 0.2	15.1 ± 0.2	6.5 ± 0.2	2.4 ± 0.1
	ZZ	77.5 ± 0.5	14.4 ± 0.2	5.7 ± 0.4	2.4 ± 0.1

Average percentage of moisture, crude protein, crude fat and ash of pooled whole fish homogenates in relation to the dietary treatment; approximately 100g pooled fresh fish per tank were used for chemical nutrient analysis; first feeding period: one tank per treatment, second feeding period: three tanks per treatment; presented are means ± SD of the triplicate tanks for the second feeding period.

3.2 Digestive enzyme activities

Amylase activities measured in egg homogenates five days prior to hatching were 0.65 ± 0.26 mU*mg⁻¹ and 0.40 ± 0.29 mU*mg⁻¹ on hatching day (data not shown). Pepsin activities measured in egg homogenates were 7.89 ± 4.58 mU*mg⁻¹ five days prior to hatching and 7.22 ± 2.53 mU*mg⁻¹ on hatching day (data not shown). Amylase activities, obtained in whole fish homogenates, indicated no differences between treatments during the

first 35 days after hatching (Fig 1-4). Furthermore, amylase activities of first feeding fry were similar to amylase activities measured in yolk sac fry. Pepsin activities, in contrast, strongly increased with feed provision 20 days post hatch (Fig 1-5). At the end of the first and the second feeding periods, amylase and pepsin activities were estimated from homogenates of the complete GI tract. On dph 89, the different first feeding diets had no influence on individual pepsin activities, except for fish fed with diet Y that exhibited significantly higher pepsin activities compared to fish fed with diet Z ($p < 0.05$). Amylase activities obtained on dph 89 did not differ between the three first feeding diets. At the end of the second feeding period, amylase activities of all fish were significantly higher ($p < 0.001$) compared to amylase activities of sampled fish at the end of the first feeding period (Fig 1-6). However, none of the dietary treatments after the diet change had a significant effect on amylase and pepsin activities and the ANOVA revealed no significant interaction between the first and the second feeding diets. However, as can be observed from Fig 1-7, inter-individual variabilities in pepsin activities were very high and could, therefore, mask possibly significant differences.



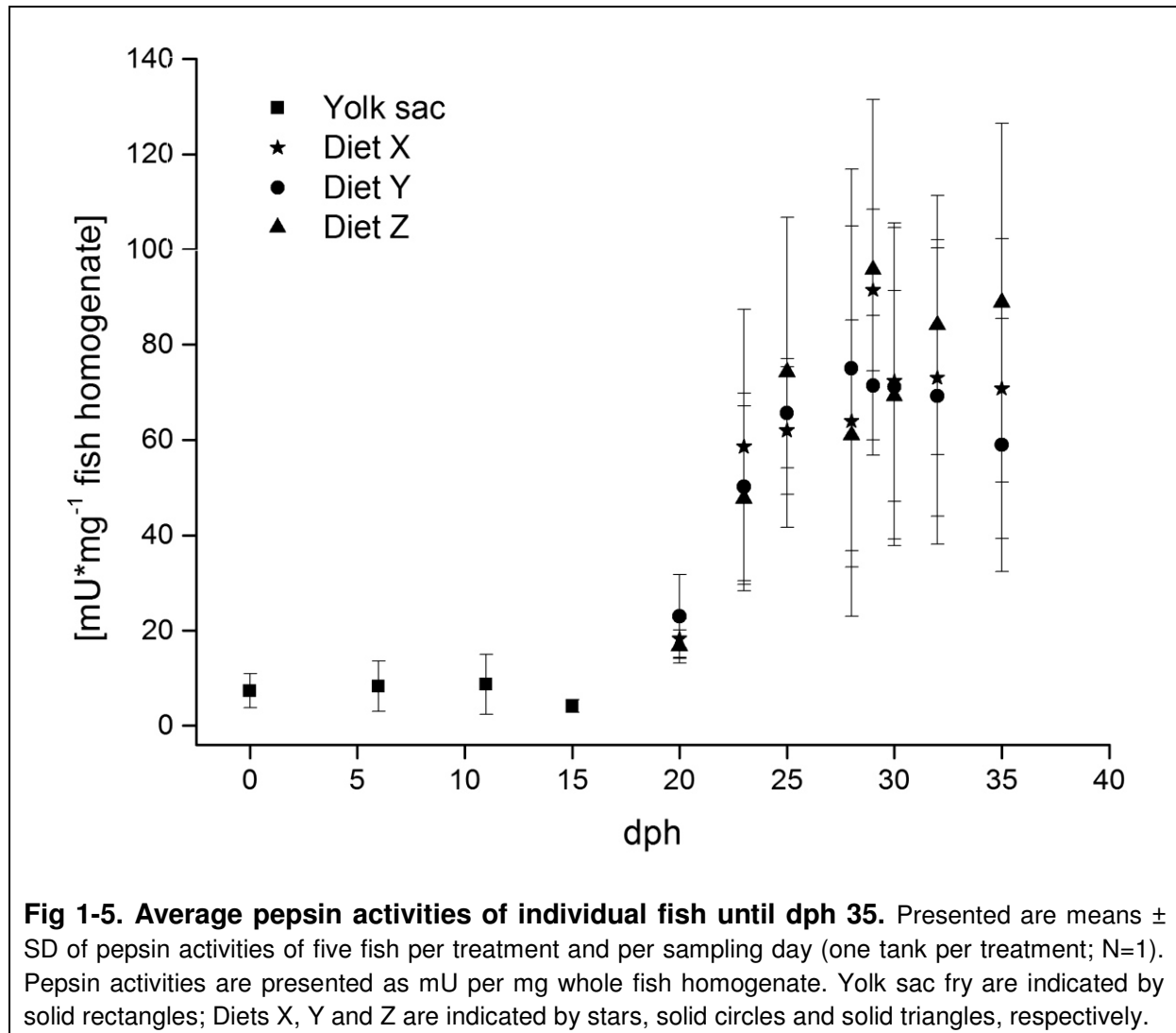
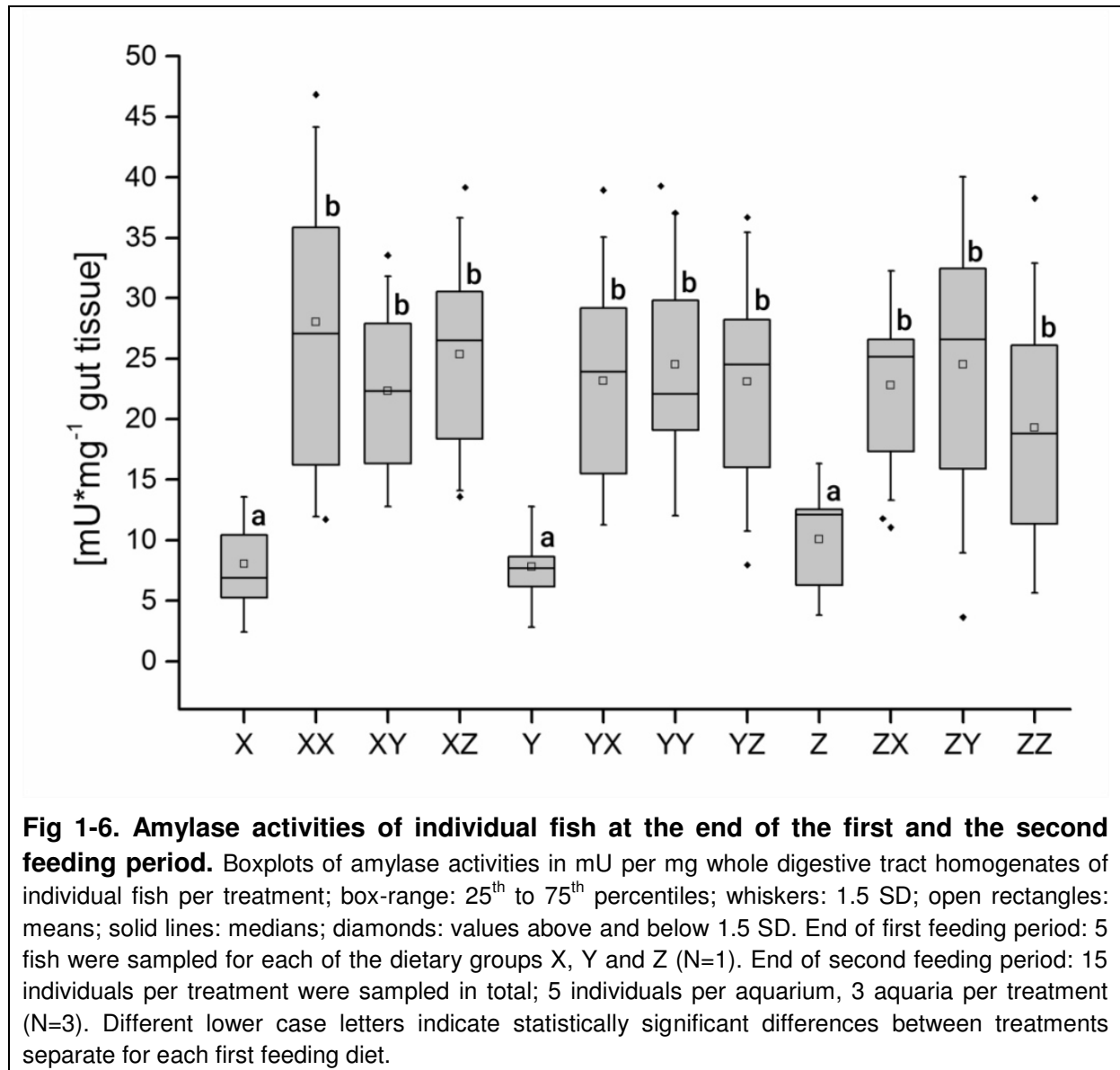
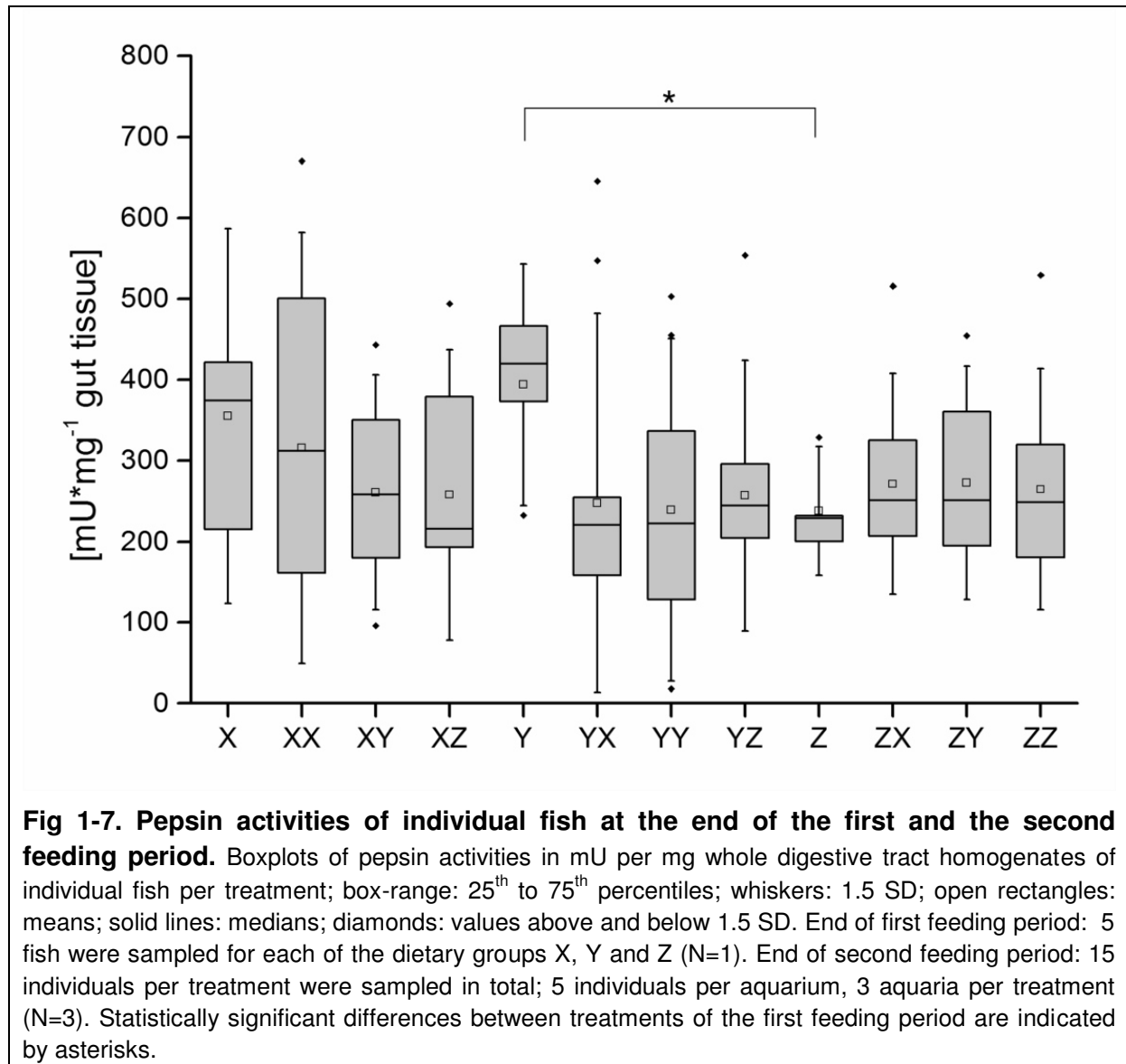


Fig 1-5. Average pepsin activities of individual fish until dph 35. Presented are means \pm SD of pepsin activities of five fish per treatment and per sampling day (one tank per treatment; N=1). Pepsin activities are presented as mU per mg whole fish homogenate. Yolk sac fry are indicated by solid rectangles; Diets X, Y and Z are indicated by stars, solid circles and solid triangles, respectively.





4 Discussion

4.1 Digestive enzyme activities in relation to dietary plant proteins

Digestive enzymes are one of the most important factors involved in the nutrient digestion in fish and are believed to be highly susceptible to external influences during early development [37, 38]. Salmonids have already a well differentiated digestive system with stomach, pyloric caeca and intestine at the onset of first feeding [39], and gastric glands are developed about twenty days post hatch [40]. Although it has been assumed that stomach pre-digestion brings only minor advantages for overall digestion [41], the acid digestion in salmonids is still the first step in the breakdown of large proteins into smaller polypeptides. We detected amylase and pepsin activities already five days prior to hatching. The presence of these two enzymes prior to feed provision could reflect intrinsic determinations within the enzymatic toolbox in

developing fish, as discussed previously [42]. Increasing pepsin-like enzymatic activities have already been detected shortly before hatching [40]. Amylase also displayed alternating activity patterns during the first days of feeding, a finding similar to that previously reported for red porgy (*Pagrus pagrus*) [19]. Nevertheless, amylase activities remained relatively stable from hatching day until 35 days post hatch and were not affected by the three first feeding diets, although the total dietary starch content in the present study was slightly increased for diet X compared to diets Y and Z, which was necessary for feed production.

It has been shown that amylase activity decreases with increasing starch levels [43]. However, experimental feeds have been extruded and extrusion is known for positively affecting carbohydrate digestibility in fish [44] because it increases the accessibility of starch molecules to amylase [45]. The different levels of plant-derived protein sources used as fishmeal replacement in the diets of the current study did not affect amylase activities, a finding similar to that already reported [46]. A significant increase of amylase activities was detected from the end of the first feeding period to the end of the second feeding period in all dietary treatments. In contrast, an extreme increase of pepsin activities already within the first ten days after first feeding reflects the digestive enzyme activities of typical carnivores with high protease and low carbohydrase levels [47] and the effect of feed provision on digestive enzyme activities. A strong increase of pepsin activities in fry of the Caspian brown trout (*Salmo caspius*) has been observed shortly before the yolk-sac was fully absorbed and exogenous feeding started [48]. In the present study, pepsin activities remained relatively constant from dph 89 to dph 137 without significant differences between treatments, which aligns well with previous findings [40]. In their study, rainbow trout fry exhibited 60 days post hatch similar pepsin and trypsin activities as juvenile trout with 70g. An *in vitro* study, using a gastrointestinal model of rainbow trout extracts, demonstrated that the efficiency of proteolytic hydrolysis in the intestine was significantly dependent on a prior peptic digestion of proteins in the stomach [26]. Furthermore, it was shown that soluble peptides from soybean meal were less efficiently absorbed compared to fishmeal and resulted in a significantly lower net release of amino acids after hydrolysis. Thus, it is possible that the equal pepsin activities of the present study resulted in a reduced digestibility of the plant-based diets Y and Z, which could have directly affected growth performance during the second feeding period. Furthermore, the significant reduction of average pepsin activities at the end of the first feeding period of fish fed diet Z compared to fish fed diet Y, could reflect a negative effect of plant proteins on the hormonal control of pepsin secretion during digestion as has been observed in humans [28]. Nevertheless, this effect could not be observed during the second feeding period.

4.2 Growth performance and nutritional programming effects

The growth performance of individual fish during the first feeding period was comparable between the fishmeal diet X and the plant-based diet Z. Dry body weights and wet body weights of fish fed the intermediate diet Y were slightly higher on day 89 post hatch compared to fish fed with diet X and Z, however, the differences between wet body weights were not statistically significant. Specific growth rates calculated for the entire first feeding period were also higher for fish fed with diet Y when compared to those fed with diets X and Z. It was demonstrated that it is possible to raise rainbow trout from first feeding on with diets completely free of marine resources (fishmeal and fish oil), however with a significant reduction in growth performance [49]. The inclusion of fish oil as lipid resource in experimental diets of the present study might therefore substantially contribute to the positive growth performance observed.

Specific growth rates were overall similar to those obtained in a study with farmed brown trout fry during the first 14 weeks after first feeding, although the used diet (raw liver) makes a direct comparison difficult [50]. That study compared wild and sea ranched brown trout fry in flow-through stream tanks for studying the effects of selection for rapid growth on agonistic behaviour. In their study, specific (weight) growth rates were obtained for fish at low (3 fry/tank) and high (50 fry/tank) stocking densities and low (1.5% BM/day) and high (3.0% BM/day) food ratios with an initial weight of about 0.17 g. In order to compare the SGRs calculated in the present study, SGRs obtained by [51] needed to be recalculated. The SGRs calculated for the entire period of the current experiment, as well as the SGRs of the intermediate periods, were consistently higher than the growth rates of both wild and sea-ranching trout in the compared study by Hedenskog et al. (2002). However, a direct comparison is again difficult because the latter fish were fed only once per day and SGRs were calculated on a feeding period of four days. In contrast, specific growth rates of about 3.18% per day were observed for farmed juvenile brown trout in a 155 day-long feeding trial [3], which is also comparable to SGRs calculated in the present study. Thus, our results indicate that feeding wild brown trout fry with plant-derived protein sources is beneficial for growth performance during the first weeks of life and that fishmeal is not necessarily needed in high dietary inclusion levels.

A cross-over diet change was applied in order to evaluate possible permanent effects of the first feeding diet, but the statistical analysis revealed no nutritional programming effect. Nutritional programming is usually defined as a response to environmental stimuli during an early developmental window with permanent physiological effects on later life-stages [15]. A significant, systematic interaction of the first and the second feeding diet affecting growth performance would have indicated such nutritional programming effects. Instead, no

interaction was detected and the second feeding diet had a significant impact on growth performance in all treatments. The best growth promoting combination of first and second feeding diet was YX, directly followed by ZX, YY and XX. Feeding diet X as second feeding diet significantly enhanced growth performance in all treatments; however, it was beneficial if plant-derived proteins were included into the first feeding diet.

It is possible that the observed differences in growth performance could originate from differences in voluntary feed intake, which could not be determined for such small fry. In a previous study with juvenile rainbow trout, voluntary feed intake was related to the nutritional state and was concluded to fit the optimal protein to energy ratio [52]. However, differences in feed intake were not expected since the different diets used in the present study have equivalent energy and protein levels. Nevertheless, the substitution of fishmeal by plant proteins has been shown to induce a reduction in voluntary feed intake of rainbow trout [7, 53] and of Atlantic salmon [54]. Thus, voluntary feed intake of fish fed the fishmeal diet during the second feeding period of the present study could have been increased, presumably because of better taste, independently of the first feeding diet and therefore provoked enhanced growth.

Nevertheless, the continuous feeding of the mixed diet Y resulted in better growth of small brown trout than the continuous feeding of the fishmeal diet X. Under natural conditions, yolk sac larvae stay in the interstitial gravel after hatching until the start of exogenous feeding. An early start of exogenous feeding before complete yolk sac depletion is crucial for the survival of the fry. Natural feed for brown trout fry mainly consists of chironomid larvae [55], which in turn feed on algae, fungi, pollen, plant particles, animal remains, detritus and silt [56], and cladocerans, copepods, as well as plecopteran larvae. The general diet composition for Plecopteran larvae includes algae, detritus, fungi and bacteria, as well as dead fish eggs or animal material [57]. Cladocerans, in addition, can be made from 28% carbohydrates, and zooplankton in general contains a lot of chitin (N-acetylglucosamine polymer) which might thus be the first carbohydrate ingested by most of the carnivorous fish in the wild [58]. The digestive capabilities (functional stomach and early activity of digestive enzymes) of trout fry seem to fit the variety of nutrients occurring in their natural diet. Although the prey is zooplankton, it is accompanied by plant particles or algae and it is beneficial if as much of the ingested feed can be digested. Larvae of herbivorous fish are strictly carnivorous during the first weeks of feeding until they undergo a trophic shift (what is also associated to a change in the intestinal microbiome [59]). They lack a stomach, as well as pharyngeal teeth, and need high amounts of nitrogen for fast growth, which can be more easily gained by animal protein [60]. For brown trout, it could be possible that a mixed diet with animal and plant-

protein sources reflects the nutrient composition of natural food and is thus beneficial for growth during the first weeks of live.

Previous studies investigating the inclusion of plant-derived proteins in salmonid diets revealed contrary results: in some experiments growth performance was significantly reduced when fish were fed plant-based diets compared to fishmeal diets [61–63]; others demonstrated equal growth performances [10, 64, 65]. Nevertheless, fish in these studies were not fed with the experimental diets already at first feeding. A meta-analysis, comparing six different plant-based feed ingredients, showed that several factors can influence the outcome of such experiments [66]. For example, it was demonstrated that the protein quality of the fishmeal control diet can affect overall results. Furthermore, the meta-analysis indicated that soy protein concentrates and canola protein concentrates had significant negative effects on growth of salmonids which was contrary to the general assumption of protein concentrates being beneficial for growth performance. The ingredients used in the present study were mainly highly purified protein concentrates and all diets were heat-extruded, which has been shown to reduce anti-nutritive factors [67]. Nevertheless, fish fed with plant-based diets during the second feeding period demonstrated reduced growth compared to those grown on fishmeal, although the continuous feeding of 50% plant proteins resulted in body weights equal to those that experienced continuous feeding of fishmeal. The continuous feeding of 90% dietary plant proteins, however, resulted in significantly reduced wet body weights.

Previous studies with rainbow trout demonstrated that, at about two months after first feeding, the development of the digestive system, which can be continuously influenced by ingested feed, is completed and digestive capabilities are established (Kawai and Ikeda, 1973a; Lazo et al., 2011). The diet change of the present experiment has been applied 61 days after first feeding, approximately when the digestive system should have been fully established. Nevertheless, a nutritional programming effect was not detected, even though previous studies have reported such effects in rainbow trout, zebrafish and Siberian sturgeon [18, 68–70]. However, the diet change to test possible programming effects in those studies was applied about four to five months after the first feeding period. Thus, it could be possible that the diet change of the current experiment was set too early and any programming effects of the first feeding diet were overlain by plastic physiological responses towards the second feeding diet. Furthermore, it remains unclear how the parental feeding history of wild broodfish had already influenced the digestive capacities of offspring used for this study. In gilthead seabream it was demonstrated that feeding broodfish with plant-based diets significantly improves the utilisation of plant-based diets later in juveniles [14]. This nutritional

programming effect might also act in different directions and impede the digestion of plant material in the present study if parental fish were strict carnivores.

5 Conclusion

In the present study, we demonstrated that the substitution of up to 90% fishmeal with plant-derived protein is possible in diets for wild brown trout fry from first feeding onwards without significant reduction of growth performance during the first nine weeks of life. In fact, the results indicated that the inclusion of plant-derived proteins into first feeding diets can be beneficial for subsequent growth performance. Later in development, however, brown trout experienced significantly reduced growth when plant proteins were subsequently introduced into their diets – independent of the first feeding diet-type. Nevertheless, a continuous feeding of 50% plant-derived dietary protein resulted in the same growth compared to a feeding of fishmeal as exclusive protein source. The continuous feeding of 90% dietary plant proteins, however, resulted in significantly reduced body weights at the end of the experiment. The present experiment could not induce a permanent nutritional programming effect of the first feeding diet; instead, the cross-over feeding design demonstrated that wild brown trout fry exhibit highly plastic responses to different feeding strategies during the first months of life. The digestive enzymes pepsin and amylase were only partly affected by the diet-type and it can be concluded that intestinal amylase and pepsin activities in juvenile brown trout are strongly regulated by intrinsic mechanisms. Furthermore, future studies should stronger address the effects of parental feeding history on the usability of plant-based diets.

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Chapter 2

The impact of dietary plant proteins on the bacterial gut community of first-feeding brown trout (*Salmo trutta*)

Stéphanie Céline Michl^{a,b,c}, Matt Beyer^d, Jenni-Marie Ratten^d, Mario Hasler^e, Julie LaRoche^d, Carsten Schulz^{a,b}

^aGesellschaft für Marine Aquakultur mbH (GMA) Büsum, Hafentörn 3, 25761 Büsum, Germany

^bInstitute of Animal Breeding and Husbandry, Department for Marine Aquaculture, Christian-Albrechts-Universität zu Kiel, Hermann-Rodewald-Str. 6, 24118 Kiel, Germany

^cGEOMAR Helmholtz Centre for Ocean Research, Wischhofstr. 1-3, 24149 Kiel, Germany

^dDepartment of Biology, Dalhousie University, 1355 Oxford Street, Halifax, Nova Scotia B3H 4R2, Canada

^eLehrfach Variationsstatistik, Christian-Albrechts-Universität zu Kiel, Hermann-Rodewald-Str. 9, 24118 Kiel, Germany

Abstract

In modern salmonid feeds, fishmeal has been significantly substituted by plant-derived proteins and, despite an increasing knowledge about the importance of the intestinal bacterial community for fish health and digestion, little is known about the effects of plant-based feedstuffs on the microbiome of juvenile brown trout (*Salmo trutta*). The aim of the present study was to investigate the impact of plant-derived dietary proteins on the gut microbiome of first-feeding brown trout originating from wild stocks and to evaluate whether the initial microbiome of early fry can be permanently manipulated by the first-feeding diet type due to nutritional programming. The results of this study demonstrate a strong influence of the plant protein inclusion levels on the trout bacterial gut community. Proteobacteria and Fusobacteria were significantly enhanced when fishmeal was integrated into the experimental diet. Firmicutes and Bacteroidetes, in contrast, were significantly promoted by plant-derived protein sources. However, no permanent effect on the intestinal microbiome of the first-feeding diet could be detected, although the microbiome of first-feeding trout was significantly shaped by the diet type. It was furthermore shown that the composition of the brown trout microbiome aligns well with the bacterial communities found in other salmonid species, supporting the concept of a salmonid core microbiome.

Key words: Brown trout; First Feeding; Intestinal Microbiome; Nutritional Programming; Plant proteins

1 Introduction

In aquaculture, rainbow trout (*Oncorhynchus mykiss*) is preferentially used for the production of consumable fish compared to brown trout (*Salmo trutta*). Nevertheless, the production of brown trout is of high commercial interest for recreational angling and restocking purposes. brown trout has a significantly longer hatching period and exhibits slower growth than rainbow trout when exposed to the same rearing conditions [1]. In modern salmonid feeds, fishmeal has been significantly substituted by plant-derived proteins due to the declining availability of fishmeal and increasing prices [2]. The usability of plant-based diets for salmonids has been evaluated in several studies and for different life stages [3, 4], but mainly for Atlantic salmon (*Salmo salar*) or rainbow trout. The digestive physiology of fish can be heavily affected by the inclusion of plant-derived proteins, such as digestive enzymes [5] or the microbial gut community [6]. Despite an increasing knowledge about the importance of the intestinal bacterial community for fish health and digestion [7–9], very little is known about specific dietary effects on the microbiome of juvenile brown trout. In rainbow trout fry it was demonstrated that first feeding initiates the gut microbiome establishment and that diet-type influences the bacterial composition [10]. It is also known that the intestinal microbiota of vertebrates is influenced not only by the environment or the diet, but also by host genetics [11]. A study conducted [12] showed that bacterial groups of the rainbow trout gut microbiota significantly correlated with individual trout families and that the dietary effect on the bacterial community structure was influenced by the individual family as well. Furthermore, it was demonstrated that nutritional programming via low-fishmeal and low-fish oil broodstock diets improved the utilisation of plant-based diets in juvenile gilthead sea bream [13]. Hence, the genetic background of juvenile brown trout (often originating from wild stocks) could affect the influence of dietary plant proteins on the bacterial gut community differently, compared to salmonids of well-established breeding lines. The present study thus aimed to evaluate effects of plant-derived dietary proteins on the gut microbiome of first-feeding brown trout, originating from wild stocks (*Salmo trutta*). It was additionally hypothesised that the initial microbiome that has been shaped during early development can be permanently manipulated by the first-feeding diet type due to nutritional programming effects.

2 Material & Methods

2.1 Experimental animals

The present experiment was conducted at the “Gesellschaft für Marine Aquakultur mbH” (Büsum, Germany). Eyed brown trout eggs (*Salmo trutta*) were reproduced from wild brown trout caught in Schleswig-Holstein (Germany) at the “Fischbrutanstalt Altmühlendorf”

(Germany). All animal handling procedures were approved by the animal welfare officer of the “Gesellschaft für Marine Aquakultur mbH” and the local authority of Schleswig-Holstein according to the German animal welfare law (TierSchG).

2.2 Experimental setup

Three isonitrogenous and isoenergetic diets (diet X, diet Y and diet Z; Table 2-1) with different plant protein inclusion levels (0%, 50% and 90%) were formulated and produced in cooperation with Skretting ARC (Stavanger, Norway). Each diet was formulated in accordance with the NRC (2011) digestible amino acid requirements for small Atlantic salmon fry (0.2 - 20.0 g) and also the composition of the vitamin and mineral premixtures were according to the NRC (2011) guidelines.

Table 2-1. Composition of experimental diets.

Ingredients (in % of dry matter)	Diet X	Diet Y	Diet Z
Fishmeal	77.56	33.50	11.00
Corn gluten		10.00	16.66
Sunflower meal		4.46	3.12
Soy protein concentrate		15.00	20.00
Wheat gluten		14.19	25.00
Faba bean meal		4.50	2.00
Wheat starch	12.97	5.00	5.00
Vitamin & Mineral Premixtures	0.72	1.04	3.11
Fish oil	8.72	12.32	14.11
Proximate composition (in % of dry matter)			
Dry matter (in % of diet)	93.43	94.69	92.93
Crude protein	57.31	58.34	57.63
Crude fat	18.04	18.74	18.84
Crude ash	9.49	6.23	4.57
Gross energy (MJ kg ⁻¹)	23.09	23.96	24.30

6000 eyed trout eggs were randomly distributed among 3 commercial hatching troughs (2000 eggs each) integrated into a recirculating tank system. Until hatching day, average water temperature was $11.2 \pm 0.3^\circ\text{C}$ and $12.4 \pm 1.3^\circ\text{C}$ until the end of the experiment. Throughout the experiment, average pH was 8.1. Fish were reared in the recirculating system for a total of 143 days and fed the three experimental diets from first feeding on. Feed was provided for the first time 20 days post hatch (dph), but active first feeding of trout fry was observed 28 days post hatch. Each of the experimental diets was provided to the fish of one hatching trough, resulting in the 1st Feeding Diet groups X, Y and Z (Fig 2-1). Feed was supplied *ad libitum* by automatic feeders once per hour for about six weeks, which was then gradually reduced until four times per day. Light was provided from 06:00 am to 09:00 pm. 61 days

post first feeding (dpff) diets were changed in a cross-over feeding design to investigate possible nutritional programming effects of the 1st Feeding Diet. Until day 109 pff, fish were fed 2.2% of the total biomass per day.

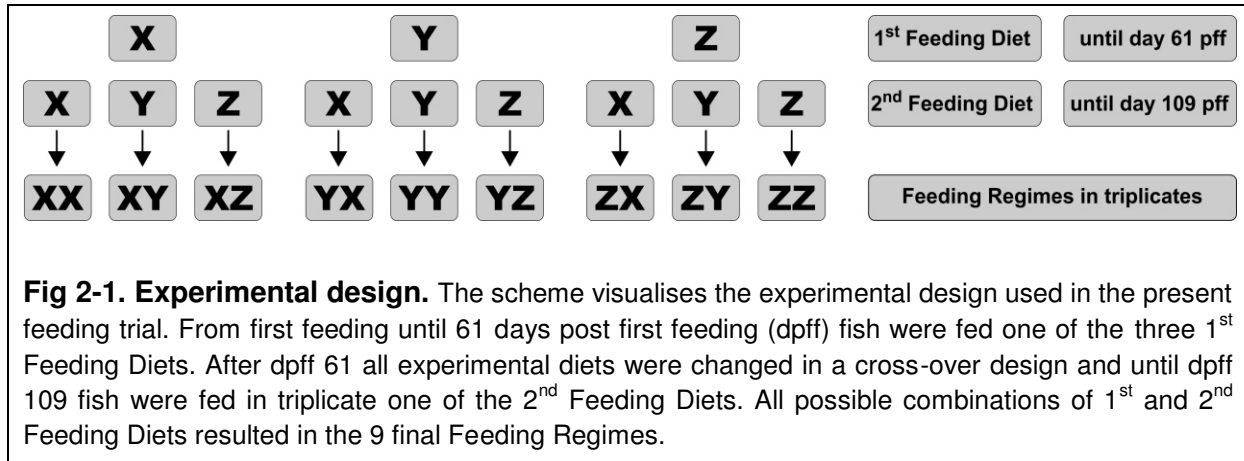


Fig 2-1. Experimental design. The scheme visualises the experimental design used in the present feeding trial. From first feeding until 61 days post first feeding (dpff) fish were fed one of the three 1st Feeding Diets. After dpff 61 all experimental diets were changed in a cross-over design and until dpff 109 fish were fed in triplicate one of the 2nd Feeding Diets. All possible combinations of 1st and 2nd Feeding Diets resulted in the 9 final Feeding Regimes.

2.3 Sampling

For microbiome analysis, 150 fish were sampled in total. Fish were starved for 12 hours before sampling. Experimental animals were narcotized with MS222 (Tricaine methanesulfonate, E10521, Sigma-Aldrich Co. LLC.) and immediately killed by cutting the gill vein. Five animals from each hatching trough were sampled on day 61 pff and five animals from each aquarium (three aquaria per treatment) were sampled on day 109 pff. The whole digestive tract was dissected on ice using sterile razor blades and instantly frozen at -80°C.

2.4 DNA extraction

The Qiagen DNeasy[®] Blood & Tissue DNA extraction kit was used according to the manufacturer's specifications to extract DNA from tissue samples. Digestive tract samples were thawed at 4°C and homogenised (KT Micra D9 homogenizer) on ice in 1 ml of a 5 mg ml⁻¹ lysozyme (8259, Carl Roth) in TE-buffer solution (10 mM Tris-HCl, 1 mM EDTA) for 30 seconds. The homogenised solution was incubated for 30 min at 37°C. Afterwards, the homogenate was gently vortexed and 80 µl were incubated for 60 min at 56°C in 200 µl of lysis buffer AL (provided in the extraction kit), 20 µl Proteinase K and 100 µl PBS (Solution without Ca-Mg, 733-2296, VWR). After incubation, 200 µl ethanol (96-100%) was added and further extraction steps were performed according to the manufacturer's protocol for purification of total DNA from animal tissue. Two extra washing steps with the provided buffers AW1 and AW2 were included into the protocol, as well as an extra centrifugation step of 1 min at maximum speed before elution, according to recommendations by Qiagen,. For purification, extracted DNA was incubated with RNase A (Qiagen) (1 mg ml⁻¹ in DEPC

water), pretreated by an inactivation of remaining microbial DNases at 70°C for 15 min. The RNase A working solution was added to each sample to a final concentration of 100 µg ml⁻¹ RNase A and incubated for 30 min at 60°C. A final DNA clean-up step was performed using the NucleoSpin[®] gDNA clean-up kit (Machery-Nagel) by following the manufacturer's protocol including all recommended steps.

2.5 16S rDNA PCR amplification

DNA amplification was performed by PCR targeting the 16S rRNA gene sequence (regions V6-V8). The final PCR reaction volume was 20 µl including 4 µl CG buffer, 0.6 µl DMSO, 0.4 µl dNTP (10 mM), 0.4 µl of each primer, 0.2 µl Phusion high-fidelity polymerase (Thermo Fisher Scientific Inc.), 12 µl DEPC H₂O and 2 µl of DNA template. The final primer concentration was 0.5 µM. Primers used were B969F (5'-ACG CGH NRA ACC TTA CC-3') and BA1406R (5'-ACG GGC RGT GWG TRC AA-3') from IDT (Integrated DNA Technologies, Inc.) according to [14]. Cycling protocol was as follows: 98°C for 3 min, 35 cycles of 98°C for 10 sec., 54°C for 30 sec. and a final extension at 72°C for 1 min, and finally 72°C for 10 min. Results of the PCR were verified on a 1.1% agarose gel. DNA samples were stained with SYBR safe DNA gel stain (Invitrogen[™], Thermo Fisher Scientific Inc.) and images were analysed using a gel imaging box (G:BOX, Syngene). Due to purchasing issues, one third of the samples needed to be amplified with the Phusion Hot Start II polymerase (Thermo Fisher Scientific Inc.). The change of polymerases had no statistically significant effect on the final results. The cycling protocol for samples amplified with the Phusion Hot Start II polymerase was as follows: 98°C for 30 sec, 35 cycles of 98°C for 10 sec, 54°C for 30 sec and a final extension at 72°C for 30 sec, and finally 72°C for 10 min. The purification of PCR samples from the gel was performed by carefully inserting a micropipette tip into the band and slowly drawing the DNA loaded agarose plug into the end of the tip. The agarose plug was then released into a well of a 96-well plate to diffuse out into 20 µl of DEPC water overnight in the refrigerator (approx. 4°C). Finally, the samples were multiplexed at equal volumes, quantified with Qubit (Invitrogen[™], Thermo Fisher Scientific Inc.) and loaded into the Illumina MiSeq platform as a 20 pM final denatured library according to the manufacturer's protocol.

2.6 Sequencing and bioinformatics

An Illumina MiSeq platform was used to sequence the amplified 16S rDNA fragments at the Integrated Microbiome Resource lab (IMR) at Dalhousie University (Halifax, Canada) following the procedure described by [15]. The analysis of raw sequences was performed with QIIME (Quantitative Insight Into Microbial Ecology) for the analysis of high-throughput

community sequencing data [16] using the 16S amplicon analysis procedure of IMR [17]. Several quality control steps were applied: forward and reverse reads were matched using PEAR (Paired-end rEAd merger; [18]), sequences with unidentified nucleotides, with mitochondrial and chloroplast DNA sequences [19], and chimeric DNA molecules (using UCHIME; [20]) were removed. Open-reference OTU (Operational Taxonomic Units) picking was performed using the picking methods *sortmerna* and *sumaclust* [21]. Reads were clustered against the reference database Greengene [22] and OTUs were grouped together based on 97% sequence similarity. Low confidence OTUs were subsequently removed and the collection of sequences was rarified to 1000 reads per sample as suggested for gut samples by Hamady & Knight [23]. In total, 122 samples remained after the quality steps during the bioinformatics workflow and were incorporated into the final statistical analysis (Table 2-2).

Table 2-2. Overview of the sample numbers used for final statistical analysis.

dpff	61			109											
Treatment	X	Y	Z	XX	XY	XZ	YX	YY	YZ	ZX	ZY	ZZ			
Replicate	I	I	I	I II III	I II III	I II III	I II III	I II III	I II III	I II III	I II III	I II III			
No. of fish	5	3	4	3 4 3	4 5 3	5 4 5	5 4 1	4 4 3	4 4 4	5 5 5	5 3 4	4 5 5			

Presented is the number of samples per treatment and replicate tank that remained after the quality steps during the QIIME workflow and were integrated into the final statistical analysis (one hatching trough per treatment on sampling day 61 pff and three replicate tanks per treatment on sampling day 109 pff). The experimental diets are X: 0% plant proteins, Y: 50% plant proteins, Z: 90% plant proteins.

2.7 Statistics

The statistical software R [24] and QIIME were used to evaluate the data. The number of observed distinct OTUs, the Chao1 richness estimator, the Simpson's evenness measure E and the Shannon diversity index H' were calculated based on the OTU table generated during the QIIME workflow. Nonmetric multidimensional scaling (NMDS) was performed to explore differences between bacterial communities on order level in relation to the dietary treatment or sampling day using the R package *vegan* [25]. Data was Hellinger-transformed and analysed by a Bray-Curtis dissimilarity matrix. The stress factor was calculated to estimate the representation of original data in the ordination space.

The following statistical procedures were performed to evaluate the impact of experimental diets on the dataset:

First, the impact of the experimental diets X, Y and Z on alpha diversity and the most abundant bacterial phyla during the first feeding period was tested. Therefore a statistical

model was established based on generalized least squares [26]. Based on a graphical residual analysis, the data were assumed to be approximately normally distributed and to be heteroscedastic. The first-feeding diet was considered as fixed factor. An analysis of variances (ANOVA) was conducted, based on this model, and in order to compare the several first-feeding diets, multiple contrast tests were performed [27] by using the R package SimComp [28].

Second, the impact of the nine feeding regimes on alpha diversity and the most abundant phyla at the end of the second feeding period was tested. A statistical mixed model was established [29, 30] with the 1st Feeding Diet and the 2nd Feeding Diet as well as their interaction term as fixed factors. The data were assumed to be approximately normally distributed and heteroscedastic. Aquaria were defined as random factor. Based on this model, an ANOVA was conducted, followed by multiple contrast tests in order to compare the levels of the fixed factors [31, 32] using the R package multcomp [33]. A significant interaction of the 1st Feeding Diet and the 2nd Feeding Diet was considered as nutritional programming effect. Data were pooled for the 1st Feeding Diet, in case of a non-significant interaction, and multiple contrast tests were rerun to compare only the three 2nd Feeding Diets (X, Y and Z).

Third, statistical differences of alpha diversity indices and bacterial phyla between the two sampling points (61 and 109 dpff) were tested for the feeding regimes XX, YY and ZZ. A statistical mixed model was established with sampling day as fixed factor and tank as random factor. An ANOVA was conducted, followed by multiple contrast tests to compare the two sampling days as described previously [31, 33].

Fourth, the impact of the experimental diets on the bacterial community structure during the first-feeding period was tested. Therefore, a Principal Component Analysis (PCA) was performed [34] with Hellinger-transformed bacterial order abundances. Order level was used, because observed OTUs on species and genera level outnumbered samples by three, which would have negatively influenced the statistical analysis. Those principal components (PC) from the PCA with the greatest influence on data variability were selected for further analysis by using the Broken-Stick-Criterion [35]. Based on the first two PC's, rotated data (i.e. pseudo-variables) were calculated and integrated into a multivariate model, established simultaneously for the two pseudo-variables. Based on this model, an ANOVA was performed. Multiple contrast tests for multiple endpoints were conducted in order to compare the experimental diets simultaneously for the two pseudo-variables [28, 36].

Fifth, the impact of the nine feeding regimes on the bacterial community structure at the end of the second feeding period was evaluated. A PCA was performed with Hellinger-transformed abundance data on order level and the PC's with the highest influence on data variability were selected as described previously. The first six PC's represented 82% of the cumulative variance. Based on these six PC's, rotated data were calculated and integrated into a multivariate mixed model, established simultaneously for the six pseudo-variables. The 1st Feeding Diet and the 2nd Feeding Diet as well as their interaction were considered as fixed factors, the tanks as random factor. Based on this model, an ANOVA was conducted. A significant interaction of the 1st Feeding Diet and the 2nd Feeding Diet was considered as nutritional programming effect. Finally, multiple contrast tests for multiple endpoints were performed to compare the levels of the fixed factors simultaneously for the six pseudo-variables [28, 36].

Sixth, the first two PC's were further examined for the individual contribution of specific bacterial orders to the cumulative variance explained of each principal component. The top-ten orders with the highest loadings on each of the two PC's were selected for further analysis. In case of a non-significant interaction of the first and the second feeding diet in the previous model, data were pooled for the first-feeding diet and multiple contrast tests as described before [28, 36] were performed to compare the three second feeding diets (X, Y and Z) simultaneously for the ten selected bacterial orders, respectively for each PC. Thus, specific bacterial orders could be identified that were significantly promoted by a certain diet-type.

Seventh, statistical differences of the bacterial community structure between the two sampling days were (61 and 109 dpff) evaluated for continuously fed fish (feeding regimes X and XX, Y and YY, Z and ZZ, respectively). Principal Component Analysis was performed for each of the three Hellinger-transformed data pairs and the first three PC's were selected as described before. Resulting pseudo-variables were integrated into a multivariate mixed model established simultaneously for the three pseudo-variables. The sampling day was integrated as fixed factor and tanks as random factor. An ANOVA was conducted, followed by multiple contrast tests for multiple endpoints to compare the two sampling points simultaneously for the three pseudo-variables as described before [28, 36].

3 Results

3.1 Alpha diversity

For all individual fish four alpha diversity indices have been calculated. In Fig 2-2a it can be seen that the number of observed OTUs slightly decreased from the first to the second feeding period when diet X or Y were fed as first-feeding diets, and for feeding regime ZX, although this was not significant. No statistically significant differences between treatments were found for the Simpson evenness measure and the only statistically significant difference between Shannon diversity indices can be observed between treatments ZX and ZY (Fig 2-2c and d). Important to consider here are the high standard deviations for some treatments, which can mask statistical significances such as for the high average Simpson's E of treatment Z (Fig 2-2c). The Chao1 richness estimator is also not generally affected by the diet type, except for the first-feeding diet Y. As can be seen in Fig 2b, richness is decreasing significantly from day 61 pff to day 109 pff. The performed multivariate ANOVA revealed no significant interaction between the first and the second feeding diet in all indices and hence data were pooled for the first-feeding diet. Overall, significantly more OTUs were observed when diet Z was fed as second feeding diet compared to diet X ($p < 0.05$). These findings are in line with significantly decreased Shannon indices once diet X was fed as second feeding diet ($p < 0.05$).

3.2 Relative abundance of bacterial phyla

The most abundant phyla present in the GI tracts of fish were analysed with regard to dietary influences. Fig 2-3 visualises the relative abundance of those phyla. For all treatments Proteobacteria and Firmicutes were the dominant phyla, followed by Bacteroidetes and Fusobacteria. The diet type had a significant influence on the relative abundance of most phyla, in contrast to the sampling point which is shown in Table 2-3. Fusobacteria significantly decreased from the first to the second feeding period when diet Y was fed continuously, but significantly increased when diet Z was fed in both feeding periods. The largest difference between sampling points can be observed for Firmicutes in fish fed with diet Z. The relative abundance increased from 8% at the end of the first-feeding period to 51% at the end of the second feeding period. The diet change applied at the end of the first-feeding period had a significant effect on the relative abundance of Proteobacteria and Firmicutes in fish of the first-feeding diet groups Y and Z. In fish of treatment X however, none of the phyla were affected by the diet change and remained relatively constant until the end of the second feeding period.

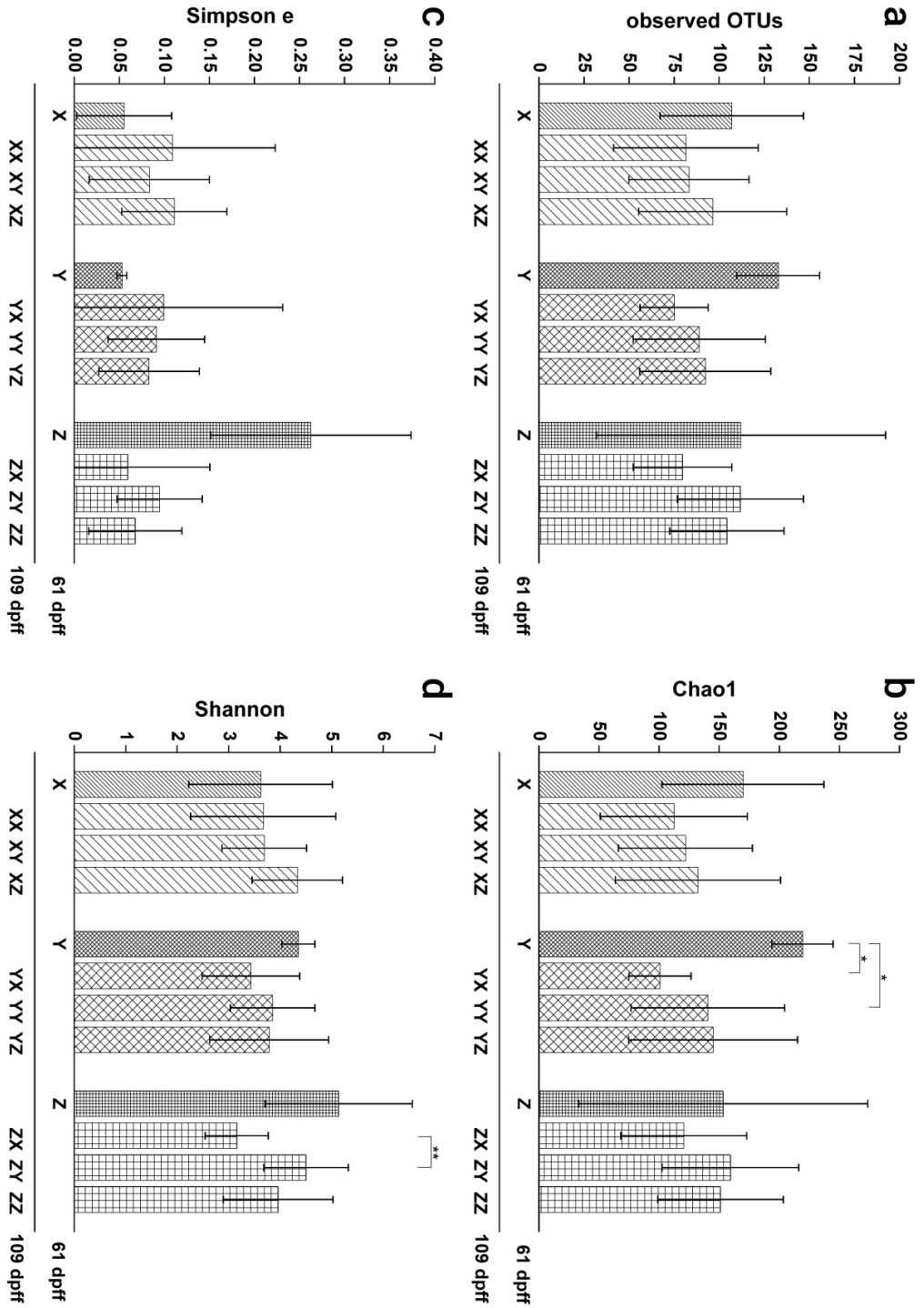


Fig 2-2. Alpha diversity indices in relation to dietary treatment and sampling point. Presented are (a) the number of observed OTUs, (b) the Chao1 richness estimator, (c) the Simpson evenness measure and (d) the Shannon diversity index as means \pm sd of individual fish per treatment and sampling day (data was obtained from one tank per treatment on day 61 pff and three tanks per treatment on day 109 pff; please see Table 2 for exact sample size). Significant differences between treatments are indicated by asterisks: $p < 0.05$ (*), $p < 0.01$ (**).

Table 2-3. The top five most relative abundant phyla in relation to the dietary treatment.

		Mean relative abundance of phyla in [%]				
Diet	dpff	Proteobacteria	Firmicutes	Bacteroidetes	Fusobacteria	Actinobacteria
X	61	74.5 ± 9.9 ^a	15.1 ± 11.8 ^a	2.2 ± 0.4	4.9 ± 3.0 ^a	0.8 ± 0.3
XX	109	58.9 ± 24.3	23.6 ± 24.2	9.0 ± 16.2	2.7 ± 2.5	4.3 ± 10.1
XY	109	47.0 ± 11.7	37.2 ± 16.3	11.4 ± 22.7	1.9 ± 1.6	1.5 ± 1.2
XZ	109	41.3 ± 23.7	37.4 ± 31.1	16.9 ± 18.5	0.3 ± 0.5	1.6 ± 1.2
Y	61	43.1 ± 2.4 ^b	48.4 ± 1.0 ^b	2.3 ± 1.9	4.3 ± 1.1 ^{a,b,*}	1.1 ± 0.3
YX	109	67.4 ± 22.6 ^A	20.1 ± 14.2 ^A	6.4 ± 9.1	4.1 ± 2.8 ^A	0.9 ± 0.8
YY	109	45.6 ± 17.1 ^{A,B}	45.2 ± 20.2 ^B	4.5 ± 9.0	2.0 ± 1.7 ^{A,*}	1.8 ± 2.8
YZ	109	29.1 ± 20.4 ^B	53.0 ± 31.0 ^B	13.5 ± 19.6	0.4 ± 0.4 ^B	1.1 ± 1.0
Z	61	46.9 ± 18.7 ^{a,b}	8.2 ± 7.2 ^{a,*}	13.2 ± 8.8	0.1 ± 0.2 ^{b,*}	16.8 ± 23.6 [#]
ZX	109	73.9 ± 9.0 ^A	18.8 ± 7.4 ^A	1.6 ± 1.5	4.6 ± 2.7 ^A	0.9 ± 1.8
ZY	109	45.5 ± 10.6 ^B	36.3 ± 20.3 ^{A,B}	13.0 ± 16.1	2.0 ± 1.2 ^B	1.4 ± 1.0
ZZ	109	29.2 ± 10.7 ^C	50.6 ± 26.2 ^{B,*}	16.0 ± 18.8	1.2 ± 1.5 ^{B,*}	1.0 ± 1.0

The means ± sd in percent for the top five most abundant phyla are presented (one tank per treatment at the end of the first-feeding period (61 dpff); three tanks per treatment at the end of the second feeding period (109 dpff); please see Table 2 for exact sample size). Statistically significant differences between first-feeding diets are indicated by lower case letters. Statistically significant differences between second feeding diets are indicated by upper case letters, separate for each corresponding first-feeding diet. Statistically significant differences between sampling points of continuously fed diets are indicated by asterisks. [#]The mean of Actinobacteria abundances, calculated for diet Z, was significantly increased by one sample identified as outlier via Grobb's test. Due to overall consistency it has not been removed from the analysis.

The results of the first ANOVA indicated no significant interaction between the first and the second feeding period. Thus the data were pooled for the first-feeding diet and a second ANOVA was performed. The statistical analysis demonstrated that Proteobacteria were significantly enhanced when fishmeal was integrated into the second feeding diet ($p < 0.01$). The same findings can be observed for Fusobacteria ($p < 0.01$). Firmicutes, in contrast, were significantly promoted by plant proteins ($p < 0.001$), and so were Bacteroidetes ($p < 0.05$). The phylum Actinobacteria, however, was not affected by the diet type at all. In Fig 2-3 it can be additionally observed that in fish of treatment Z the number of phyla and their relative abundance are higher compared to the treatments X and Y, which goes in line with the results of the diversity analysis presented in Fig 2-2.

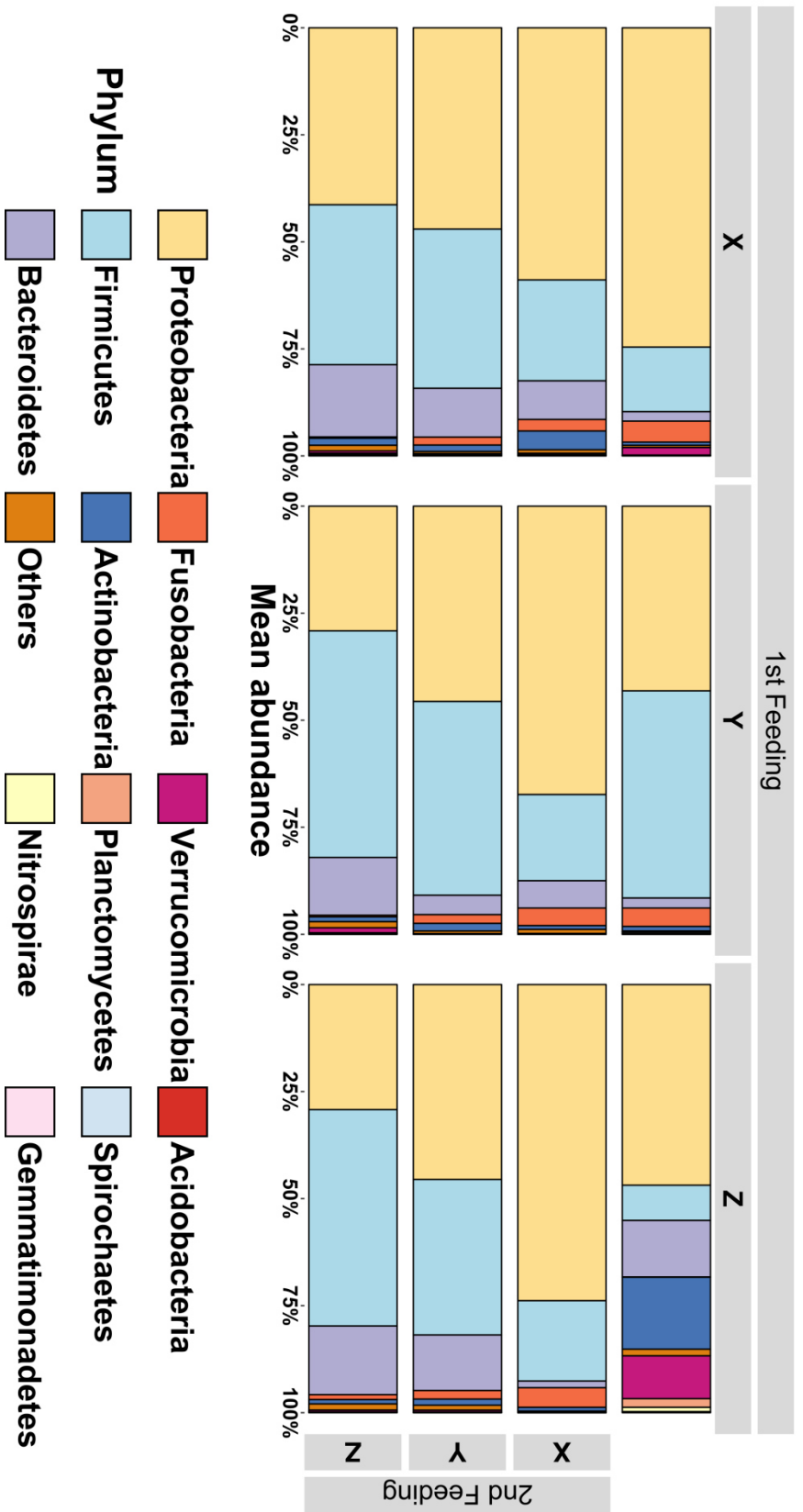


Fig 2-3. The most relative abundant phyla in relation to the dietary treatment and feeding period. The graph shows the relative abundance in percent of phyla that are present in $\geq 10\%$ of all samples and account for $\geq 1\%$ of all phyla. Phyla that did not fulfil those conditions were combined into "Others". The data presented are means calculated from individual fish (one tank per treatment at the end of the first-feeding period (61 dpff); three tanks per treatment at the end of the second feeding period (109 dpff); please see Table 2 for exact sample size).

3.3 Shaping the gut microbiome

The bacterial gut communities were explored on order level via NMDS and as it can be seen in Fig 2-4 the bacterial communities are grouping by treatment. The stress levels of all four NMDS plots are below 0.13, indicating a good representation of the original data. Fig 2-4a reveals that the microbiomes after the first-feeding period of fish fed with diet X and Y are very similar, but the microbiome of fish fed with diet Z is quite different. It can be seen in Figs 4b-d that after the diet change the microbiomes of fish clearly group by the second feeding diet. In general, those data points representing fish fed with the second feeding diet Z are more diffuse, compared to the diets X and Y. The microbiomes of fish continuously fed with diet X or Y (Figs 2-4a and 2-4b) are very similar between day 61 pff and day 109 pff. However, the microbiomes of fish continuously fed with diet Z alter from the first to the second feeding period (Fig 2-4d).

3.4 Influence of diets on specific bacterial orders

The observed alterations in the microbial gut community were further explored via Principal Component Analysis (PCA) and multivariate ANOVA. The results demonstrated a significant influence of the diet fed at the point of sampling on the general bacterial community and on individual bacterial orders. In addition, no permanent effect on the intestinal microbiome of the first-feeding diet could be detected. It can be clearly seen in all panels of Fig 2-5 that the bacterial communities strongly group by the second feeding diet and that the first-feeding diet in contrast has no influence on the community structure at the end of the second feeding period. The second feeding diet X was significantly separated from the other two diets by PC2 and the second feeding diet Z was separated from X and Y by PC1. A multivariate analysis of the bacterial orders significantly contributing to the principal components indicated a strong influence of Vibrionales, Alteromonadales, Lactobacillales and Bifidobacteriales to PC2. PC1 in contrast, is significantly influenced by the abundances of Vibrionales, Lactobacillales, Alteromonadales, Clostridiales, Fusobacteriales and Saprospirales (amongst others). The relative abundances of Vibrionales, Lactobacillales, Clostridiales and Bifidobacteriales of individual fish are exemplarily indicated by the size of points in Figs 2-5a to d and demonstrate the strong effect of individual bacterial orders on the separation and grouping of the intestinal microbiome.

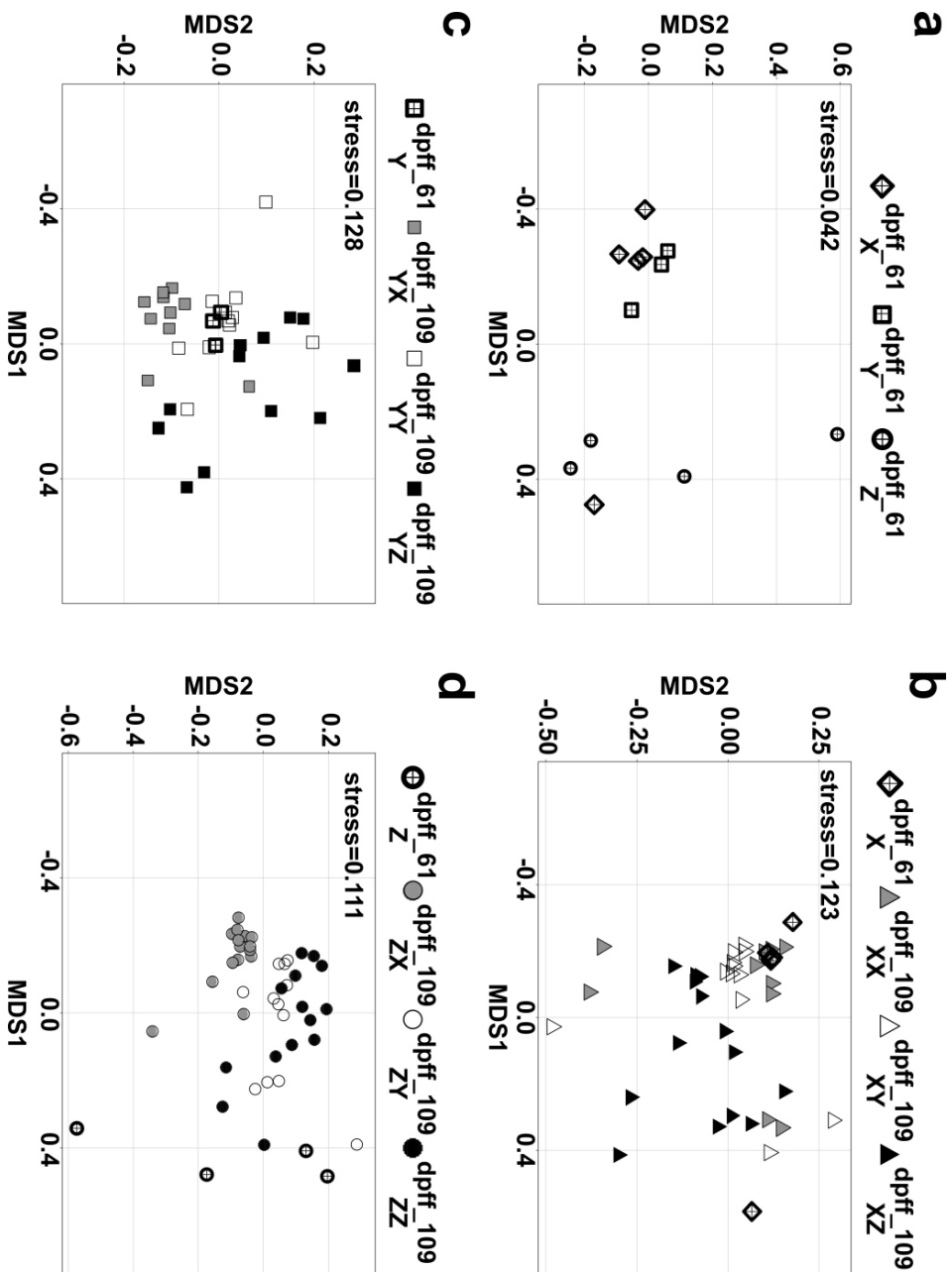


Fig 2-4. Nonmetric multidimensional scaling (NMDS) of the bacterial communities. Presented are ordination plots based on Bray-Curtis-distances between samples (calculated with relative abundances of bacterial orders). Each point in the two-dimensional space represents an individual fish and the distance between points represents the dissimilarity of the respective microbiomes. Panel (a) shows the bacterial community structure of dietary treatments X, Y and Z on day 61 pff. Panels (b), (c) and (d) present the bacterial community structure of the first-feeding diets on day 61 pff in relation to the corresponding second feeding diets on day 109 pff after the diet change. The stress level is a qualitative indicator of the original data representation. The shape of points refers to the first-feeding diet; the different shades of grey indicate the second feeding diet. Crossed points are samples obtained on day 61 pff.

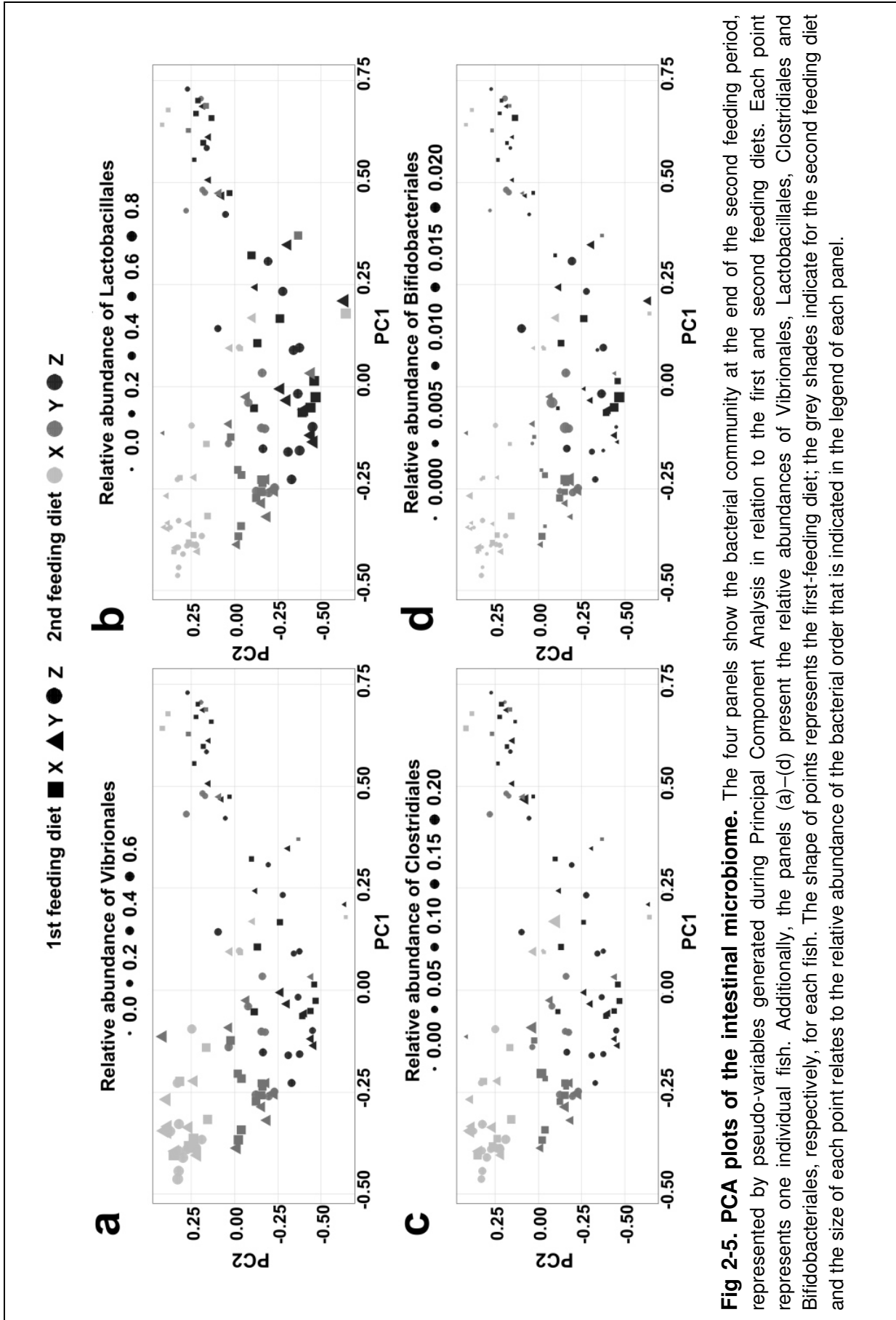


Fig 2-5. PCA plots of the intestinal microbiome. The four panels show the bacterial community at the end of the second feeding period, represented by pseudo-variables generated during Principal Component Analysis in relation to the first and second feeding diets. Each point represents one individual fish. Additionally, the panels (a)–(d) present the relative abundances of Vibrionales, Lactobacillales, Clostridiales and Bifidobacteriales, respectively, for each fish. The shape of points represents the first-feeding diet; the grey shades indicate for the second feeding diet and the size of each point relates to the relative abundance of the bacterial order that is indicated in the legend of each panel.

4 Discussion

4.1 Diversity of the intestinal microbiome

The diversity indices calculated for individual fish were investigated for dietary effects. From the results presented in Fig 2-2 it is obvious that within a treatment the individual variance of diversity indices is very high and thus statistically significant differences difficult to measure. This high inter-individual variance could reflect the various genetic backgrounds and the unknown gender of brown trout fry reproduced from wild fish. It has been demonstrated for chicken that, under identical feeding and husbandry conditions, host genotype as well as host gender significantly influence the bacterial gut community [37]. Significant associations between the microbial community and the genetic variation of individuals have also been found in humans [38]. In general, the number of observed OTUs at the end of the first-feeding period (61 dpff) is comparable to those at the end of the second feeding period (109 dpff), when the same diet was continuously fed. This could indicate an already formed intestinal microbiome on day 61 pff, especially because these observations are independent of the dietary treatment. Ingerslev et al. [10] observed only a slight increase in the amount of bacteria present until 26 days pff and concluded that the bacterial population is already well established at the onset of first feeding. The evenness measures were all relatively low with an average E of below 0.15, which indicates a high dominance of individual bacterial groups within the bacterial community. This is comparable to previous findings [39]. In this study the microbiomes of pen-raised salmon from Scotland and from Norway with wild caught Scottish salmon were compared and very high abundances of single genera were found. *Mycoplasma*, for example, accounted for about 96% of all bacteria identified from wild salmon and *Acinetobacter* for about 55% of the bacteria found in salmon of the Norwegian facility. As can be concluded from the Chao1 richness estimator (Fig 2-2b), species richness actually decreased with time and with the amount of fishmeal used in the second feeding diets. This decrease is, however, not statistically significant. Similar results have been found for two Antarctic fish species with different feeding strategies that indicated a greater gut microbial diversity of the omnivorous individual than of the carnivorous [40]. Additionally, the analysis of pooled data after the second feeding period of the current experiment revealed that significantly more OTUs were found in gut samples of fish fed the plant-based diet and that Shannon diversity was also significantly increased compared to gut samples from individuals fed the fishmeal diet. Ley et al. [41] studied the co-evolution of mammals and their indigenous microbial communities and found an increasing bacterial diversity from carnivory to herbivory, which indicated a co-diversification of the intestinal microbiome with its host.

4.2 Effects of specific bacterial groups on the gut microbial community

The most abundant phyla found in the intestinal samples of brown trout are similar to phyla found in other salmonids [42]. Proteobacteria, Firmicutes, Bacteroidetes, Fusobacteria and Actinobacteria were the most abundant phyla in samples of all treatments. The diet-type, i.e. the inclusion levels of plant-proteins or fishmeal, significantly enhanced specific phyla. This has already been demonstrated in previous studies with rainbow trout [6, 10] and is corroborated here for brown trout. In the current experiment, Proteobacteria and Fusobacteria were significantly promoted by fishmeal present in the diet. Plant-based diets in contrast, significantly enhanced the relative abundance of Firmicutes and Bacteroidetes. Firmicutes also significantly increased from day 61 pff to day 109 pff in samples of fish that were fed with the plant-based first-feeding diet Z. However, the diet-change significantly affected the observed increases. The increase was highest for the second feeding diet Z and lowest for diet X, demonstrating the strong influence of the second feeding period on the final gut microbiome. These findings were supported by the statistical analysis on order level. The ordination plots (Fig 2-4) clearly visualise that for diets X and Y the samples of day 61 pff are very similar to those of day 109 pff; however, not for diet Z. NMDS analysis shows that the points become far-scattered if diet Z was fed compared to the diets X and Y, which could be due to the already discussed inter-individual variances. Moreover, this analysis demonstrates the strong separation of the intestinal microbiome by the three experimental diets. The bacterial communities of fish fed the mixed diet Y are always located between those of fish fed the fishmeal diet X and the plant-based diet Z. These results align well with those of a comparative study on bacterial communities from different freshwater species [43]: intestinal microbiomes of those species were significantly separated by trophic status (i.e. carnivorous, herbivorous, omnivorous, filter feeders), and Principal Coordinate Analysis (PCoA) based on unweighted UniFrac distance matrix indicated a similar position of microbiomes from omnivorous fish between those from carnivorous and herbivorous individuals in the ordination space. Thus it might be possible that the wild brown trout of the present study undergo a 'temporary' trophic shift evoked by the diet-type. It has additionally been hypothesised that the first-feeding diet would have a permanent effect on the subsequent bacterial community formation in guts of early brown trout fry as known from other studies investigating the concept of nutritional programming [13, 44–46]. However, in the PCA-based analysis no permanent effects of the first-feeding diet on the intestinal microbiome were observed. Instead, fishmeal and plant-protein based diets again formed specific corresponding bacterial communities during every feeding period. Interestingly, this dietary effect is very strong, even though it is known that the host genetic background can

substantially influence the bacterial composition [12], and the experimental animals are offspring of wild fish. The results of the PCA (Fig 2-5) compare well with overall findings. Moreover the analysis strongly confirms the importance of individual bacterial groups on the formation of bacterial community structures. The orders Vibrionales, Lactobacillales, Clostridiales and Bifidobacteriales significantly separated the dietary groups according to the level of fishmeal or plant-based proteins included in the diets. As indicated already by the relative abundance of bacterial phyla, orders incorporating lactic acid producing bacteria, such as Lactobacillales and Bifidobacteriales, are mainly found in fish fed with plant-based proteins, which matches earlier findings [47]. Vibrionales and Clostridiales on the other hand are relatively more abundant in fish fed with fishmeal containing diets; however the abundance of Clostridiales is of lesser extent. Vibrionales are Gammaproteobacteria, and [48] found Proteobacteria to be the dominant phylum in all functional parts of the brown trout intestine when fed a commercial diet. Enterobacteriaceae, Gammaproteobacteria as well, have been identified as the predominant family in the intestine of wild juvenile Sea trout [49]. Thus, the relative abundance of Vibrionales, significantly enhanced by fishmeal based diets, might reflect the natural carnivorous feeding strategy of brown trout. In general, the intestinal microbiome can significantly impact the digestive physiology of fish by contributing metabolites or digestive enzymes. For example, it has been shown that intestinal bacteria substantially contributed to the cellulase activity in wood-eating catfish (*Panaque* spp.) [50]. Furthermore, the gut microflora of buffalo bream (*Kyphosus cornellii*) changes to a new bacterial community with the ability to ferment long-chain carbohydrates during developmental metamorphosis of strictly carnivorous larvae to strictly herbivorous juveniles [51]. And, in a study with Atlantic salmon [52] it was also demonstrated that lactic acid bacteria, isolated from the gut, can inhibit growth of three important fish pathogens *in vitro*. Thus, the strong influence of feed ingredients on the intestinal microbiome as observed in the present study could further have a substantial impact on host digestive capacities and should be addressed in future research.

5 Conclusion

To the authors' knowledge, this is the first study providing insight into the effects of plant-based diets on the intestinal microbiome of wild juvenile brown trout. The results confirm the strong influence of the feeding regime on the bacterial community structure in intestines of salmonids and demonstrate that the brown trout microbiome very well aligns with the bacterial communities found in other salmonid species. Proteobacteria, Firmicutes, Bacteroidetes, Fusobacteria and Actinobacteria were again the dominant phyla supporting the hypothesis of a possible core microbiome of salmonids. The microbiome of first-feeding

brown trout was significantly shaped by the diet-type, especially by certain bacterial orders, however not permanently. Vibrionales and Clostridiales were significantly associated with fishmeal diets, Lactobacillales and Bifidobacteriales with plant-based diets. Those findings emphasise the need for future studies about the metabolic contribution of intestinal bacteria associated with a specific diet-type, to understand the physiological consequences for trout.

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Chapter 3

Alternative protein sources for first-feeding fry: the potential of nutritional programming in rainbow trout (*Oncorhynchus mykiss*)

Stéphanie Céline Michl^{a,b,c}, Claudia Proksch^a, Jeffrey A. Hutchings^{d,e}, Carsten Schulz^{a,b}

^aGesellschaft für Marine Aquakultur mbH (GMA) Büsum, Hafentörn 3, 25761 Büsum, Germany

^bInstitute of Animal Breeding and Husbandry, Department for Marine Aquaculture, Christian-Albrechts-Universität zu Kiel, Hermann-Rodewald-Str. 6, 24118 Kiel, Germany

^cGEOMAR Helmholtz Centre for Ocean Research, Wischhofstr. 1-3, 24149 Kiel, Germany

^dDepartment of Biology, Dalhousie University, 1355 Oxford Street, Halifax, Nova Scotia B3H 4R2, Canada

^eCentre for Ecological and Evolutionary Synthesis (CEES), Department of Biosciences, University of Oslo, PO Box 1066 Blindern, NO-0316 Oslo, Norway

Abstract

Commercial diets for rainbow trout are increasingly based on plant-derived protein sources. Within the last few decades it has been widely demonstrated that plant-based feed ingredients can be a good substitution for fishmeal, if feedstuffs are adequately processed. Several purification steps, pre-treatments with digestive enzymes or the addition of artificial amino acids are necessary to manufacture plant-based aquafeeds with high nutritional value and low anti-nutritive effects. The present study aimed to investigate whether negative effects of plant-derived raw materials in diets for rainbow trout (*Oncorhynchus mykiss*) can be anticipated if those feedstuffs represent the initial feed fed to first-feeding fry. This concept of nutritional programming was evaluated in a cross-over feeding design with special focus on activity levels of the digestive enzymes amylase and pepsin. Three experimental diets with several plant-protein inclusion levels (0%, 50% and 97%) were applied from first feeding onward and crosswise exchanged after a first feeding period of more than two months. Amylase and pepsin activities, measured several times during the feeding trial, were not affected by the several inclusion levels of plant-based feed ingredients. Instead, we observed a strong positive relation between dietary starch levels and amylase activity. Growth of fish was accordingly determined via dry and wet body weight, but no nutritional programming effects on growth performance data were detected. In contrast, growth performance of fish from all feeding regimes at the end of the experiment was independent of the diet-type provided at first feeding. Moreover, rainbow trout fed diets with 50% plant-derived protein sources demonstrated enhanced growth throughout the experiment when compared to fish fed fishmeal as the exclusive protein source. The present study indicates that a certain percentage of plant-derived feedstuff might be beneficial for the growth of juvenile rainbow trout and suggests further reduction of fishmeal use in first-feeding diets.

Key words: Amylase; Rainbow trout; Digestion; Digestive enzymes; Nutritional programming; Pepsin; Plant proteins; Proteases

1 Introduction

The feeding of plant-derived proteins can have severe effects on health and nutrient digestibility in carnivorous fish if raw materials are not adequately processed. Plant-based feedstuff needs to fulfil particular requirements in order to successfully substitute fishmeal in aquafeeds, such as availability, pricing and especially adequate nutritional value [1]. Most plant-based raw materials need to undergo several expensive refinement steps to be of high nutritional value for fish. For example, soy protein concentrates usually have a similar amino acid profile to fishmeal and are a favorable protein source for fish, however, ash and fat concentrations are rather low and need to be supplemented by lipids and artificial minerals [1]. Another example is rapeseed or canola products, in which phosphorus is bound in phytic acid and, thus, not available for fish.

It is known that exogenous enzymes are beneficial for digesting plant-based feed ingredients [2] and phytic acid can be eliminated by adding the enzyme phytase [3]. Most legumes and seeds contain digestive enzyme inhibitors, such as trypsin-inhibitors, strongly reducing protein digestibility [4], or amylase-inhibitors, significantly affecting carbohydrate digestion [5]. These anti-nutritive factors need to be removed by, for example, heat treatment during extrusion [6]. Corn gluten meal is almost free of common anti-nutritive factors but high in non-soluble carbohydrates with little nutritional value. Although these carbohydrates can be separated from the protein fraction, they simultaneously increase production effort [7]. Thus, new and more cost-efficient approaches, such as the concept of nutritional programming, are needed to eliminate the challenges the aquaculture industry is currently facing when feeding plant-based ingredients to carnivorous fish.

Several attempts in recent years have been made to investigate the potential of nutritional programming in fish [8–10]. Feed intake, feed efficiency and growth rates of rainbow trout (*Oncorhynchus mykiss*) fed plant-based diets can be significantly enhanced, if fish experience these diets during an early-life stimulus [11]. Further evaluation of these findings revealed that nutritional programming in trout is associated with the regulation of pathways involved in, for example, sensory perception and cognitive processes [12]. However, nutritional programming can also negatively affect fish metabolism: it has been shown that gluconeogenesis regulation in Siberian sturgeon (*Acipenser baerii*) was significantly disturbed after an early exposure to high dietary glucose levels [13].

Nutritional programming is based on a nutritional stimulus experienced during early development, leading to long-term or permanent physiological effects [14]. A prerequisite of nutritional programming is the concept of developmental plasticity [14], a degree of plasticity

usually rather irreversible [15] and including responsiveness not only to the external environment, but also to genetic activity [16]. Phenotypic plasticity, in contrast, is more simply defined as “[...] the environmentally sensitive production of alternative phenotypes by given genotypes [...]” [17] and can be reversed over shorter time scales [15].

We conducted an experiment with first feeding rainbow trout fry to induce nutritional programming via first feeding diets with different plant-based protein inclusion levels. In contrast to previous studies we applied a fully cross-over diet change and sought to explore whether the first feeding diet-type resulted in permanent physiological responses after the diet-change. Those responses were hypothesized to be not only visible in growth performance, but also in digestive enzyme activities. We focused on the pancreatic enzyme amylase, which is directly involved in dietary carbohydrate breakdown, and on the stomach enzyme pepsin, the first protease to break down large proteins into smaller peptides that can be further digested by alkaline enzymatic reactions in the intestine.

2 Material & Methods

2.1 Experimental animals

The experiment was conducted at the “Gesellschaft für Marine Aquakultur mbH” (Büsum, Germany). Eyed rainbow trout eggs (*Oncorhynchus mykiss*, Kamloops strain) were purchased from the trout farm “Forellenzucht Trostadt GbR” (Reurieth-Trostadt, Germany), originating from “Troutlodge Inc.” (Sumner, Washington USA). All animal handling procedures were approved by the animal welfare officer of the “Gesellschaft für Marine Aquakultur mbH” and the local authority of Schleswig-Holstein, according to the German animal welfare law (TierSchG).

2.2 Experimental diets

Three isonitrogenous and isoenergetic (on digestible matter; Table 3-1a) experimental diets (diet A, diet B and diet C) were pelletized with different inclusion levels of plant-derived proteins (0%, 50% and 97%). Particle sizes were adjusted according to the needs of growing fish in the course of the experiment. Animal-derived feedstuffs were the exclusive protein sources for Diet A. Diet C, in contrast, contained 97% of plant-derived protein sources. Three percent of gelatin was added as hardener during the manufacturing process of the first feeding diets. Diet B contained 50% plant- and 50% animal-derived protein sources and was, thus, intermediate between diet A and diet C. The composition of amino acids of each diet conformed to NRC [18] amino acid requirements for small rainbow trout (0.2 - 20.0 g); the

composition of the vitamin and mineral premixtures were also in accordance with NRC guidelines.

Table 3-1a. Composition of experimental diets.

Ingredients (in % of dry matter)	Diet A	Diet B	Diet C
Fishmeal ¹	64.65	28.74	
Mussel meal ²	2.13	2.00	
Blood meal ³	6.14	0.96	
Shrimp meal ¹	8.65	6.00	
Corn gluten ⁴		1.00	2.00
Soy protein concentrate ⁵		5.00	5.00
Pea protein ⁶		19.86	48.19
Rapeseed concentrate ⁷		4.84	15.96
Wheat gluten ⁸		10.00	2.79
Wheat starch ⁸	6.43	3.38	2.00
Vitamin & Mineral Premixtures ⁹	4.00	4.00	4.00
Linseed oil ¹⁰	2.00	3.06	2.00
Fish oil ¹	3.00	4.78	9.07
Gelatine ¹¹	3.00	3.00	3.00
Bentonite ¹²		3.38	5.97
Tryptophan ¹³			0.03
Proximate composition (in % of dry matter)			
Dry matter (in % of diet)	88.78	92.25	91.16
Crude protein	62.72	62.81	62.84
Crude fat	12.72	13.12	17.48
Crude ash	15.83	13.00	10.36
Gross energy (MJ kg ⁻¹)	21.37	22.15	23.42
Digestible energy (MJ kg ⁻¹) ¹⁴	19.40	19.30	18.90

¹ Vereinigte Fischmehlwerke Cuxhaven GmbH & Co. KG, Cuxhaven, Germany

² CRM - Coastal Research & Management, Kiel, Germany

³ SARVAL Ouest, Issé, France

⁴ Cargill Deutschland GmbH, Krefeld, Germany

⁵ EURODUNA Rohstoffe GmbH, Barmstedt, Germany

⁶ Emsland-Stärke GmbH, Emlichheim, Germany

⁷ Helm AG, Hamburg, Germany

⁸ Kröner Stärke GmbH, Ibbenbüren, Germany

⁹ Aller Aqua Mix 7188 Micro & 7180 Vit. STD., Golßen, Germany

¹⁰ Makana Produktion und Vertrieb GmbH, Offenbach, Germany

¹¹ ARTI-Vital, Freyburg, Germany

¹² DEL LAGO Bentonite, Castiglioni Pes y Cia, Buenos Aires, Argentina

¹³ Evonik Industries AG, Essen, Germany

¹⁴ Calculated – based on ADC values available from current literature

A chemical nutrient analysis of all experimental diets was performed according to the European Commission Regulation (EC) No 152/2009 (European Union 2009). Feed samples were dried at 103°C for 4 hours (ED 53, Binder GmbH, Germany) to determine dry matter content. Crude ash content of feed was determined in a combustion oven at 550°C for 4 hours (P300, Nabertherm, Germany). Crude protein and crude lipid content of experimental

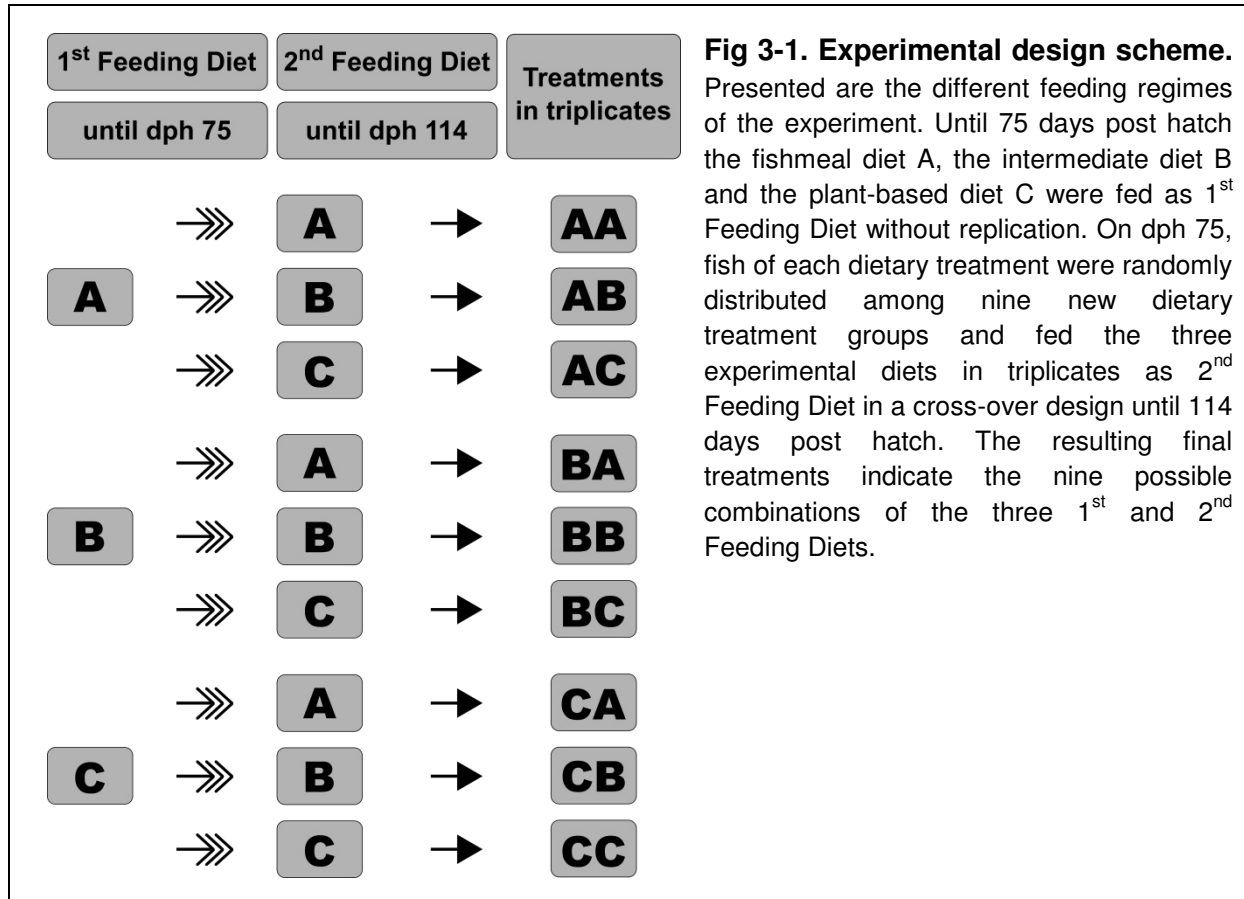
diets were determined by following the protocols of Kjeldahl (InKjel 1225 M, WD 30, Behr, Germany) and Soxhlet (Soxtherm, C. Gerhardt GmbH & Co. KG, Germany). Gross energy was measured, using a bomb calorimeter (C200; IKA, Germany). Analysis of dietary fatty acids, amino acids and mineral composition were performed by Skretting ARC (Table 3-1b).

Table 3-1b. Composition of experimental diets.

Minerals (in % of diet)	Diet A	Diet B	Diet C
Total phosphorous	1.89	1.09	0.73
Calcium	4.20	2.50	0.83
Zinc	6.43	5.88	6.17
Amino acid composition (in % of diet)			
Arginine	3.06	3.47	4.27
Histidine	1.32	1.18	1.27
Isoleucine	1.91	2.18	2.45
Leucine	3.91	4.03	4.49
Lysine	3.85	3.23	3.46
Methionine	1.30	1.05	0.78
Cystine	0.43	0.59	0.63
Phenylalanine	2.22	2.48	2.84
Tyrosine	1.01	1.48	1.58
Threonine	2.14	2.01	2.13
Valine	2.75	2.56	2.72
Alanine	3.36	2.74	2.57
Aspartic acid	4.93	4.94	5.99
Glutamic acid	6.57	9.66	9.92
Glycine	3.84	3.19	2.78
Proline	2.46	3.16	2.95
Serine	2.23	2.49	2.78
Fatty acid composition (in % of total fatty acids)			
<i>n-6 / n-3 ratio</i>	0.28	0.64	0.97
Total n-6	6.59	15.60	19.81
Total n-3	23.86	24.2	20.32
<i>ALA / LA ratio</i>	1.78	0.97	0.63
Total C18:2n-6 (LA)	5.38	14.79	19.12
Total C18:3n-3 (ALA)	9.57	14.34	12.10
<i>EPA / DHA ratio</i>	0.76	0.88	1.04
Total C20:5n-3 (EPA)	4.90	3.72	3.21
Total C22:6n-3 (DHA)	6.47	4.22	3.10

2.3 Experimental design and sampling

In total, 7500 eyed rainbow trout eggs were randomly distributed among three commercial hatching troughs that were integrated into a recirculating waterbody (2500 eggs each). Rainbow trout were reared for a total of 122 days. Until hatching, average water temperature was $9.0 \pm 0.2^\circ\text{C}$ and, by increasing water temperature to 11.0°C , hatching was induced. The mean temperature remained at $11.6 \pm 1.1^\circ\text{C}$ until the end of the experiment. The three experimental diets were supplied at 20 days post hatch (dph) for the first time and active first feeding was observed on dph 21. Each of the three experimental groups for the first feeding period was reared in one hatching trough. Trout fry were fed one of the three first feeding diets (A, B or C) *ad libitum* using automatic feeders. For 19 days, feed was supplied once per hour, but subsequently reduced and, for the remainder of the experiment, feed was supplied four times per day. In order to make feed particles visible to the fish, dimmed light was provided from 06:00 am to 09:00 pm. A cross-over diet change (Fig 3-1) was applied at dph 75 (end of the first feeding period) and the second feeding period lasted until dph 114. Six hundred trout fry from each hatching trough were randomly distributed among 27 aquaria (50 L) that were integrated into the established recirculating system. Until dph 114, each experimental group was fed 3.8% of the total biomass.



2.3.1 Sampling for growth data

Five fish from one hatching trough were sampled for determining dry body weights on hatching day and on four additional dates during the yolk sac stage. At the onset of first feeding (dph 21) and on five additional dates until dph 75 (start of second feeding period), ten fish per hatching trough were sampled for measurement of dry body weight. Wet body weight was measured on dph 75 and 114. Mean dry body weights of ten individual fish obtained separately for every sampling day were used to calculate specific growth rates (SGR) for the entire first feeding period and for three intermediate periods:

$$\text{SGR} = [\ln(m_f) - \ln(m_i)] / t * 100,$$

where m_f = dry body weight at dph f , m_i = dry body weight at dph i , $t = f - i$, $f > i$ [19].

2.3.2 Sampling for amylase and pepsin activity assays

Five eggs from one hatching trough were sampled for amylase activity assays five days before hatching and on hatching day. Five fish from one hatching trough were sampled for amylase and pepsin activity analysis on hatching day and on three additional dates during the yolk sac stage. After first feeding on dph 21, five fish per hatching trough were sampled on seven dates until dph 31. Additional samples for digestive enzyme analysis were taken on dph 75 (five fish per hatching trough) and dph 114 (five fish per aquarium, three aquaria per treatment).

2.4 Sample preparation

Rainbow trout were fed approximately one hour before sampling. Fish were narcotized with MS222 (Tricaine methanesulfonate, E10521, Sigma-Aldrich Co. LLC.) and immediately killed by cutting the gill vein. Smaller fish (i.e. before dph 31) were narcotized and killed with an overdose of MS222. Samples that were used to measure dry body weights were immediately frozen at -20°C and freeze-dried for 72 hours prior to weighing. Whole egg homogenates were used for digestive enzyme analysis before hatching and whole body homogenates were used after hatching, before first feeding and until dph 31. All samples were frozen at -80°C until analysis. For digestive enzyme analysis of fish on dph 75 and dph 114, the whole GI tract was dissected on ice and instantly frozen at -80°C .

2.5 Amylase and pepsin activity assays

Before performing amylase and pepsin activity assays samples were thawed at 4°C . The fluorescence based EnzChek® Ultra Amylase Assay Kit (E33651, Molecular Probes, Inc.) was used to determine Amylase activities, according to manufacturer's specifications. A buffer solution of 20 mM $\text{H}_2\text{PO}_4\text{Na}$, 6.7 mM NaCl and 0.5 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (pH 6.9) was

prepared for homogenising samples and fluorescence assays. Pepsin activity was measured with the EnzChek® Protease Assay Kit *green fluorescence* (E6638, Molecular Probes, Inc.) according to manufacturer's specifications. A buffer solution of 10 mM HCl (pH 2.0) was prepared for homogenisation and further reactions. Respective buffer solutions were added with a final volume to weight ratio of 3:1 (buffer:tissue). The samples were homogenised (KT Miccra D9 homogeniser, ART Prozess- & Labortechnik GmbH & Co. KG, Germany) on ice for 30 seconds at 25,000 RPM and centrifuged (Universal 320R centrifuge, Hettich Zentrifugen, Germany), at 4°C for 10 minutes with 10,000 RPM (whole body samples) and for 20 minutes with 5,000 RPM (whole digestive tract samples). The clear supernatant was transferred into a new reaction tube and glycerine was added to a final volume of 10% of the sample solution. Until performance of activity assays, samples were stored at -20°C. α -amylase from *Bacillus* spp. (A-6380, Sigma-Aldrich Co. LLC.) was used as the standard for amylase activity assays. The temperature during the assay was constantly set to 25°C. The fluorescence intensity was measured after 20 min at 485 nm (excitation) and 520 nm (emission). Pepsin from porcine gastric mucosa (P-6887, Sigma-Aldrich Co. LLC.) was used as a standard for pepsin activity assays. The reaction temperature was set to a constant 37°C. The fluorescence intensity was measured after 30 min at 490 nm (excitation) and 525 nm (emission). The activity assays were performed on a spectrophotometric microplate reader (TECAN infinite M200, Tecan Trading AG, Switzerland). The readout of each assay was in Fluorescence Units (FU). The standard curves with pepsin and amylase solutions of known activity were used to convert FU into activity units. Therefore, activity data are expressed as mU per mg of used tissue (i.e. egg homogenate, whole body homogenate or whole digestive tract homogenate). If samples had activity units below zero after the conversion from FU to mU, they were excluded from further calculations and statistics.

2.6 Influence of experimental diets on the amylase activity assay

An on-the-side experiment was conducted to evaluate possible influences of diet extracts on the activity of *B. subtilis* standard amylase. The diets A, B and C were ground and incubated either in the phosphate buffer solution or in 10 mM HCl with a final concentration of 20.0 mg ml⁻¹. Following an incubation period of 30 min at 25°C while shaking, samples were centrifuged five minutes at 5000 RPM. The supernatants were diluted 1:10 with the phosphate buffer solution to obtain a pH of 6.9 and were subsequently used for a serial dilution of the *B. subtilis* standard amylase. Thereafter, the amylase activity assay was performed as described previously. Activity assays were additionally performed with feed extracts only and with amylase standard only.

2.7 Statistical analysis

The statistical software R (2014) was used to evaluate the data. A statistical model based on generalised least squares [20] was used to describe data collected during the first feeding period. Based on a graphical residual analysis, the data were assumed to be approximately normally distributed and to be heteroscedastic. Diet was included as a fixed factor in the statistical model. Based on this model, an analysis of variance (ANOVA) was conducted and multiple contrast tests [21] were performed, in order to compare individual treatments, using the R package SimComp [22]. For data collected during the second feeding period, an appropriate statistical mixed model [23, 24] was used. Again, the data were assumed to be approximately normally distributed and to be heteroscedastic. The statistical model included diet as a fixed factor and aquarium as a random factor. Based on this model, an ANOVA was conducted, followed by multiple contrast tests (e.g. [25]), in order to compare the several levels of the influence factor, using the R package multcomp [26]. Significant interactions between the first and the second feeding diet were interpreted as nutritional programming effect. In case of a non-significant interaction, data were pooled for the first feeding diet and multiple contrast tests were rerun to compare only the three second feeding diets (A, B and C).

3 Results

3.1 Growth performance during the first feeding period

Dry body weights of individual fish obtained during the entire first feeding period were used to calculate exponential growth curves from first feeding until dph 75 (Fig 3-2). Dry body weights were not significantly different on any of the sampling dates during the first feeding period. However, as can be seen from Figure 1, on dph 59 and dph 75, dry body weights of fish fed diet C were lower than those of fish fed diet A or B. Means comparison for C – A on dph 59 resulted in $p=0.07$ and for C – B on dph 75 in $p=0.06$. The growth constant k was calculated from fitting functions for each of the three different diets: $k_A=0.037$, $k_B=0.056$, $k_C=0.039$, indicating the fastest increase of dry body weight for fish fed diet B. Specific growth rates show a similar picture for fish fed the three experimental diets (Table 3-2). Calculated for the entire first feeding period, fish fed diet B experienced the highest growth rates compared to fish fed diets A and C. In general, SGRs of fish in all treatments were highest during the first ten days of feeding. As already indicated by dry body weights, wet body weights of fish fed diet C were significantly lower ($p<0.001$) than those of fish fed diets A and B (Fig 3-3) when measured at the end of the first feeding period. Wet body weights of fish fed A and B were not significantly different on dph 75.

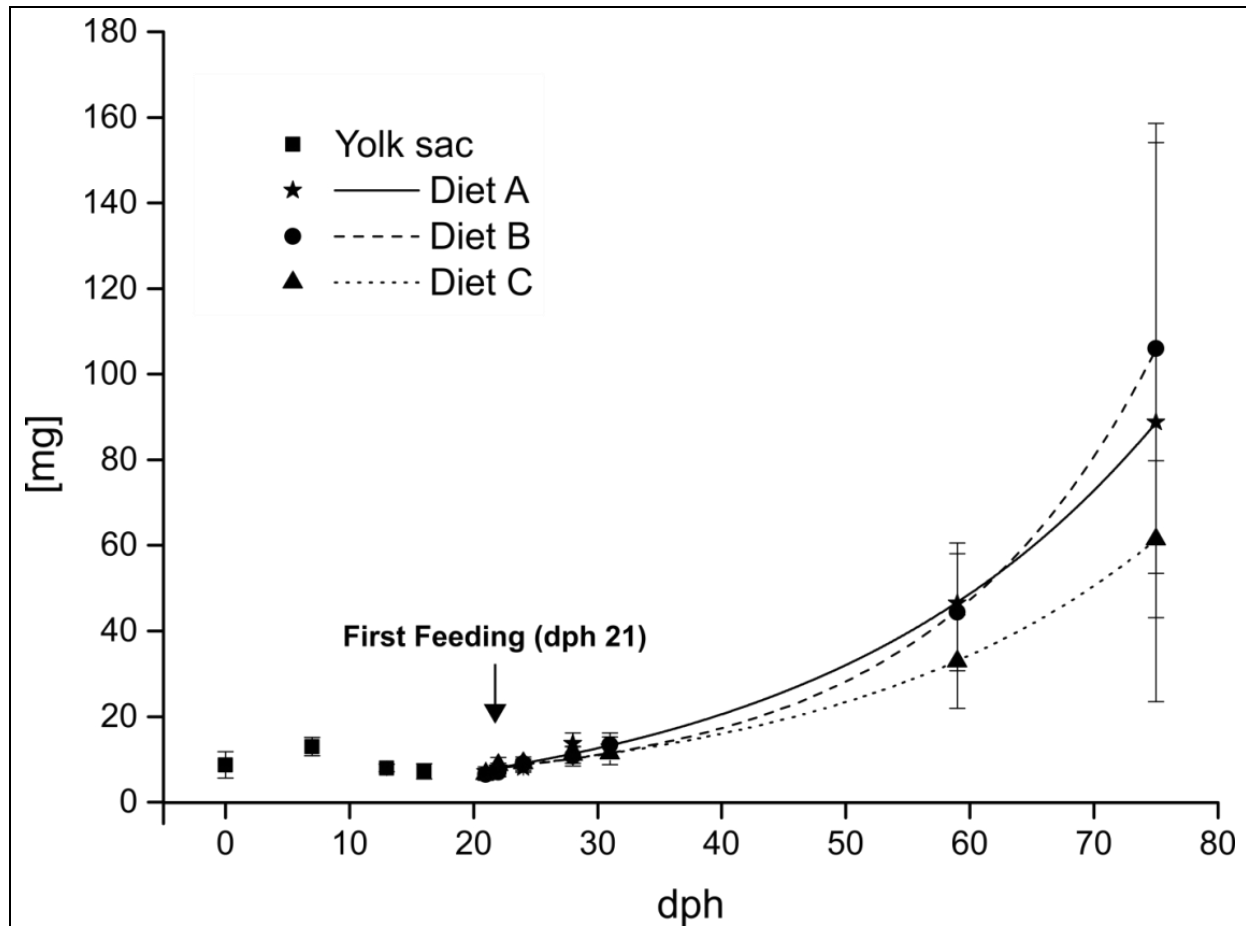


Fig 3-2. Dry body weights of fish and exponential growth curves during the first feeding period. Presented are the means \pm SD of dry body weights in mg of five fish per sampling day during the yolk sac stage and ten fish per treatment and sampling day until dph 75; Solid rectangles indicate yolk sac fry before onset of first feeding; Diets A, B and C are indicated by stars, solid circles and solid triangles, respectively. Exponential curve fitting of means was used to visualise respective growth curves; Diets A, B and C are indicated by solid, dashed and dotted lines, respectively.

Table 3-2. Specific growth rates (SGR) of rainbow trout for the first feeding period.

1 st Feeding Diet	dph 21 – 75	dph 21 – 31	dph 31 – 59	dph 59 – 75
A	4.69	5.98	4.59	4.05
B	5.17	7.24	4.27	5.45
C	4.13	5.44	3.79	3.91

SGRs were calculated from average dry body weights of ten individual fish per dietary treatment during the first feeding period. Dry body weights were obtained separately for every sampling day.

$SGR = [\ln(m_f) - \ln(m_i)] / t * 100$; m_f = dry body weight at dph f , m_i = dry body weight at dph i , $t = f - i$, $f > i$.

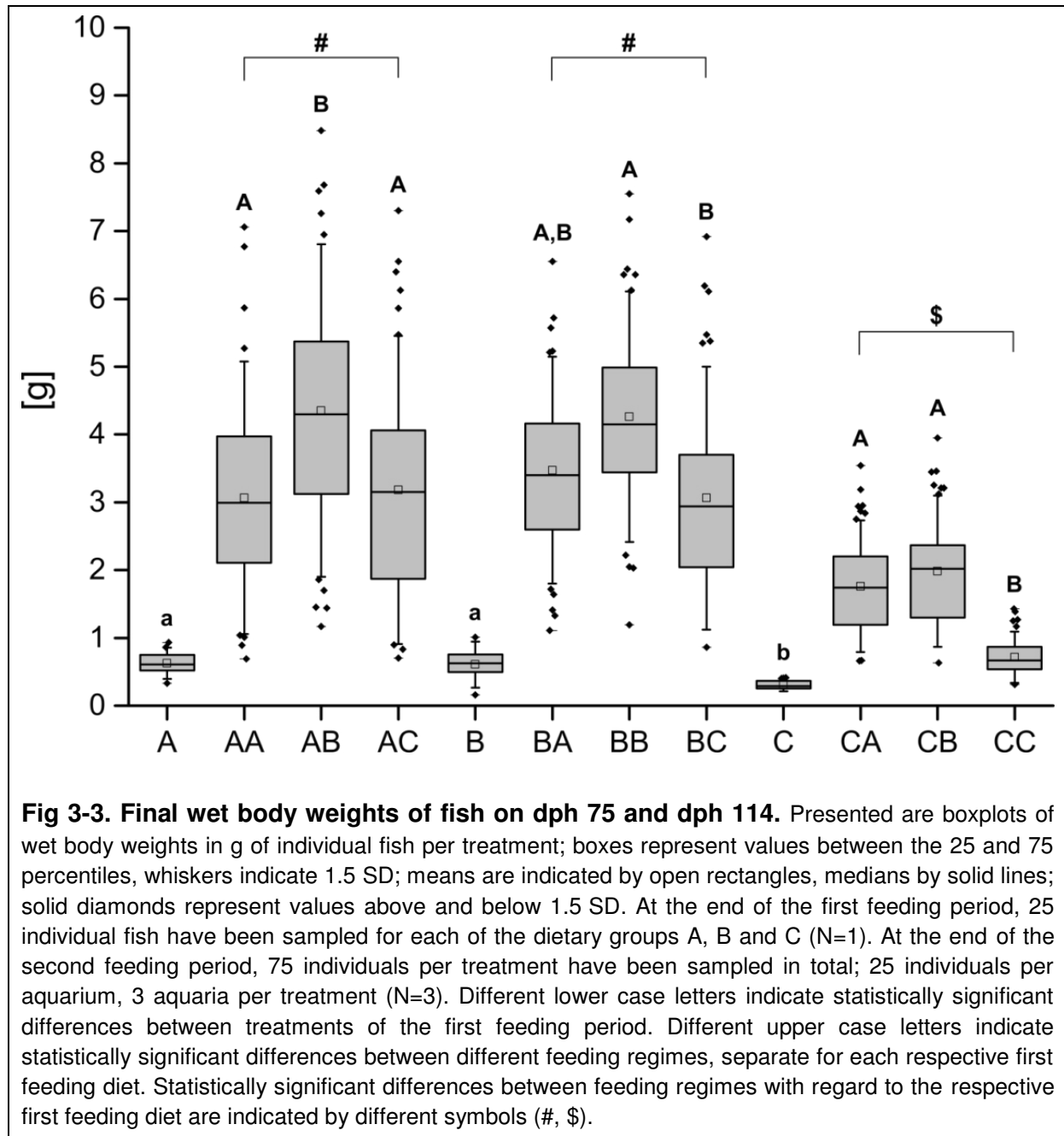
3.2 Diet change and growth performance during the second feeding period

A cross-over diet change was applied on dph 75 to evaluate possible nutritional programming effects induced by the first feeding diet. The statistical model revealed no significant interaction of the first and the second feeding diet that would affect wet body weights; however the adjusted p -value of the interaction was 0.057. By performing simultaneous comparisons of means for the different feeding regimes, it became obvious that the first feeding diet C has a strong influence on the results of the overall ANOVA, resulting in the aforementioned p -value. When diet C was fed as the first feeding diet, all wet body weights of fish were significantly reduced ($p < 0.01$) by the end of the second feeding period – independent of the second feeding diet (Fig 3-3). Furthermore, the second feeding diet B always promoted significantly higher ($p < 0.05$) wet body weights compared to those attained by fish fed diet C, independent of the first feeding diet. Compared to diet A, wet body weights were also higher for all first feeding diets, although this was only significant for fish of the first feeding diet group A. When all diets were continuously fed (feeding regimes AA, BB and CC), fish of feeding regime BB had significantly higher ($p < 0.05$) wet body weights than those associated with feeding regimes AA and CC. Moreover, fish that were continuously fed diet C had significantly lower ($p < 0.001$) wet body weights than those continuously fed diet A. The overall best growth performance was achieved with either of the two feeding regimes AB and BB. Wet body weights of fish fed these two regimes did not differ significantly.

3.3 Amylase activities

Amylase activities could be detected in egg homogenates five days before hatching ($1.7 \pm 0.5 \text{ mU} \cdot \text{mg}^{-1}$) and on hatching day ($1.2 \pm 0.1 \text{ mU} \cdot \text{mg}^{-1}$; data not shown). During the yolk sac stage, until start of feed provision on dph 20, amylase activities remained relatively stable (Fig 3-4). With the onset of first feeding, enzymatic activities showed a strong increase in inter-individual variability. Amylase activity levels slightly increased for fish fed diet A when compared to the yolk sac stage. For fish fed diet B and C, in contrast, activity levels appeared to decrease. However, when whole fish homogenates were analysed, most of the activity assays were not successful for fish that were fed diet B and for some fish fed diet C. Only one sample of treatment B showed sufficient results for each of the sampling days dph 23, 26, 28 and no measurements could be done on sampling days dph 24 and 31. For treatment C, only two samples could be measured on dph 24 and only one sample on dph 26. The problematic activity assays not only showed no increase in enzymatic activity over time, but fluorescence units (FU) were initially below the assay blank (substrate and buffer

only), which indicated a strong quenching effect by unknown biological compounds in the tissue extract. Activity assays performed with feed extracts showed neither an inhibiting effect on *B. subtilis* amylase nor amylolytic activity of the experimental diets themselves nor quenching effects of the substrate.



At the end of the first feeding period, amylase activities of fish fed the three experimental diets were significantly different (Fig 3-5). Amylase levels of fish from treatment A were significantly higher than those of fish from treatment B ($p < 0.001$) and from treatment C ($p < 0.001$). Activities of fish from treatments B and C were, however, equal on dph 75 ($p = 0.42$). Only 3 out of 5 samples from treatment C showed again sufficient results in the

activity assay. After the diet change, amylase activities were measured at the end of the second feeding period, 114 days post hatch (Fig 3-5). When these digestive tract samples were analysed, most of the activity assays were again unsuccessful for fish that were fed diet B as second feeding diet. For the feeding regime AB, only 4 of 15 samples could be analysed as well as for the feeding regime BB, and only 9 samples from feeding regime CB showed appropriate assay results. Therefore, the statistical evaluation of amylase activities obtained on dph 114 for fish fed the second feeding diet B should be interpreted with caution. Nevertheless, a comparison of activities in fish from the feeding regimes AA, BA and CA indicated a significant reduction of activity levels with an increase of plant protein inclusion levels in first feeding diets, which aligns with activity levels measured on dph 75. When the feeding regimes AC, BC and CC were compared, no significant differences in amylolytic activities were found. However, the feeding regimes AC and BC significantly reduced amylase activities compared to the feeding regimes AA ($p < 0.001$) and BA ($p < 0.01$), respectively, but remained equal when CA was compared to CC. The statistical analysis furthermore indicated significantly reduced activities in fish from feeding regime BB compared to those from feeding regime BA ($p < 0.05$), and likewise significantly increased activities in feeding regime CB, when compared to the feeding regimes CA ($p < 0.05$) and CC ($p < 0.05$).

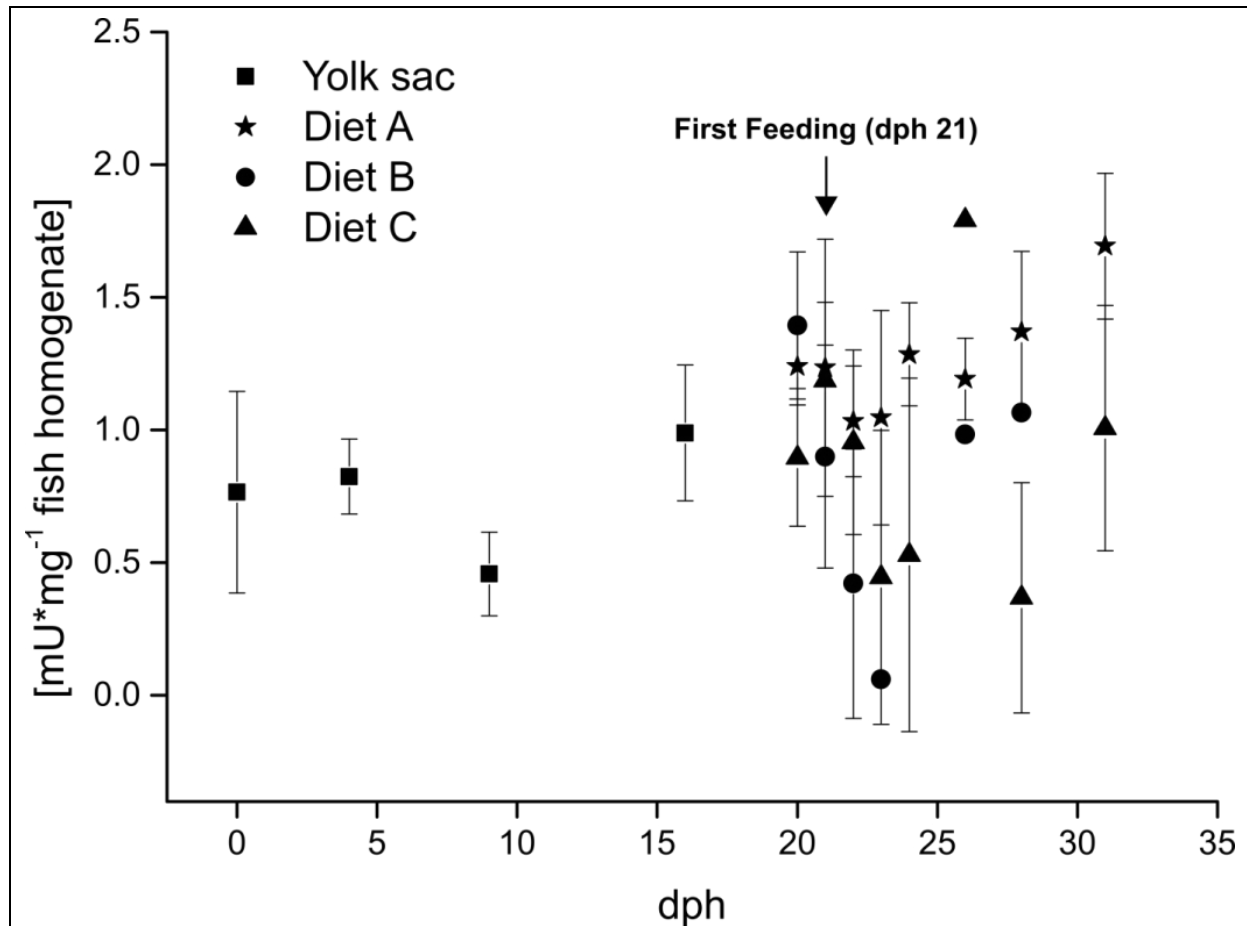
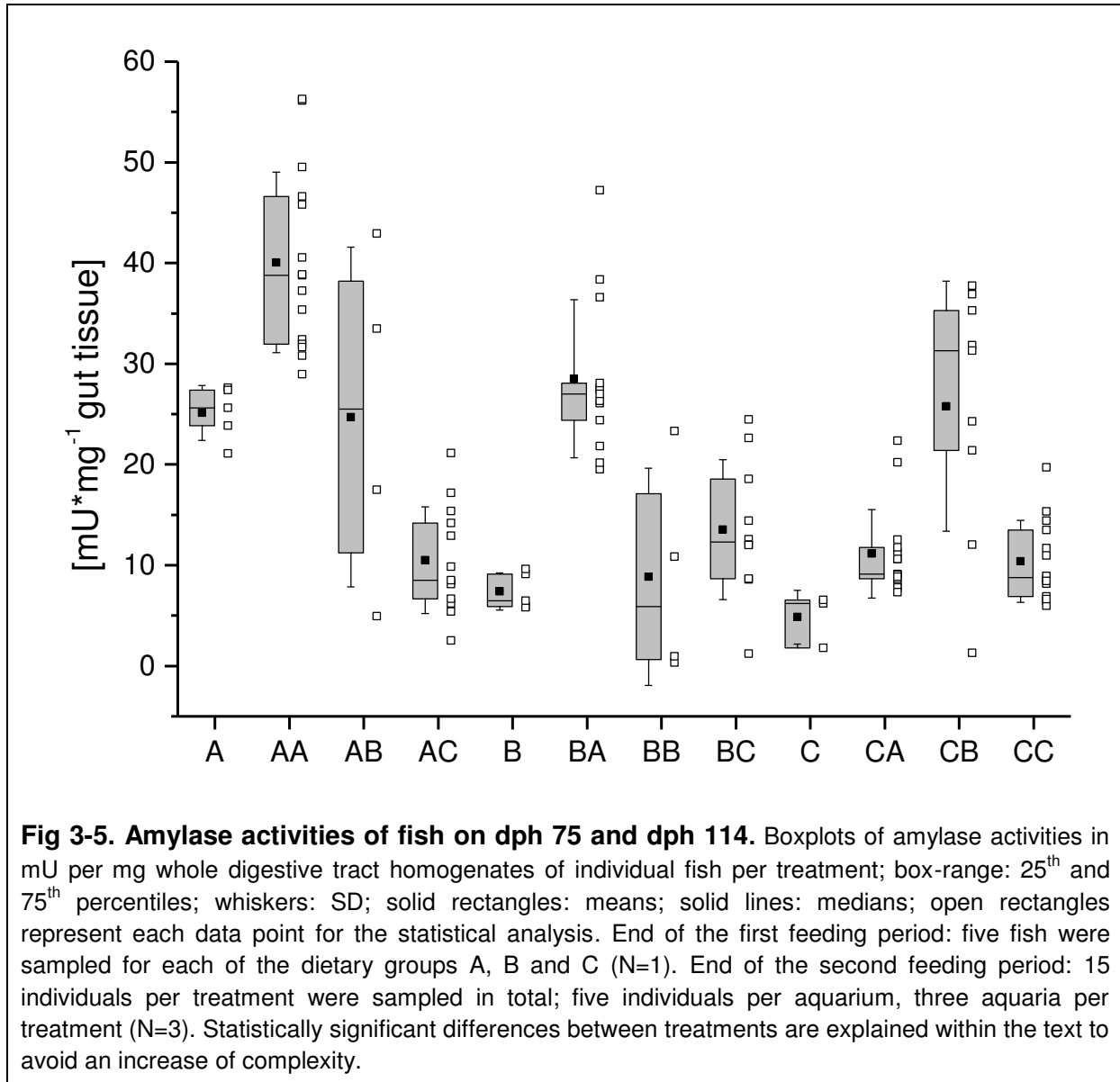


Fig 3-4. Amylase activities during the first feeding period until dph 31. Presented are means \pm SD of amylase activities of five fish per treatment and per sampling day (one tank per treatment; N=1). Amylase activities are presented as mU per mg whole fish homogenate. Yolk sac fry are indicated by solid rectangles; Diets A, B and C are indicated by stars, solid circles and solid triangles, respectively.

3.4 Pepsin activities

Pepsin activity levels measured in yolk sac fry during the first 15 days after hatching were relatively stable, albeit with high inter-individual variability (Fig 3-6). With the start of feed provision and the onset of first feeding, activity levels increased until dph 31 for fish fed diets A and B. After a short increase during the first four days following first feeding, pepsin activities of fish fed diet C remained at the same level as that measured during the yolk sac stage. Likely because of high data variability, none of the activity differences was statistically significant.



On dph 75, pepsin activities in whole digestive tract homogenates were measured for three dietary treatments and mean activity levels of fish fed diet B were higher when compared to fish fed diets A and C (Fig 3-7). However, these differences were not statistically significant due to the high standard deviations. At the end of the second feeding period, mean pepsin activities of fish from all feeding regimes were not significantly different, except for those on feeding regime CC (Fig 3-7). Fish that were fed continuously with diet C exhibited highest overall mean pepsin activities and were significantly different from the feeding regimes CB, AC, BC and BA. Comparisons of means with the feeding regimes AA, AB and BB revealed *p*-values of 0.067, 0.070 and 0.050, respectively. The ANOVA revealed no influence of the second feeding diet on pepsin activity. However, there was a significant interaction of the first and the second feeding diets, which probably resulted from the strong effects caused by pepsin activities of fish from the feeding regime CC.

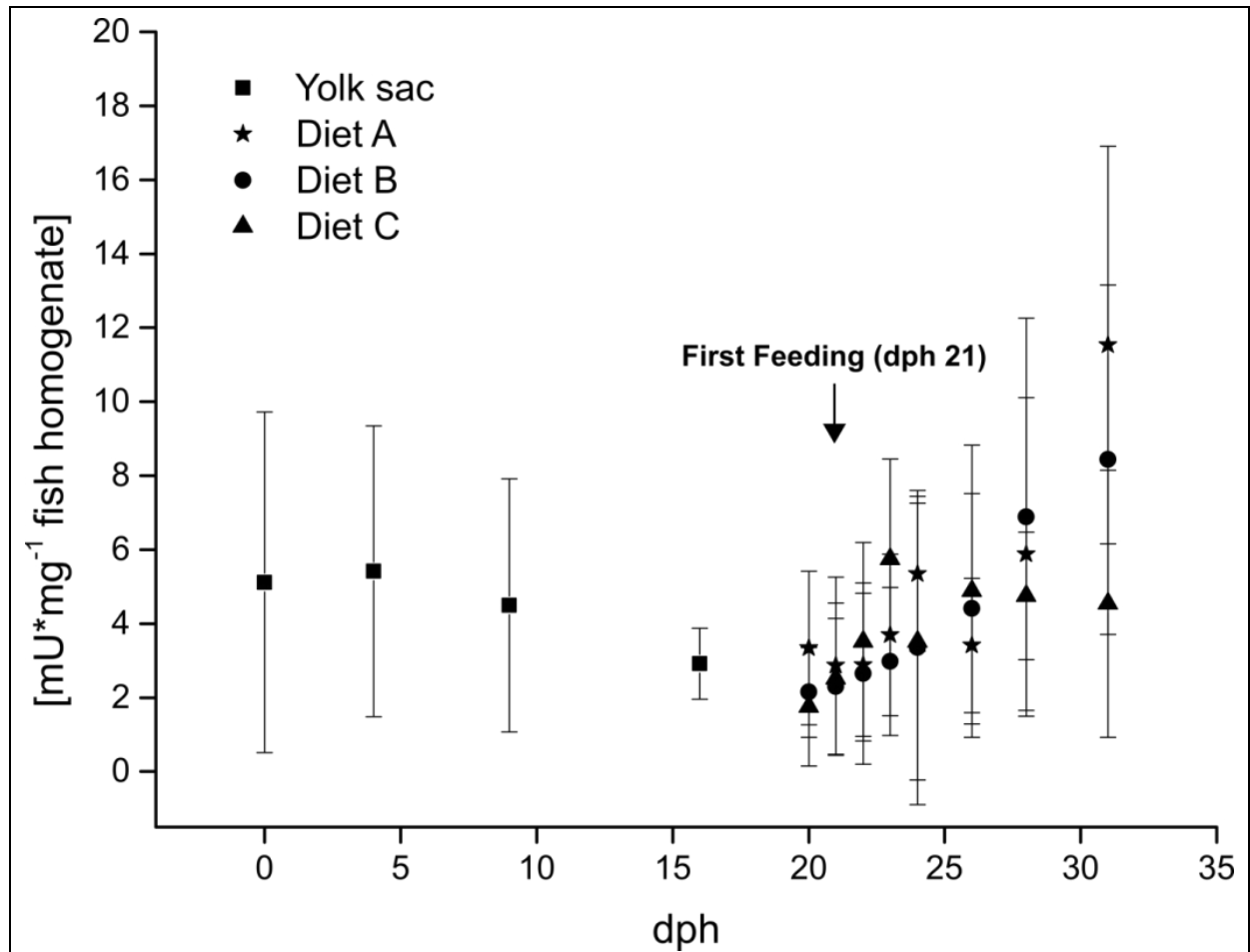
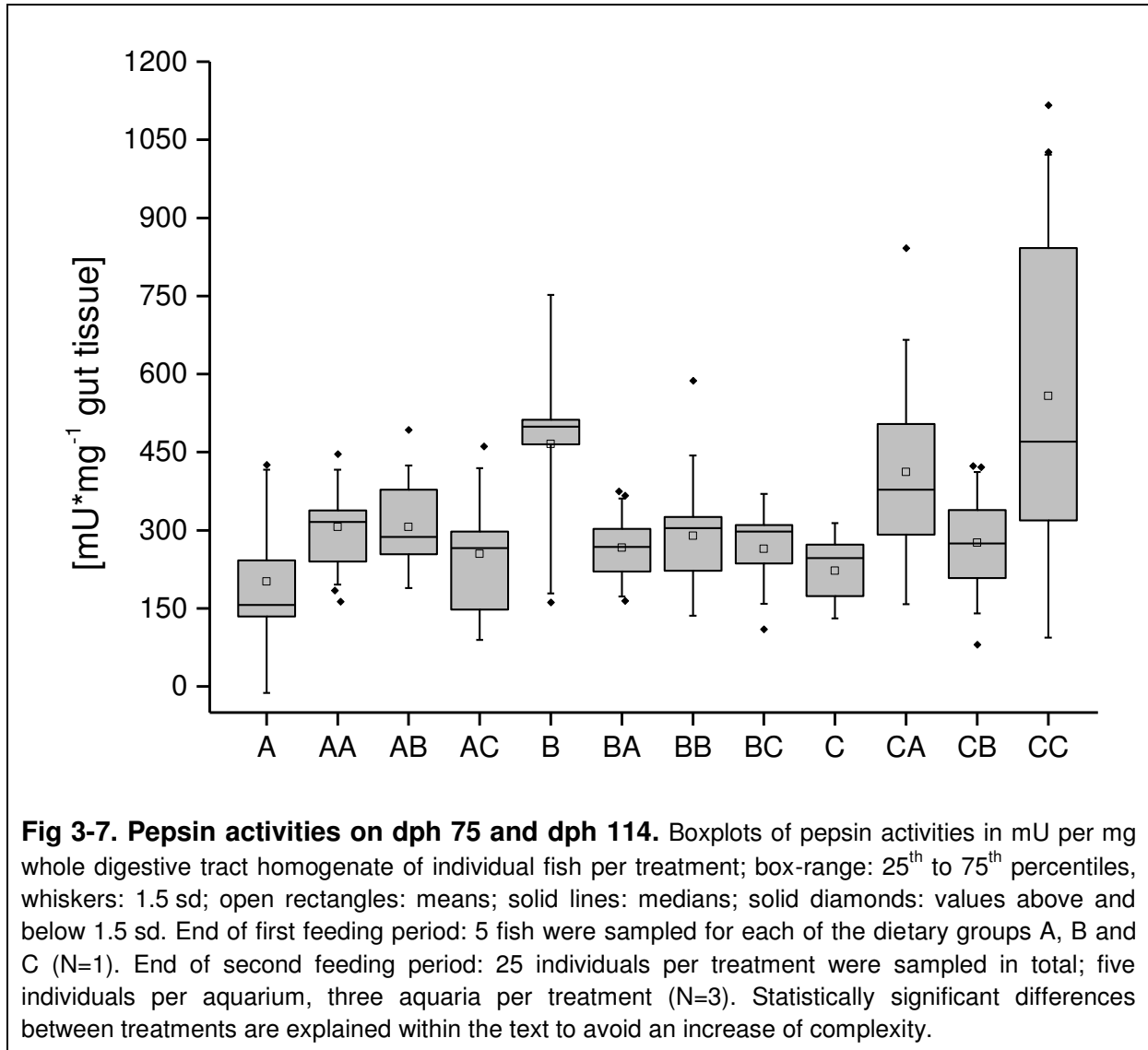


Fig 3-6. Pepsin activities of fish during the first feeding period. Presented are means \pm SD of pepsin activities of five fish per treatment and per sampling day (one tank per treatment; N=1). Pepsin activities are presented as mU per mg whole fish homogenate. Yolk sac fry are indicated by solid rectangles; Diets A, B and C are indicated by stars, solid circles and solid triangles, respectively.



4 Discussion

4.1 Growth of fish in relation to plant-based dietary proteins

Fish initially fed a diet of 97% plant-derived protein sources negatively affects growth during the fry stage. This finding aligns well with those observed in previous trials with rainbow trout fed either a fishmeal- and fish oil-based diet or a totally plant-based diet from first feeding on, exhibiting significantly reduced growth after feeding the plant-based diet [11, 27]. It has, however, also been demonstrated that growth and utilisation of plant-based diets significantly depends on family background [28]. In larger rainbow trout, it has been shown as well that a total substitution of fishmeal with plant proteins results in suppressed growth compared to a fishmeal-based diet [29]. In contrast, the feeding of 50% plant-derived protein sources enhanced growth of fish in the present study throughout the entire experiment, as evident from the wet body weights and specific growth rates.

It has been shown that specific growth rates and final body weights of young rainbow trout were not statistically different between 0% and 66% dietary plant-derived protein, but were significantly reduced with 97% dietary plant-derived protein [30]. Further studies tested various protein sources with different inclusion levels as substitution for fishmeal in juvenile rainbow trout and showed equal growth performances of fish fed the substituted diets in comparison to animals fed the fishmeal-based diets [31–33]. The analysis of growth curves during the first feeding period of the current study reveals that the three dietary treatment groups start to drift apart at ~60 days post hatch and it is known that approximately two months after first feeding the development of the digestive system, which can be continuously influenced by ingested feed, has been completed and digestive capabilities established [34]. The results of the first feeding period continued in the second feeding period with significantly improved growth performance of fish fed the 50% plant-based diet.

Interestingly, this result is independent of the first feeding diet. Nutritional programming usually operates during early development, but its effects can be manifest later in life. Thus, possible physiological alterations induced by the early feeding of a certain diet (with a direct link to growth performance) would still be obvious after a diet-change. If nutritional programming had been effective, the first and the second diet would have been directly linked in a uniform manner, which can be determined in a statistically significant interaction affecting growth. If the early feeding of a certain diet had a permanent and positive effect, the continuous feeding of plant-based diets would enhance the growth of fish that experienced plant-derived proteins during early development and would reduce growth of fish that have not. Alternatively, if the early feeding of plant-based diets had a negative effect on growth performance, we would expect this effect intensified with the continuous feeding of plant-derived proteins. In contrast, we would expect improved growth with fishmeal diets, conditional on the premise that fishmeal is the source of best growth for rainbow trout. However, none of the results accorded with these expectations. One exception might be the feeding regime CC, but the strong reduction in growth performance can more likely be attributed to a poor nutritional value of diet C.

Moreover, during the second feeding period no differences in growth performance were detected between fish fed the fishmeal diet and those fed the 97% plant-based diet. A significant reduction of growth, following the feeding of plant-derived protein sources, is commonly explained by anti-nutritive factors – ubiquitous in almost all plant-based feed ingredients [1, 4, 35], and especially in less processed ones. This explanation might be reasonable for fish initially fed diet C. For example, although total dietary phosphorous in diet C was above the requirement for small rainbow trout [18], it could be possible that some

percentage of phosphorous has been bound in phytic acid and was unavailable for fish, resulting in growth deficiencies and reduced protein digestibility [36]. Another explanation for reduced growth in relation to plant-based diets is feed intake, which could not be determined in the current experiment due to the small size of first feeding trout, feed and faeces. It has been observed in larger rainbow trout that the total replacement of fishmeal by soy protein lead to a significant reduction of voluntary feed intake [37]. However, the feeding regimes AA and AC mediated the same final body weights, as well as did the two feeding regimes BA and BC, with diets A and C being entirely contrary in their ingredient composition. It is known that fish can be imprinted by an olfactory stimulus of the first feeding diet [12] and, thus, a reduction in feed acceptance and voluntary feed intake could have produced these results. However, the feeding regime AA included no diet change and feed refusal seems therefore unlikely. The superior growth performance of fish fed diet B can already indicate a long-term breeding effect of established rainbow trout strains, which has been observed earlier [27], suggesting another form of nutritional programming: via broodstock diets as has been demonstrated in gilthead sea bream [9].

4.2 Digestive enzyme activity levels

The fluorescence based assays used to measure amylase activity were not successful for many samples when diet B or C was fed, although the success rate was higher for fish fed diet C. The general problem was the strong quenching effect of the assay substrate, a special starch molecule (DQ™ starch) with an attached fluorescent dye (BODIPY®FL). The excitation maximum of this dye is at 502 nm and the emission maximum at 512 nm. In the present study the best fluorescence readouts were obtained with Ex/Em values of 485/520 nm. A quenching effect would have been observed if molecules present in the sample absorbed at the same wavelength as the emission wavelength of the fluorescent dye, meaning that the originally emitted light of the dye would not have been detected. There are several biological compounds that are either present in plants or in the digestive tract that would have an excitation spectrum around the emission spectrum of the dye, however, this remains purely speculative. For example, yellow carotenoids that were extracted from green plants absorb in a range of 400 to 500 nm [38]. However, no quenching effects were detected in the assay with feed extracts only. Since whole digestive tracts were excised about one hour after feeding, it could also be possible that bile salts were influencing the assay. Bilirubin, the main pigment in bile and resulting from the breakdown of haem, has absorption maxima around 420 to 450 nm [39], but also other pigments present in bile could have exerted an inhibitory effect. Nevertheless, all fish had been fed before sampling and no issues were detected with tissue samples of fish fed diet A and only with some samples of fish fed diet C. Another possibility could be the bacterial gut community. It has been

demonstrated, that the dietary treatment significantly separates the intestinal microbiome of individual fish and it is well known that several bacteria contain colour pigments, such as carotenoids with absorption maxima around 500 nm [40]. However, it can be speculated whether the amount of pigments found in intestinal bacteria is high enough to disturb the spectrophotometric assay. Probably the most obvious reason for the unsuccessful assay is the likely presence of free amino acids from the extracted digestive tract. It has been demonstrated that the fluorescence of BODIPY[®]FL is quenched by tryptophan, tyrosine and phenylalanine [41]; thus an increased release of these amino acids during homogenisation could result in unsuccessful activity assays.

Nonetheless, before hatching and during the yolk sac phase, amylase activity levels were rather stable, indicating that amylase is a basic component of the digestive enzymes present in carnivorous fish already in early developmental stages [42]. The same is true for pepsin, which is already present before first feeding, displaying the early functional stomach of salmonids [43]. With the onset of first feeding, inter-individual variation of amylase and pepsin activities increases without significant influence of the experimental diets. Feeding of diet A slightly enhanced amylase activities of juvenile trout after first feeding. The dietary starch content of diet A was about 6.4%, twice as high as in diets B and C, and it is known that increasing dietary carbohydrates can result in increasing amylase activities [5], although it has also been observed in rainbow trout that dietary starch can inhibit amylase molecules [44]. Pepsin activities increased with the onset of first feeding in all dietary treatment groups about 20 days post hatching, which aligns well with previous studies about pepsin activities in Caspian brown trout (*Salmo caspius*) and rainbow trout [45, 46]. At the end of the first feeding period, amylase activities of fish fed diet A were significantly higher when compared to fish fed diets B and C, and treatment B slightly enhanced amylase activity levels in comparison to treatment C, which reflects the composition of experimental diets: amylase activities are increasing with increasing dietary starch contents and with decreasing plant protein inclusion levels. Pepsin activities in contrast were not significantly different between dietary treatments, however, inter-individual variabilities, especially for fish fed diet B, were high.

At the end of the second feeding period, highest amylase activities could be observed in fish from the feeding regime AA. A comparison of the feeding regimes AA, BA and CA showed that, although the three feeding regimes received the same second feeding diet A, amylase activity levels were dependent on the first feeding diet-type and were significantly higher if dietary starch levels had been high as well. Similar effects have been demonstrated in rainbow trout: feeding a hyperglucidic diet as an early nutritional stimulus to first feeding fry,

resulted in significantly increased amylase expression when the same diet was fed again later in life [47]. A comparison of the feeding regimes AC, BC and CC, in contrast, demonstrated no significant differences of amylase activities between treatments, which could be a sign of additional inhibitory effects [48]. The comparisons of feeding regimes separate for the specific first feeding diets reveal very similar results. Amylase activity levels significantly decreased from feeding regime AA to AB and to AC. The same results can be found for the first feeding diet B: Amylase activity levels decreased from feeding regime BA to BB and to BC. Only the comparison of CA to CC and of CA to CB showed no significant differences, due to the high standard deviations. All these results indicate that the dietary starch level has potentially a higher influence on intestinal amylase activities of trout than the dietary inclusion levels of plant-derived proteins. In contrast to amylase, pepsin activities showed no significant differences between dietary treatments on dph 114, except for the strong differences when compared to the feeding regime CC.

5 Conclusion

The aim of the present study was to evaluate the potential of nutritional programming through plant-based feedstuff in first-feeding rainbow trout. Although earlier studies have reported positive effects from similar experiments, it was not possible to induce a permanent and general improvement in the utilisation of plant-based diets in the current experimental setup. The analysis of digestive enzyme activities indicated that amylase activities can be permanently influenced by the starch content of the first feeding diet. Nevertheless, the results suggest that rainbow trout at the same time exhibit a high potential of nutritional programming for dietary starch levels. In contrast, none of these effects could be detected in growth data. The non-, but almost, significant statistical interaction of the first and the second feeding diet is only based on the significantly reduced growth of fish from all feeding regimes with the first feeding diet C. Moreover, the feeding of 50% plant-derived protein sources as second feeding diet resulted in significantly improved growth performance, indicating that the inclusion of plant-based feedstuff is generally beneficial for the growth of juvenile rainbow trout, which could be an effect of already well-established breeding strains.

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Chapter 4

The malleable gut microbiome of juvenile rainbow trout (*Oncorhynchus mykiss*): diet-dependent shifts of bacterial community structures

Stéphanie Céline Michl^{a,b,c}, Jenni-Marie Ratten^d, Matt Beyer^d, Mario Hasler^e, Julie LaRoche^d, Carsten Schulz^{a,b}

^aGesellschaft für Marine Aquakultur mbH (GMA) Büsum, Hafentörn 3, 25761 Büsum, Germany

^bInstitute of Animal Breeding and Husbandry, Department for Marine Aquaculture, Christian-Albrechts-Universität zu Kiel, Hermann-Rodewald-Str. 6, 24118 Kiel, Germany

^cGEOMAR Helmholtz Centre for Ocean Research, Wischhofstr. 1-3, 24149 Kiel, Germany

^dDepartment of Biology, Dalhousie University, 1355 Oxford Street, Halifax, Nova Scotia B3H 4R2, Canada

^eLehrfach Variationsstatistik, Christian-Albrechts-Universität zu Kiel, Hermann-Rodewald-Str. 9, 24118 Kiel, Germany

Abstract

Plant-derived protein sources are the most relevant substitutes for fishmeal in aquafeeds. Nevertheless, the effects of plant based diets on the intestinal microbiome especially of juvenile rainbow trout are yet to be fully investigated, although it is well known that health and digestive capacity of trout can be strongly connected to the intestinal microbiome. This study demonstrates, based on 16S rDNA bacterial community profiling, that the intestinal microbiome of juvenile rainbow trout is strongly related to dietary plant protein inclusion levels. After the first feeding of juveniles on diets in which 0%, 50% and 97% of total dietary protein were derived from plants, statistically significant differences of the bacterial gut community for the three diet-types were detected both at the phylum and order level. The microbiome of juvenile fish consisted mainly of the phyla Proteobacteria, Firmicutes, Bacteroidetes, Fusobacteria and Actinobacteria. Dietary plant proteins significantly enhanced the relative abundance of the orders Lactobacillales, Bacillales and Pseudomonadales. Animal proteins in contrast significantly promoted Bacteroidales, Clostridiales, Vibrionales, Fusobacteriales and Alteromonadales. The overall alpha diversity significantly decreased with increasing plant protein inclusion levels and with age of experimental animals. In order to investigate nutritional programming effects of the first feeding diet type on the development of the microbiome, a diet change was included in the study after 54 days. No such programming effect could be detected. Instead, the microbiome of juvenile trout fry was highly dependent on the actual diet fed at the time of sampling.

Key words: First feeding; Gut microbiome; Nutritional programming; Plant proteins; Rainbow trout

1 Introduction

It is generally accepted that the gut microbiome of fish is involved in a number of important physiological processes, for example immune response, pathogenic defense or nutrient digestion. Some microorganisms are able to produce inhibitory compounds that can control the colonization of pathogens in the gastro-intestinal (GI) tract [1]. Lactic acid bacteria in Atlantic salmon for example, were shown to inhibit the intestinal colonization of the fish pathogen *Aeromonas salmonicida* [2] and plant-based diets seem to promote a protective effect against *Yersinia ruckeri* in juvenile rainbow trout [3], possibly due to increased numbers of *Lactobacillaceae*. In recent years the development of rapid and precise culture-independent techniques like next-generation sequencing (NGS) provided better insight into the diversity of intestinal bacterial communities and generated further research interest in the microbiome of aquaculture species [4]. The NGS approach has led to the discovery that enzyme producing bacteria within the fish intestinal microbiome can positively influence nutrient digestibility in several fish species by excreting digestive enzymes like amylase, cellulase, lipase or even phytase [5]. For example, Fusobacteria and Bacteroidetes played an important role in fermentation of plant material in Common carp [6] and Firmicutes found in the GI tract of Grass carp could utilize various polysaccharides, such as cellulose, xylan and hemicelluloses [7].

Plant material as an alternative protein source is a crucial issue in modern aquaculture feed production. With significantly decreasing marine fish stocks and an increasing demand for fish as a protein source for human consumption, plant-derived proteins as an alternative feed source for fishmeal have been of high commercial and scientific interest during the past decades [8]. The central problem – to successfully feed carnivores with plant meals – has been overcome for many species and feeds for growing and adult salmonids currently contain less than 10% fishmeal. However, although several studies demonstrated that juvenile and adult rainbow trout can be successfully reared with plant material [9-13], the amount of fishmeal as protein source in salmonid fry feed is still greater than 50% [14]. This is probably due to the fact that very high inclusion levels of plant proteins in first feeding diets led to reduced growth accompanied by inflammation of the gastro-intestinal tract in most studies.

Even though the importance of dietary effects on the gastro-intestinal microbiome of fish has been demonstrated [15], little is known on how early feeding of plant proteins is affecting the developing intestinal microbiome of juvenile carnivores. A recent study [16] revealed that first feeding initializes the colonization of the gut in rainbow trout fry and the subsequent development of the bacterial community structure is influenced by the diet itself: plant-based

diets significantly separated the microbiome from fishmeal diets, with a prevalence of the phylum Firmicutes, which has been observed for adult trout as well [17]. Thus, the microbiomes of both trout fry and adults can be manipulated by diet-type. Nevertheless, the magnitude of this manipulation and its relation to the initial juvenile microbiome remain uncharacterized.

During the early development of vertebrates, nutritional programming “as a result of early life experience” plays an important role for long-term effects on several subsequent physiological processes [18]. The question arises whether the intestinal microbiome can be imprinted as well. In zebrafish, intrinsic factors play a role in the initial development of the microbial community structure [19], but whether these can outplay dietary modulations is unknown. The purpose of the current study was therefore to evaluate effects of first feeding diets with various levels of plant-derived proteins on the initial gut microbiome development of rainbow trout (*Oncorhynchus mykiss*) fry and to examine whether or not a subsequent diet change during the juvenile stage would influence the diversity of the microbial community structure. It was hypothesized that due to the concept of nutritional programming the first feeding diet would promote an initial microbial community that is subsequently influencing diet-dependent alterations of the intestinal microbiome at later stages in life.

2 Material & Methods

2.1 Experimental Animals

The experiment was conducted at the “Gesellschaft für Marine Aquakultur mbH” (Büsum, Germany). Eyed rainbow trout eggs (*Oncorhynchus mykiss*, Kamloops strain) were purchased from the trout farm “Forellenzucht Troststadt GbR” (Reurieth-Troststadt, Germany), originating from “Troutlodge Inc.” (Sumner, Washington USA). All animal handling procedures were approved by the animal welfare officer of the “Gesellschaft für Marine Aquakultur mbH” and the local authority of Schleswig-Holstein according to the German animal welfare law (TierSchG).

2.2 Experimental Diets

Three isonitrogenous and isoenergetic (on digestible matter; Table 4-1) experimental diets (diet A, diet B and diet C) were pelletized in different particle sizes according to the needs of first feeding and growing fry. Diet A consisted exclusively of animal derived protein sources, contrasting diet C, which contained 97% of plant derived protein sources. Three percent of gelatin was needed for hardening during the manufacturing process of the first feeding diets.

Diet B was intermediate between diet A and diet C with 50% plant and 50% animal protein sources. Amino acid composition of each diet was formulated according to the NRC [20] amino acid requirements for small rainbow trout (0.2 - 20.0 g); likewise was the composition of the vitamin and mineral premixtures in accordance with NRC guidelines.

Table 4-1. Composition of experimental diets.

Ingredients (in % of dry matter)	Diet A	Diet B	Diet C
Fishmeal ¹	64.65	28.74	
Mussel meal ²	2.13	2.00	
Blood meal ³	6.14	0.96	
Shrimp meal ¹	8.65	6.00	
Corn gluten ⁴		1.00	2.00
Soy protein concentrate ⁵		5.00	5.00
Pea protein ⁶		19.86	48.19
Rapeseed concentrate ⁷		4.84	15.96
Wheat gluten ⁸		10.00	2.79
Wheat starch ⁸	6.43	3.38	2.00
Vitamin & Mineral Premixtures ⁹	4.00	4.00	4.00
Linseed oil ¹⁰	2.00	3.06	2.00
Fish oil ¹	3.00	4.78	9.07
Gelatine ¹¹	3.00	3.00	3.00
Bentonite ¹²		3.38	5.97
Tryptophan ¹³			0.03
Proximate composition (in % of dry matter)	Diet A	Diet B	Diet C
Dry matter (in % of diet)	88.78	92.25	91.16
Crude protein	62.72	62.81	62.84
Crude fat	12.72	13.12	17.48
Crude ash	15.83	13.00	10.36
Gross energy (MJ kg ⁻¹)	21.37	22.15	23.42
Digestible energy (MJ kg ⁻¹) ¹⁴	19.40	19.30	18.90

¹ Vereinigte Fischmehlwerke Cuxhaven GmbH & Co. KG, Cuxhaven, Germany

² CRM - Coastal Research & Management, Kiel, Germany

³ SARVAL Ouest, Issé, France

⁴ Cargill Deutschland GmbH, Krefeld, Germany

⁵ EURODUNA Rohstoffe GmbH, Barmstedt, Germany

⁶ Emsland-Stärke GmbH, Emlichheim, Germany

⁷ Helm AG, Hamburg, Germany

⁸ Kröner Stärke GmbH, Ibbenbüren, Germany

⁹ Aller Aqua Mix 7188 Micro & 7180 Vit. STD., Golßen, Germany

¹⁰ Makana Produktion und Vertrieb GmbH, Offenbach, Germany

¹¹ ARTI-Vital, Freyburg, Germany

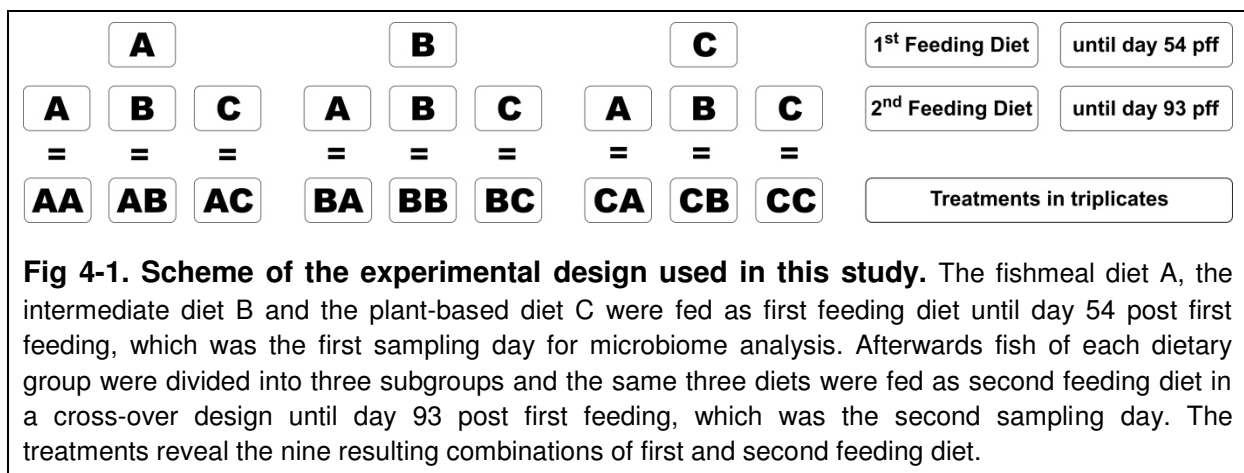
¹² DEL LAGO Bentonite, Castiglioni Pes y Cia, Buenos Aires, Argentina

¹³ Evonik Industries AG, Essen, Germany

¹⁴ Calculated – based on ADC values available from current literature

2.3 Experimental Setup

7500 eyed rainbow trout eggs were randomly distributed into three commercial hatching troughs that were integrated into a recirculating waterbody. Average water temperature was $9.0 \pm 0.2^\circ\text{C}$ until hatching day. Hatching was induced by an increase in water temperature to 11.0°C and remained at $11.6 \pm 1.1^\circ\text{C}$ until the end of the experiment. First feeding was initiated on day 21 post hatch. Each hatching trough represented one of the three experimental groups for the first feeding period. Trout fry were fed with the first feeding diets A, B or C *ad libitum* with automatic feeders. Feeding frequency was once per hour for 19 days and subsequently reduced to a frequency of four times per day until 54 days post first feeding (pff). Dimmed light was provided from 06:00 am to 09:00 pm in order to make feed particles visible to the fish. On day 54 pff 600 trout fry from each hatching trough were randomly transferred as triplicates into 50 L aquaria integrated in the established recirculating system. All diets were changed in a cross-over design (Fig 4-1) and until day 93 pff each experimental group was fed four times per day with their second feeding diet - in total 3.8% of the total biomass. On days 54 and 93 pff, samples for microbiome analysis were taken and bodymass of experimental fish were determined.



2.4 Sample preparation

In total, 150 fish were sampled for microbiome analysis. Fish were fed two hours before sample collection. Five animals were collected from each tank (one hatching trough per treatment on day 54 pff and three tanks per treatment on day 93 pff) and individual body weight was measured. Prior to sampling, experimental animals were narcotized with MS222 (Tricaine methanesulfonate, E10521, Sigma-Aldrich Co. LLC.) and immediately killed by cutting the gill vein. The whole gastrointestinal tract was dissected on ice using sterile razor blades and instantly frozen at -80°C .

2.5 DNA extraction and purification

DNA extraction was performed with the Qiagen DNeasy[®] Blood & Tissue DNA extraction kit according to the manufacturer's protocol. First, gastrointestinal tissue samples were thawed at 4°C and homogenised (KT Micra D9 homogenizer) on ice for 30 seconds in 1 ml of a 5 mg ml⁻¹ lysozyme (8259, Carl Roth) in TE-buffer solution (10 mM Tris-HCl, 1 mM EDTA). The homogenised solution was incubated for 30 min at 37°C. Next, the homogenate was gently vortexed and 80 µl were incubated for 60 min at 56°C in 200 µl of lysis buffer AL (provided in the extraction kit), 20 µl Proteinase K and 100 µl PBS (Solution without Ca-Mg, 733-2296, VWR). After incubation, 200 µl ethanol (96-100%) was added and further extraction steps were performed according to the manufacturer's protocol for purification of total DNA from animal tissue. According to recommendations by Qiagen, two extra washing steps with the provided buffers AW1 and AW2, as well as an extra centrifugation step of 1 min at maximum speed before elution were included in the procedure. DNA purification was initiated with RNase A (Qiagen) digestion (1 mg ml⁻¹ in DEPC water), followed by inactivation remaining microbial DNases at 70°C for 15 min. This working solution was added to each sample to obtain a final concentration of 100 µg ml⁻¹ RNase A and incubated for 30 min at 60°C. This was followed by a DNA clean-up step using the NucleoSpin[®] gDNA clean-up kit (Machery-Nagel) following the manufacturer's protocol including all recommended steps.

2.6 16S rDNA PCR amplification

All DNA samples were amplified by PCR targeting the 16S rRNA gene sequence (regions V6-V8). The final PCR reaction volume was 20 µl including 4 µl CG buffer, 0.6 µl DMSO, 0.4 µl dNTP (10 mM), 0.4 µl of each primer, 0.2 µl Phusion high-fidelity polymerase (Thermo Fisher Scientific Inc.), 12 µl DEPC H₂O and 2 µl of DNA template. The final primer concentration was 0.5 µM. Primers used were B969F (5'-ACG CGH NRA ACC TTA CC-3') and BA1406R (5'-ACG GGC RGT GWG TRC AA-3') from IDT (Integrated DNA Technologies, Inc.) according to [21]. Cycling protocol was as follows: 98°C for 3 min, 35 cycles of 98°C for 10 sec., 54°C for 30 sec. and a final extension at 72°C for 1 min., and finally 72°C for 10 min. Results of the PCR were verified on a 1.1% agarose gel. DNA samples were stained with SYBR safe DNA gel stain (Invitrogen[™], Thermo Fisher Scientific Inc.) and images were analysed using a gel imaging box (G:BOX, Syngene). Afterwards, samples were gel extracted using the QIAquick Gel Extraction Kit (Qiagen) following the manufacturer's protocol and DNA concentrations were determined using a NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific Inc.). Amplified products were cleaned and normalized using the SequalPrep Normalization Plate Kit (Invitrogen[™], Thermo Fisher

Scientific Inc.). Subsequently, the samples were multiplexed at equal volumes, quantified with Qubit (Invitrogen™, Thermo Fisher Scientific Inc.) and loaded into the Illumina MiSeq platform as a 20 pM final denatured library according to manufacturer's specifications.

2.7 Sequencing and bioinformatics workflow

The amplified 16S rDNA fragments were sequenced at the Integrated Microbiome Resource lab (IMR) at Dalhousie University (Halifax, Canada) using an Illumina MiSeq platform and following the procedure described in [22]. Raw sequences were analysed with QIIME (Quantitative Insight Into Microbial Ecology) for the analysis of high-throughput community sequencing data [23] using the 16S amplicon analysis procedure of IMR [24]. The following quality control methods were applied: forward and reverse reads were matched using PEAR (Paired-end rEAd merger; [25]), sequences with unidentified nucleotides, with mitochondrial and chloroplast DNA sequences [26], and chimeric DNA molecules (using UCHIME; [27]) were removed. Open-reference OTU (Operational Taxonomic Units) picking was performed using the picking methods *sortmerna* and *sumacrust* [28]. Reads were clustered against the reference database Greengene [29] and OTUs were grouped together based on 97% sequence similarity. Low confidence OTUs were subsequently removed and the collection of sequences was rarified to 1000 reads per sample as suggested for gut samples by Hamady & Knight [30].

2.8 Statistical analysis

The statistical software R [31] and QIIME were used to evaluate the data, generated on days 54 and 93 post first feeding. Important to note here is the small number of samples obtained for diet C at sampling day 54 post first feeding: from five GI-tract samples extracted, only three samples showed substantial sequencing results and could thus be integrated into the statistical analysis. Four alpha diversity measures were calculated based on the OTU table generated during the QIIME workflow. The number of observed distinct OTUs, the Chao1 richness estimator, the Simpson's evenness measure E and the Shannon diversity index H' were selected for the purpose of estimating alpha diversity of microbial species within the GI tract samples. Differences between bacterial communities in relation to the dietary treatment or sampling day were visualised by nonmetric multidimensional scaling (NMDS) based on a Bray-Curtis dissimilarity matrix of Hellinger-transformed data using the R package *vegan* [32]. A stress factor was calculated to provide a way of determining how well original data is represented in the ordination space.

Further statistical analysis of the data was performed as follows:

First, the influence of the three first feeding diets (A, B and C) on the four alpha diversity indices and on the top-five most abundant bacterial phyla at the end of the first feeding period was tested. Therefore a statistical model based on generalized least squares was established [33]. The data were assumed to be approximately normally distributed and to be heteroscedastic. These assumptions were based on a graphical residual analysis. The first feeding diet was considered as (fixed) factor. Based on this model, an analysis of variances (ANOVA) was conducted and multiple contrast tests were performed [34] using the R package SimComp [35] to compare the three different first feeding diets.

Second, the influence of the nine dietary treatments on alpha diversity indices and on the top-five most abundant phyla at the end of the second feeding period was tested. Thus, a statistical mixed model was defined [36, 37] with the first feeding diet and the second feeding diet as well as their interaction term as fixed factors. Again, the data were assumed to be approximately normally distributed and heteroscedastic. The individual aquaria were included as random factor. Based on this model, an ANOVA was conducted followed by multiple contrast tests to compare the levels of the fixed factors [38, 39] using the R package multcomp [40]. A significant interaction of the first feeding diet and the second feeding diet was considered as nutritional programming effect of the first feeding diet. In case of a non-significant interaction, data were pooled for the first feeding diet and multiple contrast tests were rerun to compare only the three second feeding diets (A, B and C).

Third, statistical differences of alpha diversity indices and bacterial phyla between the end of the first feeding period (day 54 pff) and the end of the second feeding period (day 93 pff) were evaluated for continuously fed fish (treatments A, AA, B, BB, C and CC). A statistical mixed model was established with the sampling day as fixed factor and the hatching troughs and aquaria as random factor. An ANOVA was conducted, followed by multiple contrast tests to compare the two sampling days as described before [38, 40].

Fourth, the influence of the three first feeding diets on the bacterial community structure at the end of the first feeding period was tested. Bacterial abundance data on order level were Hellinger-transformed and a Principal Component Analysis (PCA) was performed [41]. The data were evaluated on order level, because the number of observed OTUs on species and genera level was approximately three times the number of samples, which would have negatively affected the outcome of the statistical models. The Broken-Stick-Criterion [42] was used to select those principal components (PC) from the PCA with the greatest influence on data variability. The first two PC's represented 85% of the cumulative variance. Based on these two PC's, rotated data (i.e. two pseudo-variables) were calculated and integrated into a multivariate model, established simultaneously for the two pseudo-variables. An ANOVA was

performed based on this model and multiple contrast tests for multiple endpoints were conducted in order to compare the three diets simultaneously for the two pseudo-variables [35, 43].

Fifth, the influence of the nine dietary treatments on the bacterial community structure at the end of the second feeding period was tested. A PCA was performed with Hellinger-transformed abundance data on order level and the PC's with the highest influence on data variability were selected as described before. The first six PC's represented 84% of the cumulative variance. Based on these six PC's, rotated data were calculated and integrated into a multivariate mixed model, established simultaneously for the six pseudo-variables. The first feeding diet and the second feeding diet as well as their interaction were considered as fixed factors, the aquaria were considered as random factor. Based on this model, an ANOVA was conducted. Again, a significant interaction of the first feeding diet and the second feeding diet was considered as nutritional programming effect of the first feeding diet. Afterwards, multiple contrast tests for multiple endpoints were performed to compare the levels of the fixed factors simultaneously for the six pseudo-variables [35, 43].

Sixth, the first two PC's were further examined for the individual contribution of specific bacterial orders to the cumulative variance explained of each principal component. The top-ten orders with the highest loadings on each of the two PC's were selected for further analysis. In case of a non-significant interaction of the first and the second feeding diet in the previous model, data were pooled for the first feeding diet and multiple contrast tests as described before [35, 43] were performed to compare the three second feeding diets (A, B and C) simultaneously for the ten selected bacterial orders, respectively for each PC. Thus, specific bacterial orders could be identified that were significantly promoted by a certain diet-type.

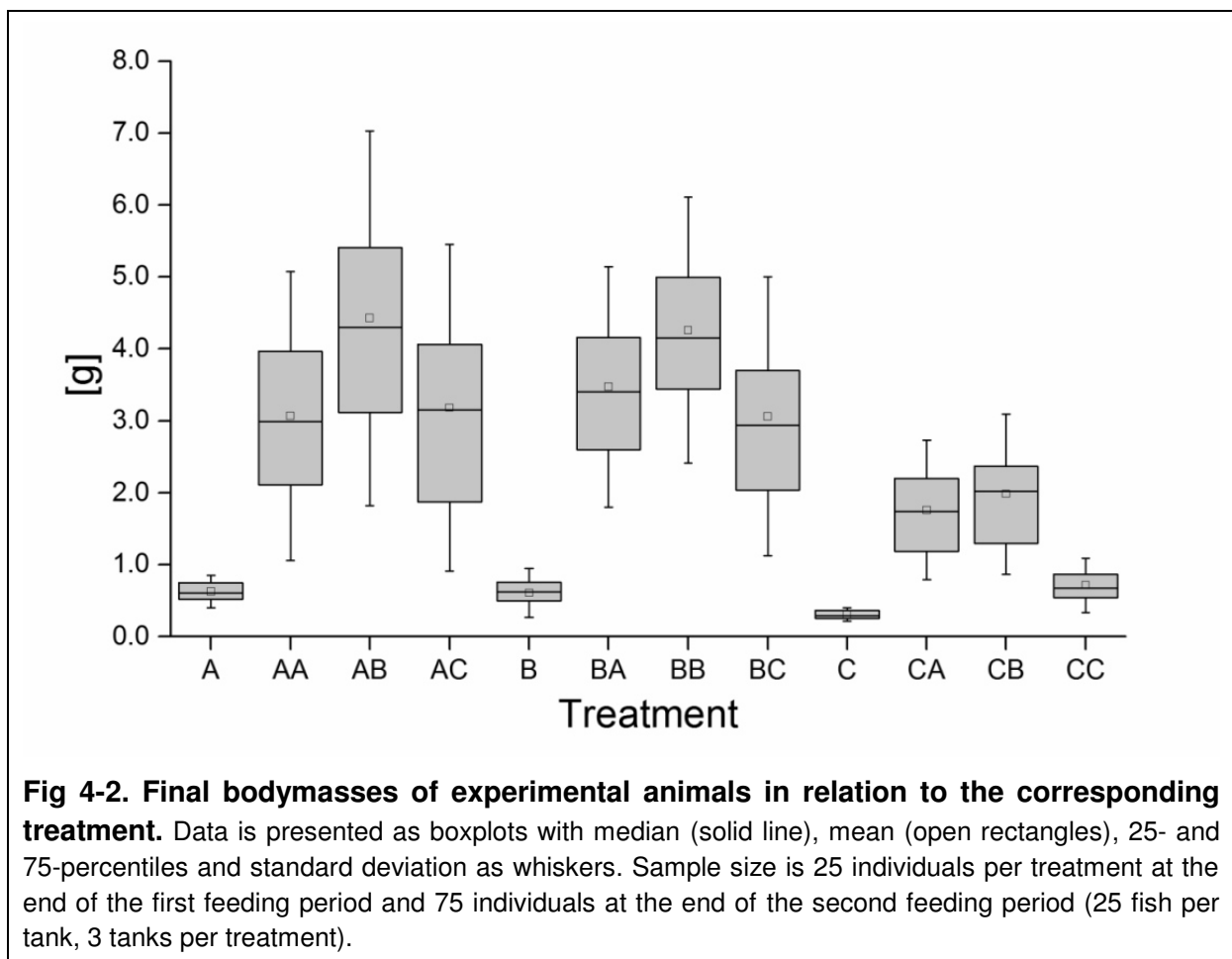
Seventh, statistical differences of the bacterial community structure between the end of the first feeding period (day 54 pff) and the end of the second feeding period (day 93 pff) were evaluated for continuously fed fish (treatments A and AA, B and BB, C and CC, respectively). A PCA was performed for each of the three Hellinger-transformed data pairs and the first three PC's were selected as described before. Resulting pseudo-variables were integrated into a multivariate mixed model established simultaneously for the three pseudo-variables. The sampling day was integrated as fixed factor and the hatching troughs and aquaria as random factor. An ANOVA was conducted, followed by multiple contrast tests for multiple endpoints to compare the two sampling days simultaneously for the three pseudo-variables as described before [35, 43].

Finally, a correlation analysis based on Spearman ranks was conducted in order to evaluate a possible relation between bodymass and one of the principal components. The correlation analysis was repeated for each of the second feeding diets. The top ten orders with the highest loadings on PC2 were used again in a Spearman ranks correlation analysis to test possible relations of a specific bacterial order to bodymass.

3 Results

3.1 Growth performance

Fig 4-2 shows the final bodymass of fish at the end of the second feeding period. The body mass of fish continuously fed with diet C is significantly reduced in comparison to the other treatments. Interestingly, the second feeding diet B also promoted better growth than the second feeding diet A.



3.2 Alpha diversity of the GI tract microbiome

Alpha diversity indices were calculated for data of both sampling days (Fig 4-3). At the end of the first feeding period, only the Shannon diversity index differed significantly between the three diets. The index was significantly higher ($p < 0.05$) for fish fed with diet C than for fish fed with diet A or diet B. All calculated indices increased significantly from day 54 to day 93 pff for individuals of the treatments AA and BB. In contrast, diversity indices remained constant between the two sampling points for fish of treatment CC. The statistical model neither revealed a significant effect of the first feeding diet on any of the calculated indices, nor a significant interaction between the first and the second feeding diet; however, the second feeding diet was significantly influencing all alpha diversity indices. Multiple contrast tests revealed a significant decrease of all indices in fish of the second feeding diet C compared to the second feeding diets A and B.

3.3 Relative Abundance of phyla

Fig 4-4 presents the relative mean abundance of all phyla accounting for at least 1% of all observed OTUs and present in at least 10% of all samples. The top five most abundant phyla were Proteobacteria, Firmicutes, Bacteroidetes, Fusobacteria and Actinobacteria (Table 4-2). On average they represented 98% of all sequences obtained. At the end of the first feeding period the abundance of individual phyla differed. Diet C promoted significantly higher abundances of Proteobacteria ($p < 0.05$) in comparison to diet B and diet A, in which Firmicutes dominated instead ($p < 0.05$). Abundances of Actinobacteria were significantly increased ($p < 0.05$) in fish of diet group A compared to fish of the diet groups B and C. The relative abundances of the phyla Bacteroidetes and Fusobacteria however did not differ between diets at the end of the first feeding period.

After the diet change, no significant interaction of the top five phyla between the first feeding diet and the second feeding diet was detected. Instead, the mean abundance of several phyla was again significantly influenced by the second feeding diet. In individuals of the treatments AA and BB, Proteobacteria, Bacteroidetes and Fusobacteria significantly ($p < 0.001$) increased in relative abundance from day 54 pff to day 93 pff. Firmicutes in contrast significantly decreased in both treatments, but Actinobacteria decreased only for fish of treatment AA. No differences were found between sampling days for fish of treatment CC. The relative abundance of Proteobacteria did not differ significantly between most of the second feeding diets, even though Proteobacteria were generally less abundant in fish fed with diet A as second feeding diet. Similar effects could be observed for the phylum Firmicutes: the relative abundance significantly increased ($p < 0.05$) when diet C was used as

second feeding diet in contrast to diet A and diet B. Bacteroidetes in contrast were significantly more abundant ($p < 0.05$) when fish were fed with diet A as second feeding diet. Alike Bacteroidetes, abundances of Fusobacteria were significantly higher ($p < 0.01$) when diet B or diet A were fed as second feeding diet. Abundances of Actinobacteria however were low and not significantly different for any of the treatments.

3.4 The influence of diets and bodymass

Four NMDS-plots are presented in Fig 4-5. Fig 4-5a shows that the ordination distances between objects of the same experimental group result in distinct grouping. Additionally, the gut microbiome of fish fed with diet A is very similar to the microbiome of fish fed with diet B. In contrast, the data points of fish fed with diet C are significantly offset, indicating a microbiome composition that is different from diet A and B as confirmed by a statistical model ($p < 0.01$).

Figs 4-5a-d illustrate the microbial communities at the end of the first feeding period in relation to their corresponding communities at the end of the second feeding period. The stress level is less than 0.2 for all three plots and microbiomes from the same treatment clearly cluster together. The proximity between objects of the second feeding diets A and B is smaller than to those of the second feeding diet C – independent of the first feeding diet. Furthermore, fish of the treatments AA and BB are clearly separated by the two sampling days which was further verified by the statistical model. Interestingly, fish of treatment CC had a similar gut microbiome on both sampling days.

The Principal Component Analysis revealed that PC1 already explained 41% and PC2 22% of the total variance observed in the dataset. The first six principal components explained together 84% of the variability. From the statistical model, no interaction between the first and the second feeding diet could be observed and only the second feeding diet had significantly influenced ($p < 0.001$) the microbial community composition. Multiple contrast tests revealed significant ($p < 0.001$) differences of the gut microbiome for the three second feeding diets (Fig 4-6). Furthermore, the following orders were identified to be strongly influenced ($p < 0.001$) by the second feeding diets A and B: Bacteroidales, Clostridiales, Lactobacillales, Bacillales and Pseudomonadales. Three additional orders were significantly different between diet A and diet C: Vibrionales, Fusobacteriales and Alteromonadales, and except for Lactobacillales, the same orders were found significantly altered in their abundance between diet B and diet C.

The PCA plot additionally indicates that the final bodymass plays a role for the data separation along the axis of PC2. The correlation analysis revealed a strong correlation ($\rho = -0.476$; $p = 1.4e^{-8}$) between PC2 and the bodymass of individual fish. This correlation was persistent when dietary subgroups were tested. For all three second feeding diets PC2 remained significantly correlated to bodymass (diet A and B: $p < 0.001$, diet C: $p < 0.05$). From the ten orders with the highest loadings on PC2, especially Lactobacillales was highly correlated ($\rho = 0.400$; $p = 2.9e^{-6}$) to bodymass.

Table 4-2. Mean relative abundances in [%] of the top five phyla found in GI tract samples of fish for each treatment at two different sampling days.

Diet	dpff	Proteobacteria	Firmicutes	Bacteroidetes	Fusobacteria	Actinobacteria
A	54	4.3 ± 5.6 ^{a,*}	84.9 ± 4.2 ^{a,*}	0.6 ± 0.4 [*]	0.2 ± 0.3 [*]	9.6 ± 4.3 ^{a,*}
AA	93	43.6 ± 8.8 [*]	11.9 ± 8.1 ^{*,A}	27.1 ± 11.0 ^{*,A}	12.7 ± 7.9 ^{*,A}	0.8 ± 1.3 [*]
AB	93	52.5 ± 9.6	17.1 ± 6.6 ^A	19.2 ± 10.6 ^{A,B}	8.0 ± 4.3 ^A	0.9 ± 2.0
AC	93	52.6 ± 19.2	38.7 ± 23.4 ^B	7.0 ± 12.7 ^B	0.0 ^B	1.0 ± 2.5
B	54	6.4 ± 9.1 ^{a,*}	91.5 ± 11.9 ^{a,*}	0.6 ± 0.8 [*]	0.0 [*]	0.6 ± 0.6 ^b
BA	93	43.9 ± 13.9	10.9 ± 6.2 ^A	30.9 ± 12.5 ^A	12.0 ± 6.7 ^A	0.4 ± 0.3
BB	93	55.1 ± 16.8 [*]	22.4 ± 22.6 ^{*,A,B}	12.6 ± 4.6 ^{*,B}	8.1 ± 4.8 ^{*,A}	0.8 ± 0.9
BC	93	56.8 ± 12.1	31.7 ± 14.0 ^B	9.0 ± 11.5 ^B	0.0 ^B	0.5 ± 0.9
C	54	57.9 ± 11.9 ^b	39.5 ± 12.4 ^b	1.9 ± 1.2	0.0	0.4 ± 0.1 ^b
CA	93	46.9 ± 11.3	10.4 ± 5.2	27.2 ± 9.0 ^A	12.5 ± 5.9 ^A	1.4 ± 3.4
CB	93	55.2 ± 11.7	18.8 ± 16.2	16.2 ± 13.5 ^{A,B}	7.7 ± 5.2 ^A	0.5 ± 0.4
CC	93	68.0 ± 20.2	24.1 ± 20.1	5.4 ± 8.0 ^B	0.0 ^B	1.3 ± 2.2

5 intestinal samples per treatment were analysed at 54 dpff (only 3 samples could be analysed for diet C) and 15 intestinal samples (3 tanks per treatment, 5 fish per tank) per treatment at 93 dpff (only 14, 11 and 13 samples could be analysed for treatment BB, CB and CC, respectively). Statistically significant differences between the three first feeding diets A, B and C are indicated with different lower case letters. Statistically significant differences between treatments after the second feeding period are indicated with different upper case letters, separate for the respective first feeding diet. Statistically significant differences between sampling days for continuously fed diets are indicated with asterisks.

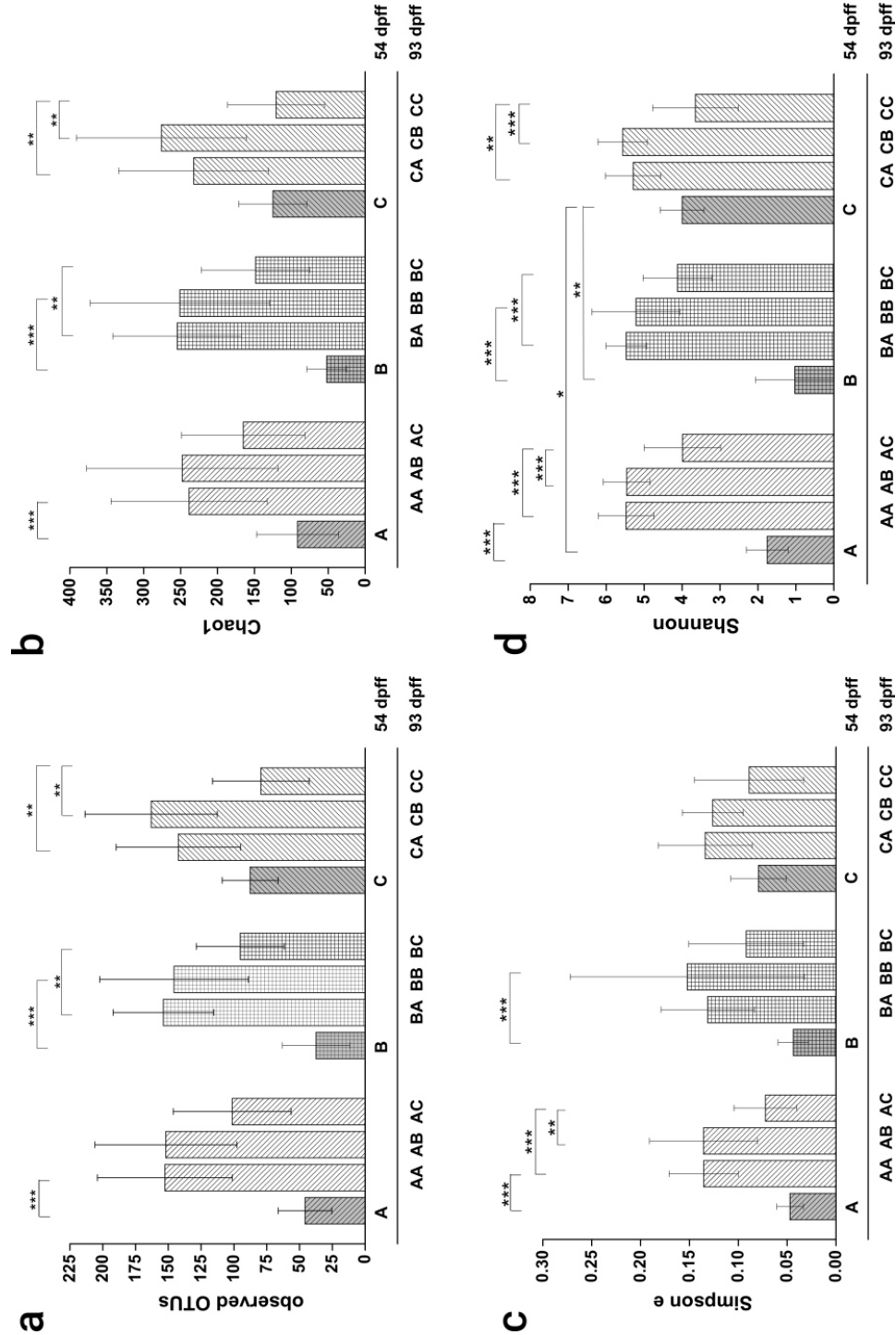


Fig 4-3. Alpha diversity indices in relation to the dietary treatment and sampling day. The number of observed OTUs (a), Chao1 richness estimator (b), Simpson's evenness measure (c) and Shannon diversity index (d) are presented. Indices were calculated for 5 intestinal samples per treatment at day 54 pff (only 3 samples could be analysed for diet C) and for 15 intestinal samples (3 tanks per treatment, 5 fish per tank) per treatment at day 93 pff (only 14, 11 and 13 samples could be analysed for treatments BB, CB and CC, respectively). Statistically significant differences between treatments or between sampling days for continuously fed diets are indicated with asterisks: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

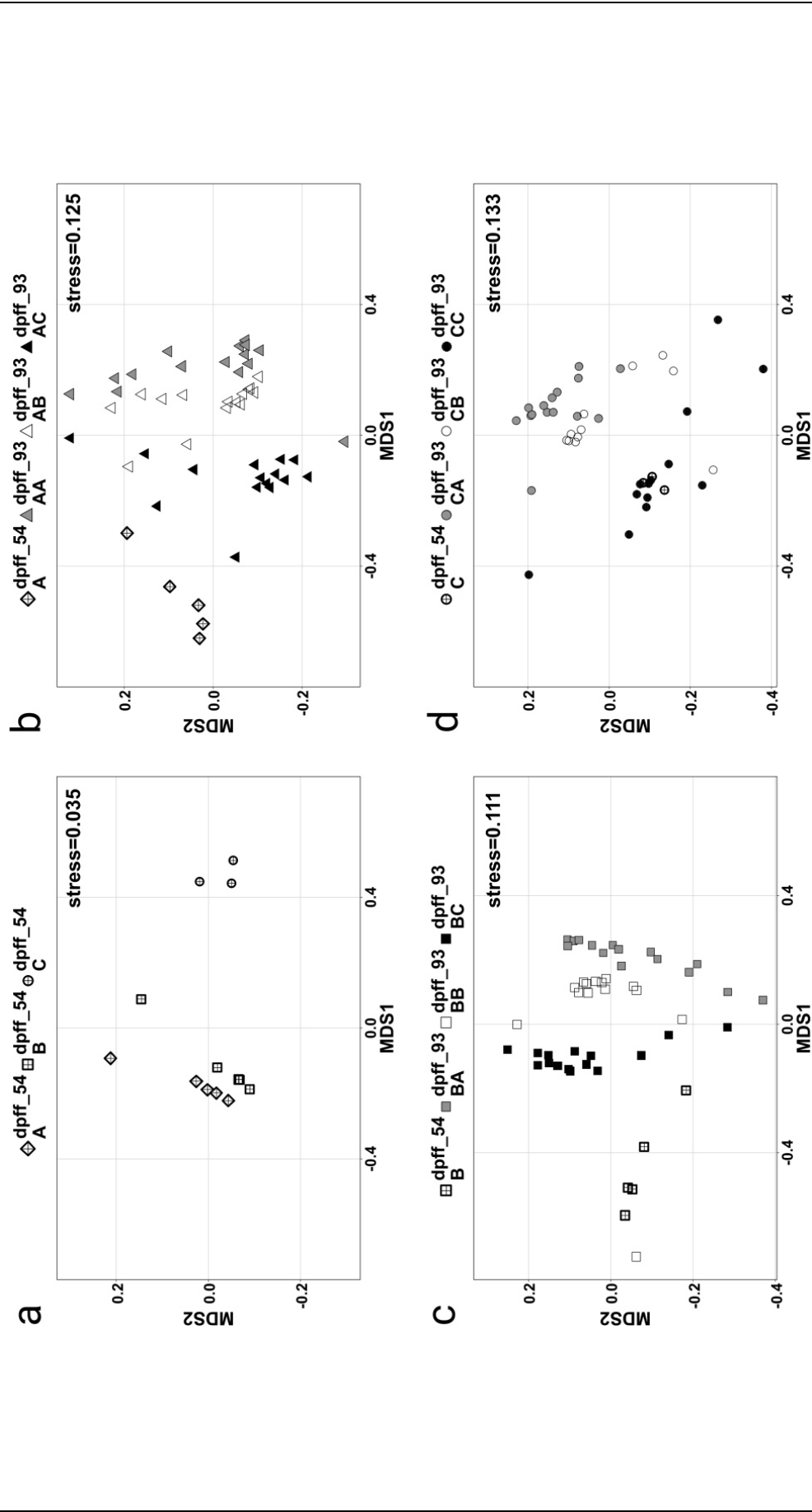
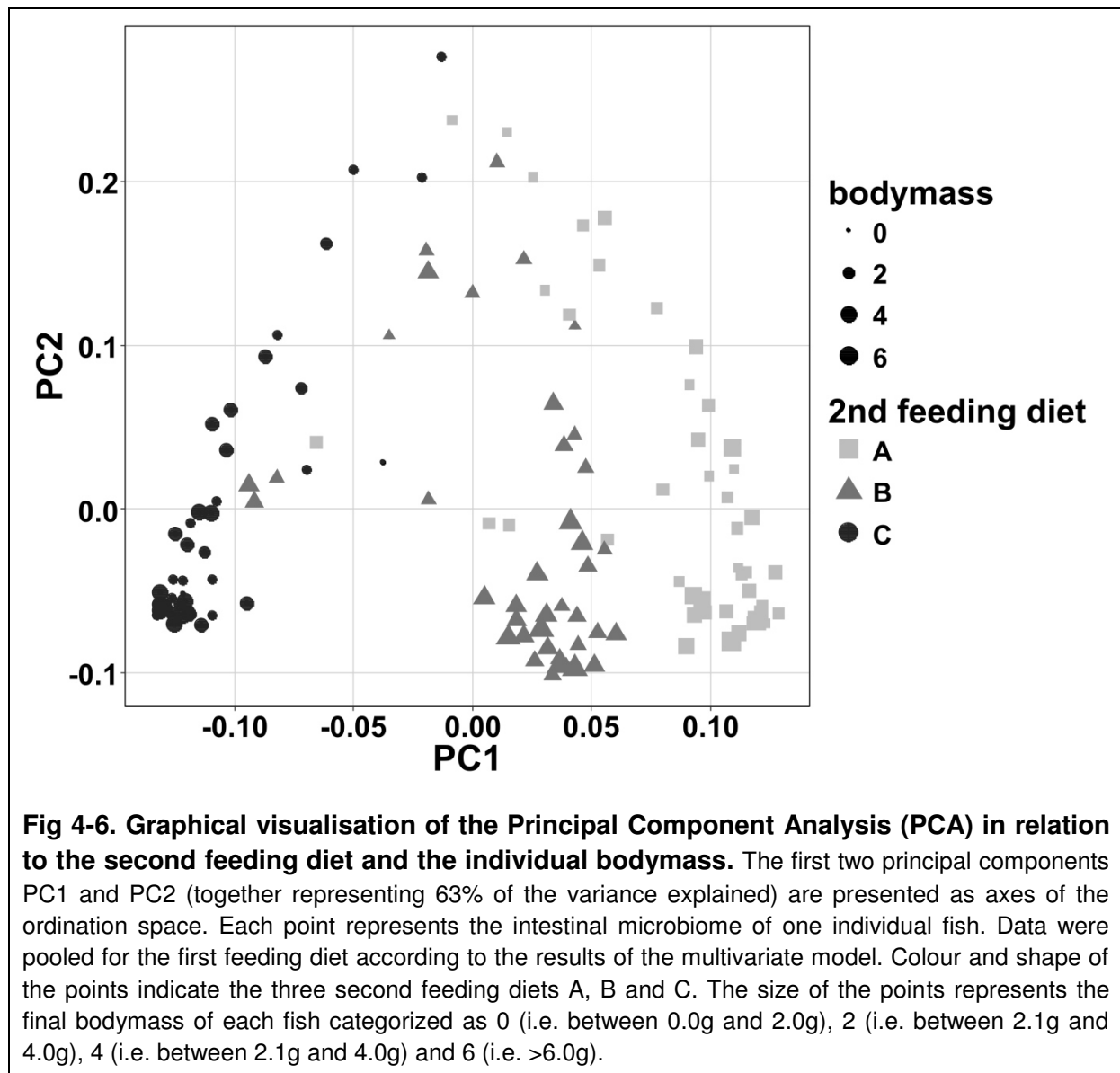


Fig 4-5. Nonmetric Multidimensional Scaling (NMDS) plots of the microbiomes of individual intestinal samples in relation to treatment and sampling day. MDS1 and MDS2 represent the two axes of the two-dimensional ordination space. Each point represents the microbiome of one individual fish. The plots result from a Bray-Curtis dissimilarity matrix based on Hellinger-transformed bacterial abundance data on order level. The stress-level shown in each plot indicates how well the individual distances between objects are represented (between 0 and 1; the closer to 0, the better). Plot (a) shows a comparison of samples by first feeding diet on day 54 pff. Plots (b), (c) and (d) compare the samples by second feeding diet on day 93 pff and continuously fed fish for both sampling days. The proximity between points represents the similarity of their microbiomes.



4 Discussion

4.1 Alpha diversity values

In this study different dietary plant protein inclusion levels were hypothesized to affect the intestinal environment of fish and thus alter the conditions for bacterial communities present in the gastro-intestinal tract. As a consequence, not only the bacterial community structure in general is strongly affected by the level of plant proteins used, but also the species diversity of this community. The number of observed OTUs was statistically not influenced by the type of diet at the end of the first feeding period, which might however be due to the reduced sample size for diet C. The diet change and the following second feeding period however

generated significant differences between treatments. On the second sampling day (93 dpff) the number of observed OTUs significantly increased when fish were continuously fed with diet A or diet B. It can be speculated whether the stable number of OTUs present in samples of fish continuously fed with diet C is related to the significantly reduced growth or to the already higher number of observed OTUs after the first feeding period. Nevertheless, species richness is increasing after the diet change – independent of the first feeding diet. Species richness can only be affected by three processes [44]: speciation, extinction and dispersal. An increase in species richness could therefore be the result of colonization by rare bacterial species or taxa. Within the second feeding period new bacteria colonized the GI tract of all fish; the amount however was determined by the inclusion level of plant proteins. From the analysis of phyla abundances it is obvious that the taxonomic dominance shifts towards Proteobacteria, Fusobacteria and Actinobacteria in fish that were fed with A and B as first feeding diet. In trout fry, grown on the first feeding diet C, Proteobacteria were already present in a relative abundance of about 50% and thus the richness was already higher in comparison. There are several factors that could have influenced the increase of species: First of all, the diet change could have induced an alteration of the established intestinal environment and thus attracted additional bacteria on top of the ones that colonized the GI tract during the first feeding period. Second however, species richness also increased when no diet change was performed and so intrinsic factors could be emerging during the morphological development of young trout fry, like changes in the pH or the growth of the GI tract and thus increased residence time or simply more space. In a study on the bacterial community turnover within developing zebrafish [45] it was found that time was generally a better predictor for species richness and bacterial turnover than was the intestinal volume, which would go in line with the assumption of ontogenetic factors being involved. The Chao1 richness estimator reveals the same findings of increasing species richness with time. The Simpson's evenness measures follow a similar pattern as the species richness indicators: a significant increase of evenness with time for fish of treatment groups AA and BB, a stable evenness measure for fish of the treatment group CC, and a reduced (however not throughout significantly) evenness for all fish fed with diet C during the second feeding period. This is not surprising since Firmicutes were present in 85% and 90% of all sequences obtained for diet A and diet B, respectively, after the first feeding period. The Shannon diversity index was significantly higher in samples of fish fed with the plant protein based diet C at the end of the first feeding period. At the end of the second feeding period however, the Shannon index decreased significantly in all samples of fish from the treatment groups AC and BC, whereas CC remained constant. The statistical model conducted did not reveal any nutritional programming effect of the first feeding diet on the calculated indices. Thus, the large difference between diets with and without animal protein, in combination with a stable

richness for all fish fed without animal protein reflects the strong influence of both the second feeding diet and intrinsic factors on the development of specific OTUs, and on the overall diversity in the digestive tract of trout fry.

4.2 Relative Abundance of phyla

The development of diet dependent OTUs can be observed in the analysis of relative abundances of phyla. As previously stated, Firmicutes played a significant role in the early intestinal microbiome of trout fry after the first feeding period. The abundance of Firmicutes was significantly higher when animal protein was used in the first feeding diets. In second feeding diets however, the abundance of Firmicutes was strongly reduced. These findings appear to be contrary to previous studies: Ingerslev et al. [16] for instance found Proteobacteria being significantly more abundant in early trout fry that have been fed with a fishmeal based diet until 49 days post first feeding. Nevertheless, their study also revealed the ontogenetic influence on the abundance of phyla, since 26 days post first feeding Bacteroidetes represented the group of most abundant OTUs. Animal proteins used in the current study promoted a significant increase of Proteobacteria, Bacteroidetes and Fusobacteria during the second feeding period until day 93 post first feeding and displaced the high abundance of Firmicutes. Tenericutes had been found being the most abundant phylum in Rainbow trout [46], but the study already stated that for example the applied method (DGGE vs. 16S rDNA vs. cpn60 gene analysis, or the amplified regions V1-V3 vs. V5 vs. V6-V8) could have an effect on the differences in gut bacterial communities. Furthermore, the diets used in the current study not only contained fishmeal as animal protein source, but also shrimp meal, mussel meal and blood meal – together 26% of the amount of fishmeal. Since the focus of this study was not to evaluate individual protein sources, but the nutritional programming effect of first feeding diets, specific differences to other studies could also result from the used protein sources. The observed phyla in general were however quite consistent with other studies and a core intestinal microbiota has been postulated [47] which covers most of these phyla found. On genera level however they concluded that the core microbiome is possibly not present in all Rainbow trout.

4.3 The influence of diets and bodymass on the microbiome

Nutritional programming effects of the first feeding diet on the intestinal microbiome and the influence of a subsequent diet change have been evaluated. The results of the analysis revealed that the microbial community is significantly determined by the diet fed at the point of sampling and that the first feeding diet is not programming the intestinal microbiome of trout fry. The present study clearly demonstrated that the microbiome can be manipulated

during several life stages of Rainbow trout, which has already been suggested [16] and what is well-known from human studies. It has been demonstrated [48] that even a short-term consumption of a new diet alters the human gut microbiome rapidly. Order-specific analysis conducted in the current study revealed that Lactobacillales, Bacillales and Pseudomonadales were specifically promoted when plant proteins were included in the diet. In contrast, Bacteroidales, Clostridiales, Vibrionales, Fusobacteriales and Alteromonadales were significantly promoted when animal proteins were included in the diet. Lactobacillales for example produce lactic acid as a final metabolic product from the fermentation of carbohydrates and thus it is not surprising that plant meals promote an increase of this order. Bacteroidales on the other hand include several microorganisms that are bile-resistant and related to protein fermentation. The mean differences between diet B and diet A were generally smaller than between diet C and diet A. Accordingly, the PCA plot revealed that the microbiomes of fish cluster by the second feeding diet and all NMDS plots indicated that the distances between objects reflect the inclusion level of plant proteins: individuals fed with the mixed diet were placed in-between both extreme diets, with shorter distances towards the animal protein based diet and with longer distances towards the plant protein based diet. The final bodymass also significantly correlated with the intestinal microbial community at the end of the second feeding period. It can only be speculated what influencing factors cause this correlation. One possibility could be stress: Even though the stocking density in each tank was according to approved recommendations for small trout fry [49], high variation in growth rates between individual fish could have caused aggression, territorial or dominant behavior and thus lead to a higher stress level in smaller fish. Since it is well known, that stress has a strong effect on the intestinal microbiome of fish [50] this could be a reason for bodymass dependent microbial community differences. It was not possible to monitor this problem quantitatively during the experiment, but obviously big fish controlling the rest of the group (i.e. preventing from feeding or aggressive biting) were removed from the experimental group.

5 Conclusions

The intestinal microbiome is substantially involved in metabolism, and increasing plant protein levels in fish feed are significantly affecting this microbiome. This study has furthermore demonstrated that the intestinal microbiome of Rainbow trout can be adapted several times towards a given diet and that dietary plant proteins modulate this adaptation. Thus nutritional programming mechanisms of diets on the bacterial community could not be detected – within the presented experimental setup. Early fry feeding with plant proteins will therefore not positively influence the relation between the intestinal microbiome and plant

proteins fed in later life stages. However, early fry feeding with fishmeal (representing the current state of work) will certainly not affect the subsequent replacement with plant proteins as well. The phyla and orders found in this experiment agree very well with bacteria that have been found in previous studies and thus the idea of a trout specific core microbiome on higher taxonomic levels is still valid. These findings should therefore play a key role in further research about fishmeal substitution by including specific bacterial strains commonly present in the GI tract of trout into digestibility analysis.

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General Discussion

This thesis aimed to investigate if nutritional programming can be a potential approach for the improvement of utilising plant-based diets by carnivorous fish species. Farmed rainbow trout and wild brown trout were used to study the mechanistic relationships and the degree of plasticity responding to an early nutritional stimulus. Although the inclusion levels of fishmeal into salmonid diets rapidly declined within the last years, other marine species and shrimps are still highly dependent on fishmeal and fish oil. The aquaculture sector accounted for 68% of global fishmeal and for 74% of global fish oil use in 2012 [1]. Average inclusion levels of marine protein and oil sources are about 30% for marine and carnivorous fish species and about 19% for penaeid shrimp species [1]. Plant proteins are, for various reasons, the most interesting substitutes for fishmeal, however, their use in aquafeeds is facing several constraints regarding nutrient digestibility and fish health [2].

The physiological effects of food, that exceed the basic nutrient utilisation for metabolism and growth, have primarily been observed in humans. One example is the strong increase of obesity already in young children that has been linked to nutrition and the hormonal status of their mothers during pregnancy, although exact mechanisms still remain poorly understood [3]. The potential of a permanent and directed alteration of physiological responses of fish towards a certain diet-type has already been discussed as approach in aquaculture research [4]. The effects of high dietary carbohydrate levels during early development on subsequent glucose metabolism have been investigated for zebrafish [5, 6], as well as for rainbow trout [7] and for Siberian sturgeon [8]. The feeding of rainbow trout fry with plant-based diets induced a permanent improvement of feed intake later in life [9], which was linked to alterations in metabolic pathways involved in feed acceptance [10]. Influencing the fatty acid metabolism of juvenile fish via stimuli during the larval stage was investigated in European sea bass [11] and in Senegalese sole via broodstock diets [12]. Broodstock diets for gilthead sea bream that were based on plant-derived protein sources and plant oils also improved the utilisation of such diets by juveniles [13]. Albeit the positive effects of previous studies, the experimental setups used in this thesis did not provoke nutritional programming effects comparable to those observed in earlier investigations.

Phenotypic plasticity as response to the early feeding of plant-derived protein sources

The aim of this thesis was to explore the several physiological impacts of an early feeding with plant-derived proteins on trout and to evaluate whether these impacts contribute to a

nutritional programming effect. The production of different phenotypes that emerge from one genotype as response to environmental change is defined as *phenotypic plasticity* [14]. Applying different diets to fish of the same species under identical rearing conditions was hypothesised to induce plastic responses of digestive enzyme activities and of the intestinal microbiome. The present thesis demonstrated that pepsin is neither responsive to the experimental diets in rainbow trout (Chapter 3) nor in brown trout (Chapter 1). Amylase activity levels were not affected by the diet-type in brown trout (Chapter 1), however, by the dietary starch content of rainbow trout diets (Chapter 3). Strong plasticity as response to diet-type was, in contrast, demonstrated by the intestinal microbiome in both species (Chapters 2 and 4). The next step to nutritional programming would be *developmental plasticity*, a response to the environmental change on a genomic level [15] with persistent changes in the phenotype [16]. Thus, the dietary stimulus that induced the observed phenotypic plasticity of amylase activities or of the bacterial gut community would also have to act on a genomic (or epigenomic) level. During an early developmental stage, an organism is highly susceptible to developmental plasticity [14], for example at the onset of first feeding. In the case of developmental plasticity, the physiological responses observed after first feeding of trout fry would lead to the so-called “match-mismatch paradigm of the predicted and the actual mature environment” [14]. This concept is used in human medicine to explain the effects of malnutrition during pregnancy on the foetus resulting in higher risks for metabolic diseases later in life. If the environmental stimulus during early development, i.e. the first feeding diet for trout fry, matches the environment afterwards, i.e. the second feeding diet during the juvenile stage, no impact is expected. However, if the early stimulus mismatches the later environment, i.e. after a change in diet, strong effects would be expected. From the results of the cross-over feeding design that was applied in the rainbow trout experiment of this thesis (Chapter 3) it could be concluded that amylase activity levels were dependent on the starch levels that fish had experienced during the first feeding period – regardless of the starch content applied during the second feeding period. This effect could be regarded as nutritional programming, although this was not the desired target variable (the starch content was highest in the fishmeal diet and fish were supposed to be programmed for the protein source). As an example, fish of the feeding regime CA experienced low starch contents during the first feeding period and high starch contents during the second feeding period. Fish of the feeding regime AA experienced high starch contents throughout the entire experiment. Amylase activities were significantly lower in fish of the feeding regime CA compared to fish of the feeding regime AA, which implies a mismatch of the experienced and the actual starch content and could result in a disadvantage. The intestinal microbiome of rainbow trout and of brown trout reacted highly plastic towards the different diets. This plastic response was however observed in the same strength after the diet-change without obvious

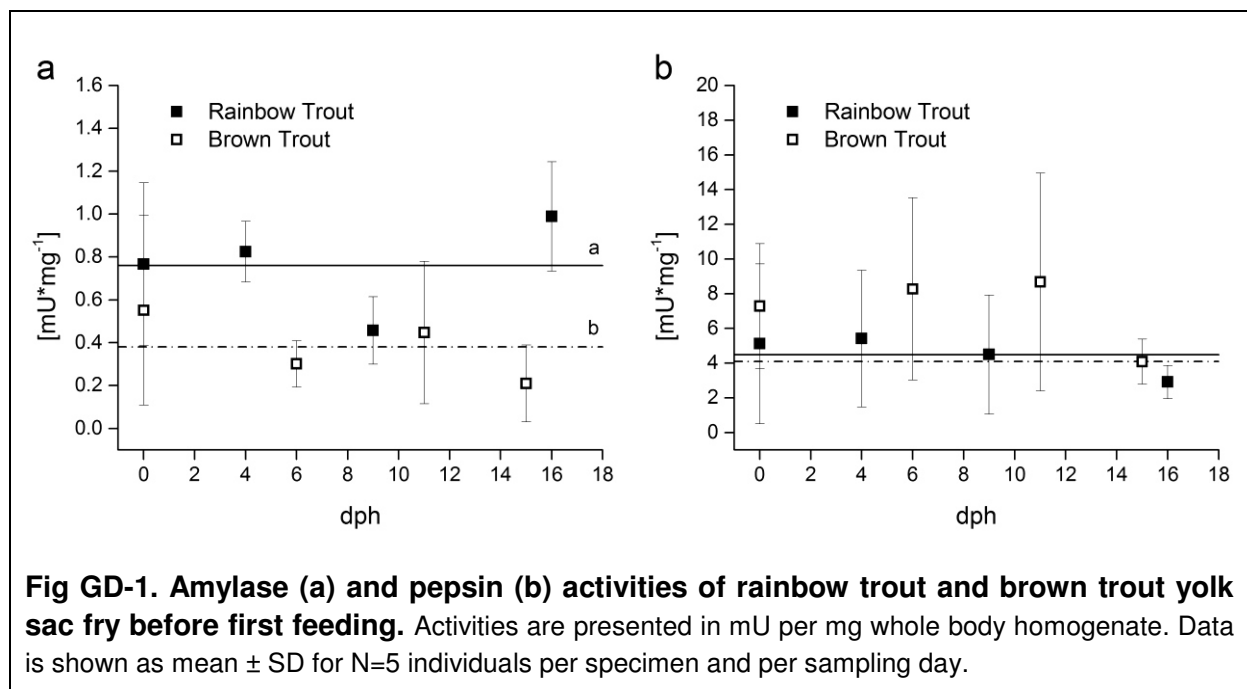
nutritional programming effects and indicates a very high degree of phenotypic plasticity of the bacterial community which allows no persistent impact of the diet, but rather demonstrates the ability of the intestinal microbiome to quickly respond to environmental changes.

The early development of digestion in trout

Salmonid embryos need about three weeks of development inside the egg from fertilisation to hatching, although this timeframe varies strongly with surrounding water temperatures [17]. Shortly after fertilisation, the gastro-intestinal tract of fish is a rudimentary tube, which slowly develops into its functional compartments [18]. After hatching, in contrast to several other fish larvae, a large yolk sac ensures high survival rates of salmonid fry until the onset of exogenous feeding [19]. Moreover, salmonids not only have a fully developed intestine and pancreas but possess a functional gastric digestion at first feeding which favours higher protein assimilation rates [20]. An essential component of gastric digestion is the digestive enzyme pepsin. Feed entering the stomach triggers the release of pepsinogen (the precursor enzyme of pepsin) and of hydrochloric acid (HCl) by the oxynticopeptic cells of the gastric glands in the stomach [21] via the myenteric reflex and an acetylcholine induced cascade [22]. The hydrochloric acid contributes to the conversion of pepsinogen to the active form of pepsin. In this thesis, pepsin activities were detected in both species already before exogenous feeding started (Chapters 1 and 3). When pepsin activity levels of the two experiments were compared to each other (Fig GD-1b) they were not significantly different, however, brown trout tend to show higher pepsin activities than rainbow trout. This could reflect the carnivorous characteristics of wild brown trout, because typical carnivores exhibit higher protease and lower carbohydrase levels [23]. Moreover, activity assays were performed in presence of an acidic buffer solution (pH 2.0) and thus pepsinogen from the gastric glands could have also been converted to pepsin. Therefore, the relatively stable pepsin activities from hatching to first feeding in both trout species (Fig GD-1b) could also indicate a basal amount of pepsinogen that is present in the gastric glands of early trout fry. This hypothesis is supported by similar pepsin activities found in wild brown trout eggs already 5 days prior to hatching (Chapter 1, this thesis) and could be verified by studying the expression of pepsinogen in trout eggs and newly hatched fry.

The average activity of amylase from hatching until first feeding is significantly higher ($p=0.03$) in rainbow trout than in brown trout (Fig GD-1a) and the presence of amylase activity without feed provision again reflects a fundamental enzymatic composition of developing fish [18]. The secretion of amylase is located in the exocrine glands of the pancreas (alike all pancreatic digestive enzymes). Via ducts, amylase is transported from the

exocrine glands to the gut lumen and the secretion of pancreatic enzymes can be stimulated by nutrients in the gut lumen [24]. The pancreas furthermore consists of endocrine cells that produce hormones (mainly glucagon, insulin, somatostatin or peptide YY). Those hormones can modulate the activity of the exocrine glands and thus influence the production and secretion of pancreatic digestive enzymes [24]. The hormone and digestive enzyme secretion of the pancreas is mainly controlled by sympathetic and parasympathetic nerves, and in rainbow trout the endocrine cells are distributed in the adipose tissue surrounding the pyloric caeca and the spleen [24].



Digestive enzymes are one of the most important factors involved in the nutrient digestion in fish and are believed to be highly susceptible to external influences during early development [25, 26]. Thus, the application of an early nutritional stimulus in form of plant-derived feedstuffs that are fed from first feeding onwards was hypothesised to permanently alter digestive enzyme activities, for example by targeting the endocrine control mechanisms of digestive enzyme secretion. In Chapter 1 of this thesis, on the contrary, it has been demonstrated for brown trout that the early feeding of plant-based diets neither affected amylase nor pepsin activities later in life and that activity levels of amylase and pepsin during the different life-stages are not influenced by the diet-type. Acute changes of enzymatic activity at the onset of first feeding were rather related to the presence of feed and to active ingestion than to the experimental diets. In contrast, a small programming effect in the rainbow trout experiment presented in Chapter 3 was observed, because amylase activities that were measured during the second feeding period were significantly dependent on the first feeding diet type. This dependency, however, was strongly related to the dietary starch

content rather than to the inclusion levels of plant-derived proteins. Alike the results of the brown trout experiment, no nutritional programming effects were detected for pepsin activity levels.

Important to consider is that a direct comparison of the two species after first feeding started needs to be taken with care due to the large differences between dietary ingredients and feed manufacturing. A meta-analysis about the impact of plant proteins on salmonid growth additionally revealed that the results of such studies strongly depend on the specific ingredients and that fishmeal quality, for example, can have a severe effect on the outcome of experiments [27].

Plant-based diets for carnivorous fish

The results of the feeding trial with brown trout presented in Chapter 1 and the results of the feeding trial with rainbow trout presented in Chapter 3, both demonstrate that the inclusion of plant-derived proteins into first feeding diets for salmonids not necessarily results in poor growth performance. Especially during the first feeding period, the substitution of 50% dietary fishmeal content by plant-derived protein sources significantly enhanced growth performance of trout in both experiments. Furthermore, growth of wild brown trout fry fed with 90% plant-derived dietary protein from first feeding onwards was equal to that of fish fed with fishmeal as exclusive protein source (Chapter 1). Although the fishmeal reduction in modern salmonid feeds for growing fish has been reduced to less than 15%, the diets for first feeding fry still contain 68% fishmeal and diets for fingerlings contain about 46% fishmeal [28]. With regard to the results of Chapter 1, the amount of fishmeal used in diets for early fry and juveniles could be further reduced. Important, however, is again the need for feed ingredients that are very low in antinutritive factors, since they seem to account for the poor growth of rainbow trout fed with totally plant-based diets (Chapter 3). For example, a reduced bioavailability of phosphorous because of phytic acid has been proposed to severely affect growth performance of salmonids [29, 30]. Phytic acid not only binds phosphorous but also calcium, and dietary deficiencies of both minerals can affect skeletal development and mineral deposition in rainbow trout fry [31].

Differences in growth performance can also originate from differences in voluntary feed intake. Due to the small size of experimental diets and the automatic feeding during the first feeding period of both experiments presented in this thesis, it was very difficult to determine voluntary feed intake of fish. However, in a previous study with juvenile rainbow trout it was concluded that voluntary feed intake is related to the nutritional state and thus adapted to ingest the optimal protein to energy ratio [32]. However, differences in feed intake were not

expected since the different diets used in the present thesis have equivalent energy and protein levels. Nevertheless, the substitution of fishmeal by plant proteins has been shown to induce a reduction in voluntary feed intake of rainbow trout [33, 34] and of Atlantic salmon [35], although an increase in protein to energy ratio increased feed intake of rainbow trout [34]. Furthermore, feed intake of Atlantic salmon was negatively related to dietary phytic acid contents [29]. The hypothalamus is the central controlling system of feed intake, but strongly connected to other physiological signals, such as the secretion of hormones. In a study on rainbow trout, aiming to induce nutritional programming via early feeding of plant-based diets, it was also observed that plant-based diets induce a significant reduction in feed intake of juvenile fish, although this reduction is smaller, if fish had experienced plant-based diets during early development [9]. This is contrasting the present results of the rainbow trout experiment (Chapter 3), in which fish from the feeding regime AB showed the same growth as fish from the feeding regime BB. No differences in growth performance were also observed between trout of the feeding regimes AA and AC, and thus nutritional programming effects on subsequent feed intake through the olfactory system as described previously [9] had presumably no impact on growth performance of rainbow trout. In contrast, wild brown trout (Chapter 1) exhibited enhanced growth performance when the diet changed to the fishmeal-based diet and reduced growth performance when the diet changed to the plant-based diet. This could indicate an influence of voluntary feed intake (which is presumably higher for the fishmeal-based diet) on growth performance of wild brown trout.

However, why is the feeding with a certain percentage of plant-based proteins beneficial for small trout fry? After hatching, yolk sac larvae stay in the interstitial gravel until the start of exogenous feeding. An early start of exogenous feeding before complete yolk sac depletion is crucial for the survival of the fry. Natural feed for brown trout fry mainly consists of chironomid larvae [36], which in turn feed on algae, fungi, pollen, plant particles, animal remains, detritus and silt [37]. Furthermore, cladocerans and copepods, as well as plecopteran larvae are part of the feed for brown trout fry and plecopteran larvae were found to represent about a fourth of the total stomach content volume due to their large size [36]. Plecopteran larvae can either stem from a herbivorous or a carnivorous family [38]. The general diet composition for Plecopteran larvae includes algae, detritus, fungi and bacteria, as well as dead fish eggs or animal material [38]. Cladocerans, in addition, can be made from 28% carbohydrates, and zooplankton in general contains a lot of chitin (N-acetylglucosamine polymer) which might thus be the first carbohydrate ingested by most of the carnivorous fish in the wild [19]. The digestive capabilities (functional stomach and wide range of digestive enzymes) of trout fry seem to fit the variety of nutrients occurring in their natural diet. Although the prey is zooplankton, it is accompanied by plant particles or algae. A

comparison of hatchery-reared and wild brown trout also demonstrated a higher amount of plant particles in the stomachs of hatchery-reared fish, which was related to a lack of experience in catching the right prey [39]. Nevertheless, it is beneficial if as much of the ingested feed can also be digested. Larvae of herbivorous fish are strictly carnivorous during the first weeks of feeding until they undergo a trophic shift (what is also associated to a change in the intestinal microbiome [40]). They lack a stomach, as well as pharyngeal teeth, and need high amounts of nitrogen for fast growth, which can be more easily gained by animal protein [41]. For salmonids, it could be possible that a mixed diet with animal and plant-protein sources reflects the nutrient composition of natural food and is thus beneficial for growth during the first weeks of live. This hypothesis could be addressed, for example, by expression studies of digestive enzymes in wild salmonid fry feeding on natural food.

The growth (in terms of dry body weight) of fish from the different dietary treatments in the experiments of Chapter 1 and 3 started to develop apart about two months after hatching. In a study with two strains of Arctic charr (*Salvelinus alpinus*), differing in their growth potential, the differences in growth performance with identical rearing and feeding conditions became obvious around the same point in time [42]. In their study the authors linked growth performance to a specific ratio of digestive to catabolic enzyme activities, before fry is able to increase its bodymass. Thus, further research should also include the determination of key enzymatic activities involved in nutrient metabolism, in order to investigate the reasons for the positive effects of plant-derived dietary protein sources on growth of trout fry.

The formation of the intestinal microbiome

With increasing knowledge about the effects of the gut microbiome on human health and nutritional status [43], the interest for studying the effects of the fish microbiome strongly increased as well [44]. After fertilisation the egg chorion is colonised by bacteria that are present in the surrounding water and soil, and are hypothesised to already interact with the egg via precursors of the innate immune system [45]. At the moment of hatching, the fish larva is exposed to the bacteria that have already colonised the egg. The formation of the intestinal bacterial community in fish is not yet fully understood. It has been shown that the fish microbiome is very similar to the bacterial community found in the surrounding environment [46]. However, it has also been shown in zebrafish that some bacteria are present in both, wild and farmed fish, promoting the idea of a *core gut microbiome* for a specific fish species [47]. In addition to this, some bacterial groups were found to be significantly associated with a certain trout family and dietary effects on the gut microbiome were dependent on the trout family as well [48]. In general, the microbes of a gut community compete for nutrients, however, they can also live in symbiosis by using the metabolites of

another microbe [49]. The intestinal microbiome is usually classified into autochthonous and allochthonous bacteria. Autochthonous bacteria are attached to the microvilli of the gut epithelial surface and allochthonous bacteria are part of the ingested food, passage the intestinal tract or remain in the gut lumen [50]. It is obvious that feed can have a tremendous effect on the intestinal microbiome. The results from Chapter 2 and Chapter 4 clearly demonstrate that the bacterial gut community of fish is separated by the diet-type. However, Fig GD-2 addresses the idea of a core microbiome by comparing the rainbow trout and the brown trout microbiome. The experiments with the two species were too different for a direct comparison of the dietary influence, but as can be seen in the plot of a Principle Coordinate Analysis (PCoA; a very similar procedure to NMDS, see Appendix A) the microbiomes of the two experiments separate along one axis in space, however are very alike along another. A change in diet is not only affecting the metabolism of fish, but it also changes the substrate for the intestinal microbiome.

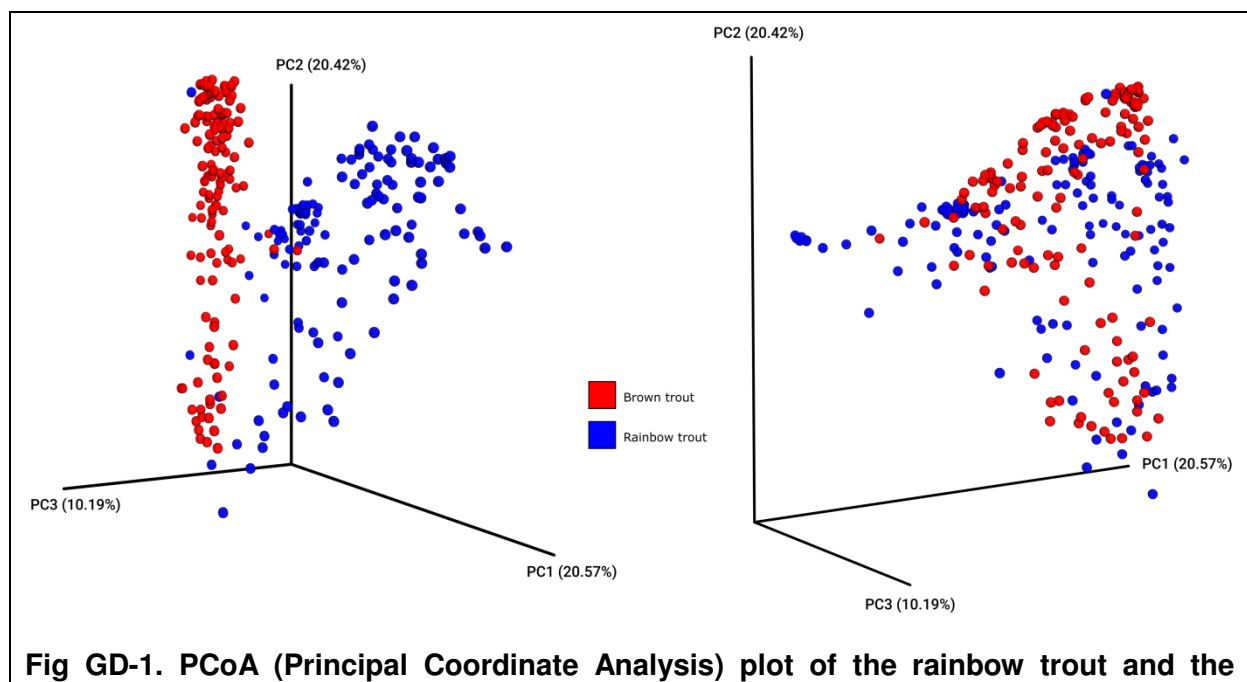


Fig GD-1. PCoA (Principal Coordinate Analysis) plot of the rainbow trout and the brown trout microbiomes. Each globe represents the microbiome of an individual fish placed as object in a 3-dimensional space. The two panels represent the same dataset, but were rotated around PC2. The red globes are fish from the brown trout experiment (Chapter 2), the blue globes from the rainbow trout experiment (Chapter 4). The percentage next to each axis (the coordinate axis) is the percentage of total variance that can be explained by each specific principal coordinate (PC). The PCoA was based on a weighted UniFrac distance matrix [51] and visualised with Emperor [52].

Discussing the concept of a core microbiome leads to at least two possibilities. First, if the main bacterial community present in the gut is autochthonous, then diet would act as environmental pressure, with specific ingredients promoting the growth of a specific bacterial taxon, while other ingredients promote the growth of a different taxon. Thus the bacterial gut community of one trout species comprises specific bacterial taxa, but the relative abundance

of each taxon is shaped by an external stimulus – such as feed. A similar thought was addressed when a zebrafish microbiome was transplanted into germ-free mice. The microbiome that colonised the mouse was very alike the zebrafish microbiome, however, it developed back into the direction of a mouse microbiome [53], which underlines the importance of the host for the intestinal bacterial community. Second, bacteria that enter the fish digestive tract with feed could establish in addition to the present bacterial gut community, though only if the intestinal habitat is suitable. The colonisation of the GI tract by microbes is significantly determined by several physiological factors, such as gastric acidity, bile salts, peristalsis, digestive enzymes, immune response and the already present bacteria [54].

The microbial gut community of first feeding rainbow trout had been examined one day before first feeding and afterwards [55]. The authors observed a significant increase in bacterial diversity with the first ingestion of exogenous food; however, all bacteria found belonged to the same phyla that were observed in previous studies on salmonids. The bulk of bacteria that were identified in brown trout (Chapter 2) and in rainbow trout (Chapter 4) belonged to the phyla Proteobacteria, Firmicutes, Bacteroidetes, Fusobacteria and Actinobacteria, with differing abundances according to the diet fed. The same phyla were found in rainbow trout [48, 55, 56], in brown trout [57] and in zebrafish [47]. A comparative study on the microbiome of eight freshwater fish species [58] that differed in their trophic levels demonstrated that Proteobacteria were present across all trophic levels as the most abundant phylum, followed by Firmicutes, Actinobacteria and Chloroflexi (herbivorous), by Firmicutes, Bacteroidetes and Acidobacteria (carnivorous), by Acidobacteria, Planctomycetes and Bacteroidetes (omnivorous) or by Acidobacteria, Bacteroidetes, and Firmicutes (filter-feeders). The results of this thesis very well align with those findings. The strong separation of intestinal microbiomes of rainbow trout (Chapter 4) and of brown trout (Chapter 2) by the inclusion level of plant-derived protein sources indicates an occurring trophic shift, which is expressed in the alteration of relative abundances of diet-specific bacterial phyla and orders. As a consequence, it would be beneficial to investigate how quickly this trophic shift would occur and what role the allochthonous and the autochthonous bacteria play for the outcome.

The contribution of bacteria to trout digestive physiology

The digestive capacity of fish not only depends on the physiological characteristics of the gastro-intestinal system, but also on the metabolic activity of the intestinal microbiome. From several investigations in fish [59] and in humans [60] it is known that metabolites excreted by the gut microbiome significantly contribute to the host metabolism. Those metabolites have

several functions, for example inhibitory effects against colonising pathogens [61], but also the secretion of digestive enzymes such as amylase, cellulases, proteases, lipases, chitinases or proteases [59]. The experimental setups used in this thesis are not appropriate to describe the metabolic contribution of the present microbiome, nevertheless specific bacterial groups significantly associated with a certain diet-type (Chapters 2 and 4) usually exhibit characteristic properties that could contribute to the growth performance of rainbow trout and brown trout. For example, the pre-treatment of soybean meal with lactic acid bacteria reduced anti-nutritive factors and improved overall digestibility in Atlantic salmon [62]. It is known that Bacteroidetes ferment oligosaccharides from plant material [63], although this is mainly the case for carp. Nevertheless, the relative abundance of Bacteroidetes increased in brown trout with increasing plant-protein inclusion levels (Chapter 2). In rainbow trout (Chapter 4), Bacteroidetes were decreasing with increasing plant-protein inclusion levels, however, Firmicutes were significantly enhanced, which also incorporate several groups of lactic acid bacteria and fermentative processes. Nevertheless, it is important to discuss the influence of diets also on a deeper taxonomic level. The relative abundance of Lactobacillales, for example, significantly separated the microbiomes of both, rainbow trout and brown trout that were fed a diet including plant-based proteins from those of fish fed exclusively animal protein sources (Chapters 2 and 4). Clostridiales, an order of the phylum Firmicutes as well, in contrast contributed stronger to the microbiome of brown trout and rainbow trout that were fed diets with animal protein. It is known from humans that *Bifidobacteria*, belonging to the order Bifidobacteriales, can have several physiological effects, such as an additional source of vitamins which positively affects the immune system or the production of lipopolysaccharides which can induce inflammation [60]. Bifidobacteriales were found to be significantly enhanced in brown trout fed diets with plant-based ingredients. The specific contribution of the intestinal microbiome to growth of fish presented in this thesis remains speculative; however, the importance of investigating such contributions is obvious. The growth of rainbow trout and of brown trout was significantly enhanced during the first weeks of life when plant-proteins were included in the diet. Furthermore, at least for rainbow trout the feeding of a mixed diet was also enhancing growth later in life (Chapter 3). In brown trout, the feeding of a plant-based diet during the early development was beneficial for subsequent growth, although the feeding of a fishmeal diet during the second feeding period lead to a significantly better growth performance (Chapter 1). Relating the intestinal microbiome to growth performance is extremely difficult due to the high complexity of interactions and functions of the bacterial gut community. Nevertheless, what is known from the literature and from the results of this thesis a certain microbial composition might be beneficial at a certain age or developmental status. If lactic acid bacteria are enhanced by plant-based diets during the first weeks of feeding and if these

bacteria are positively impacting the immune system or the carbohydrate-energy-metabolism, this might result in enhanced growth. Thus, further research should strongly focus on the physiological functions of the microbiome present in fish, for example via metagenome studies, to understand in what way the microbiome influences digestion and utilisation of plant-derived protein sources. Moreover, with regard to the strong impact of feed ingredients on the intestinal microbiome, there is need for an accurate synchronisation of available pre- and probiotics with feed ingredients to increase effectiveness of such additives and to avoid unwanted interferences.

Conclusion

This thesis provided new insight into the physiological responses of first-feeding trout fry towards an early nutrition with plant-based diets and contributed to a better understanding of nutritional programming effects in fish. Nutritional programming by an early nutritional stimulus has been proposed as being beneficial for an improvement in utilising plant-derived feedstuffs, however, this hypothesis could not be corroborated with the experiments presented in this thesis. Nevertheless, it was demonstrated that wild brown trout and farmed rainbow trout responded similarly positive to dietary plant-based proteins during early life stages.

When wild brown trout fry (Chapter 1) were fed diets with either 0%, 50% or 90% plant-proteins, no differences in body weights of fish between the three experimental diets could be observed at the end of the first feeding period, indicating an equal utilisation of the different protein sources. After the diet change, however, wild brown trout of all feeding regimes exhibited enhanced growth when fed the 0% plant-protein diet, which could be a relic of the parental feeding history. However, the continuous feeding of 0% plant-protein diets resulted in the same body weight as the continuous feeding of 50% plant-protein diets. Furthermore, despite being hypothesised, amylase and pepsin activities of wild brown trout were only slightly affected by the different protein sources.

The intestinal microbiome of wild brown trout (Chapter 2) has been examined for the impact of an early feeding with plant-based diets and it was observed that the bacterial community is significantly influenced by the inclusion level of plant-derived proteins. However, this influence induced no permanent shape of the microbiome. After the diet-change, the bacterial community formation proceeded again according to the fed diet-type, indicating a high plasticity of the microbiome towards environmental change. Alpha diversity was not significantly affected by the inclusion of plant-derived proteins, but the general evenness was relatively low, indicating a high contribution of a few individual bacterial groups to the

microbial community. It was also observed that certain bacterial groups associated with a specific teleost feeding strategy were significantly promoted by the diet-type and might thus contribute to a better utilisation of this diet. Nevertheless, all bacterial groups observed align well with those observed in previous studies on the intestinal microbiome of salmonids.

Rainbow trout originating from a well-established breeding strain (Chapter 3) exhibited significantly reduced growth when fed a diet almost entirely based on plant-proteins during the first feeding period, probably because of anti-nutritive effects. In contrast, no differences were observed between fish fed a 50% plant-protein diet and fish fed a 0% plant-protein diet. Best growth was also observed for rainbow trout fed the 50% plant-protein diet after the diet change, independent of the first feeding diet-type, and a continuous feeding of 50% plant-derived proteins promoted significantly higher body weights when compared to a continuous feeding of 0% plant-derived proteins. Amylase activities of rainbow trout were significantly enhanced with increasing dietary starch levels, however not with increasing inclusion levels of plant-based protein sources. Pepsin activities were not influenced by the diet-type either.

The feeding with either 0%, 50% or 97% plant-derived dietary proteins (Chapter 4) also significantly affected the intestinal microbiome of rainbow trout. The bacterial community structure was significantly different according to the diet-type and it was observed that alpha-diversity decreased with increasing plant-protein inclusion levels, but increased with age of the experimental animals. The intestinal microbiome of rainbow trout could furthermore not be programmed for the first feeding diet. Nevertheless, the bacterial community exhibited a significant relation to the individual bodymass of fish, which could be an indicator for inter-individual stress during the feeding trial.

From the results of this thesis it can be concluded that trout fry exhibit a high phenotypic plasticity of physiological components towards nutritive stimuli during the first months of life. However, this plasticity has not reached the next level of developmental plasticity, which is required for nutritional programming. In contrast, trout fry have the potential to quickly respond towards different diet-types, which could underline the growth-advantage of salmonid fry in contrast to other fish larvae during early development. The high plasticity of the intestinal microbiome is, however, comprehensible, because an organism that is strictly specialized to a specific environment experiences strong disadvantage with environmental change, according to the match-mismatch paradigm. Aquaculture would, thus, significantly profit from future research, studying in greater detail the intrinsic mechanisms that control the digestive physiology of aquaculture species and the intestinal microbiome formation. Furthermore, on the basis of the present results, feed formulations that are aligned with the intestinal microbiome of fish will positively contribute to future aquafeeds.

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Summary

Aquaculture is one of the fastest growing agricultural sectors in the world. Within the last 20 years, global aquaculture production has doubled and is still growing by 7% each year. Global capture fisheries, in contrast, is stagnating at about 80 million tonnes of annual production since the 1980s, partly because of a significant overfishing of marine stocks. With an annual production of about 90 million tonnes, aquaculture has by now outnumbered capture fisheries. Although during the last decades increasing costs for, and simultaneously decreasing availability of fishmeal and fish oil, generated huge research effort to investigate suitable substitutes, aquaculture is still highly dependent upon wild fish stocks due to its fast growth. In the 1990s, commercial Norwegian salmon feed contained about 90% marine ingredients that are usually produced from wild stocks, such as anchovies, mackerels, sand eels, herring, etc. Especially in feeds for carnivorous species fishmeal is used as highly digestible protein source. The most promising substitutes for fishmeal and fish oil are derived from plants, such as wheat, soya, corn, pea, rapeseed or bean products. In several studies it has been demonstrated that fishmeal and fish oil can be successfully substituted by plant-derived feedstuffs, although some constraints remain. Most plant-derived resources are not generally suitable for their use in aquafeeds. For example, some plant-derived protein sources lack a sufficient amount of specific amino acids and thus the addition of artificial amino acids is needed. Moreover, the crude protein content of most plant meals is rather low, compared to fishmeal, which requires further processing, for example to protein concentrates. In addition, most land-based plants produce secondary compounds as defence mechanism against pathogens. Those secondary plant compounds are associated with health benefits in human nutrition; however, in fish secondary plant compounds can negatively affect fish health and nutrient digestion. It was observed that such *anti-nutritive factors* from plant ingredients induced enteritis in Atlantic salmon, reduced overall protein digestibility in fish or inhibited the availability of dietary minerals, such as phosphorous or calcium. Modern commercial feedstuff is highly processed to avoid anti-nutritive effects, which is, however, costly.

A concept that could counteract these problems is *nutritional programming* and was examined in this thesis. Nutritional programming is currently of high interest in human medicine as one potential cause for the strong increase of diabetes and obesity, already observed in young children. Nutritional programming is based on an early external stimulus, which induces a permanent physiological effect and thus affects the long-term physiological responses of an organism to precisely that stimulus. In animal nutrition, the knowledge about the impacts of such early stimuli becomes of increasing importance. Little is known about the

effects of nutritional programming in fish. However, it has already been shown that components of trout carbohydrate metabolism could be positively influenced when certain carbohydrates were already present in first-feeding diets. The ability to digest especially long-chain carbohydrates, such as fibres, is strongly reduced in most carnivorous fish species. However, such long-chain carbohydrates are a natural constituent of many plants and therefore also found in plant-based feedstuff. The exact mode of nutritional programming and the physiological impact on fish remains unknown. The aim of this thesis was, therefore, to investigate the potential of nutritional programming during the early developmental phase of carnivorous fish to improve the utilisation of plant-based feedstuff. For this purpose one experiment was carried out with rainbow trout and one with wild brown trout. For each experiment, three diets were formulated differing in their percentage of plant-based raw materials. The experimental diets were subsequently administered from the onset of exogenous feeding and effects on the growth of animals, on the activity of the digestive enzymes amylase and pepsin, and on the composition of the gastrointestinal microbiome were investigated.

The first two chapters of this dissertation describe the structure and results of the feeding experiment with wild brown trout. For this experiment, three experimental diets containing 0%, 50% and 90% of plant-based raw materials were developed and produced together with the Skretting Aquaculture Research Centre in Stavanger, Norway. For protein supply, a mixture of different plant raw materials was used to ensure optimal nutrient composition of the diets. All experimental diets were produced in different grain sizes to meet the needs of growing trout. Chapter 1 focused on the growth of fish and on activity levels of the digestive enzymes amylase and pepsin. Amylase is a carbohydrate-cleaving enzyme which mainly degrades starch and is therefore an important enzyme for the digestion of plant raw materials. Pepsin is a protein-cleaving enzyme and secreted in the stomach. It digests dietary proteins to peptides, which are subsequently broken down to amino acids in the intestine by other proteases, such as trypsin. Since pepsin is the first enzyme in the gastrointestinal tract to break down plant proteins, it was of particular interest for this thesis. Digestive enzymes are very target specific and for this reason, it was hypothesised that the various forms of plant and animal proteins can have an effect on the activity of pepsin. However, the results of the first chapter clearly show that, although feed intake per se produced an increase in the amylase and pepsin activities, the nature of the feed and the associated percentage of plant or animal protein sources had no effect on the activity of the two digestive enzymes. Instead, the activities of these enzymes seem to be intrinsically controlled and dependent on the particular state of development of the animals. The aim of the experiment was to induce nutritional programming with respect to the protein source by a

cross-over feeding design. Thus, a total of nine feeding regimes resulted from the respective combinations of the three experimental diets. However, no nutritional programming effects on amylase or pepsin activities could be detected. The increase in body weight of individual fish was further monitored during the course of the feeding trial. It was shown that 50% plant-based raw materials in experimental diets had a positive effect on growth performance during the early development stages of wild brown trout, compared to fishmeal-based feed. After the diet change, during the juvenile stage, the fishmeal-based diet, in contrast, promoted increased growth. However, the growth-enhancing effect from the previous feeding period was preserved and had a positive effect on final body weights. Nevertheless, as with the digestive enzymes, no nutritional programming effect could be observed. In contrast, individual fish exhibited a high plasticity with regard to the experimental diet.

However, growth is only the result of various metabolic pathways. Besides digestive enzymes, the intestinal microbiome plays an important role in the digestion of nutrients, too. In several studies it has been shown that the fish microbiome is not only important for the immune system and the defence of pathogenic bacteria. It has also been observed that intestinal bacteria in fish can secrete digestive enzymes or provide metabolites that allow the fish to utilise otherwise indigestible feed ingredients. Chapter 2 of this dissertation, thus, discussed in detail the effects of plant-based raw materials on the bacterial community of brown trout. It was observed that the diet-type has a strong effect on the bacterial gut community. Further analysis demonstrated that the bacterial composition of the gastrointestinal tract has strongly adapted to the respective diet composition. Interestingly, it was observed that such bacteria are specifically established that were associated with the feeding strategy of fish. For example, the proportion of lactic acid bacteria strongly increased when juvenile trout were fed with a high proportion of plant protein sources. Lactic acid bacteria are especially associated with the fermentation of long-chain carbohydrates or plant fibres in herbivorous or omnivorous fish. Those findings clearly demonstrate that the nature of the diet has a great influence on the intestinal microbiome, however, as already observed with digestive enzyme activities and growth performance data, no nutritional programming of the bacterial community by the first feeding diet can be assumed.

Similar research questions were addressed in the second experiment, which is presented in Chapters 3 and 4 of this thesis. In this experiment, rainbow trout from an established North American broodstock were used as experimental animals. The diets used in this experiment were produced manually and with rather un-processed raw materials. Again, three experimental diets were formulated, containing either 0%, 50% or nearly 100% plant-based protein sources. Those diets were produced in various sizes, correspondingly to the growth

of fish during the feeding trial. The aim of this experiment was again to establish nutritional programming effects by the means of an early stimulus in the form of first-feeding diets. Chapter 3 discusses the results of the analysis of growth data as well as the influence of feed on the two digestive enzymes amylase and pepsin. The results indicated that not only the fry but also the juvenile rainbow trout showed a significantly better growth performance if the feed contained at least 50% plant-based raw materials. In contrast, the diet consisting of almost exclusively plant-based raw materials induced a significant reduction in growth performance, presumably due to the known anti-nutritive effects. As already observed with brown trout, it was not possible to induce nutritional programming effects and the amylase and pepsin activities were again not correlated with the proportion of plant-based raw materials in the experimental diets.

Interestingly, a clear positive relationship between amylase activity and dietary starch content was found. Starch is an important component in the production of fish feed because it provides stability for the feed pellet. Unlike extruded feed, which has been used in the first experiment, pelletized feed was used for the second experiment. Extruding (a process involving heat and pressure) has a positive effect on the digestibility of starch, because the pure form of starch - as is normally found in a feed pellet - is of low digestibility for salmonids. The positive relationship between amylase activity and dietary starch content during the first feeding period also had an effect on the amylase activities measured after the feed conversion. Here a clear nutritional programming effect was observed.

Possible effects of experimental diets on the intestinal microbiome should also be investigated in this experiment. The results are presented in detail in Chapter 4 and are consistent with the results of the experiment presented in Chapter 2. Interestingly, strong differences between sampling at the end of the first feeding period and sampling at the end of the second feeding period were observed. For example, the number of observed taxonomic units (OTUs) and alpha diversity increased markedly with increasing age. The strict separation of the bacterial composition depending on the diet-type was observed as well.

In summary, it can be stated that, with the present experimental parameters, no nutritional programming of trout fry could be induced - with the exception of amylase activities in the rainbow trout experiment. As a result, no positive effects on the subsequent utilisation of plant raw materials could be generated. Instead, it was observed that rainbow trout and wild brown trout respond highly plastic during the first months towards nutritive stimuli. This was observed especially in the composition of the intestinal microbiome. The positive effect of plant-based raw materials on growth performance of fish in both experiments during the early

stages of development should be addressed in future research. Generally said, aquaculture would strongly benefit from knowledge about the metabolic contribution of the intestinal microbiome to digestive physiology. With regard to the large number of commercially available pro- and prebiotics and the strong effect of feed on the intestinal microbiome, it would be advantageous to synchronise those products more closely with the respective feed. Thus, possible negative interactions could be avoided and positive interactions generated.

Zusammenfassung

Die Aquakultur ist einer der am schnellsten wachsenden Sektoren der Agrarwirtschaft unserer Zeit. Innerhalb der letzten fünfzehn bis zwanzig Jahre hat sich die globale Produktion verdoppelt und wächst jedes Jahr um fast sieben Prozent weiter. Im Gegensatz dazu stagniert die weltweite Fischerei seit Jahrzehnten bei etwa 80 Millionen Tonnen Jahresertrag, vor allem aufgrund der starken Überfischung mariner Bestände. Die Aquakultur hingegen liegt derzeit bei einer Jahresproduktion von etwa 90 Millionen Tonnen und obwohl sie die Fangfischerei mittlerweile an Produktionsvolumen überholt hat, ist sie immer noch stark von ihr abhängig. Die stetig wachsende Aquakultur hatte immer einen hohen Bedarf an günstigen und hochwertigen Proteinquellen für die Futterproduktion, jedoch erzeugte der Kollaps vieler Fischbestände (und damit einhergehend steigende Preise für marine Rohstoffe) ein Umdenken. In den neunziger Jahren bestand beispielsweise das Futter für Norwegischen Lachs noch zu neunzig Prozent aus Fischmehl und Fischöl, hauptsächlich gewonnen aus marinen Fischbeständen. Allerdings werden bereits seit einigen Jahren alternative Rohstoffe zu fischbasierten Produkten erforscht. Am vielversprechendsten ist dabei der Einsatz pflanzlicher Protein- und Ölquellen. Vor allem Landpflanzen wurden auf ihre Eignung für den Einsatz in hochwertigen Futtermitteln hin untersucht und zahlreiche Studien konnten belegen, dass Fischmehl durch pflanzliche Proteinquellen ersetzt werden kann. Dabei werden hauptsächlich Produkte aus Weizen, Mais, Soja, Erbsen, Raps oder Bohnen genutzt. Allen Rohstoffen gemein ist, dass sie im Vergleich zum Fischmehl nicht *per se* optimal für die Fischernährung geeignet sind. So entspricht beispielsweise das Aminosäureprofil nicht dem Bedarf der Fischart oder der Rohproteinanteil ist zu niedrig, weshalb viele Pflanzenmehle zu Proteinkonzentraten weiterverarbeitet werden müssen. Außerdem haben fast alle Landpflanzen natürliche Abwehrmechanismen gegen Schädlinge entwickelt. Diese sogenannten sekundären Pflanzenstoffe werden in der Humanernährung häufig mit positiven Effekten auf die Gesundheit assoziiert, bei Fischen können sie sich jedoch negativ auswirken. So konnte man zum Beispiel zeigen, dass pflanzliche Substanzen Darmentzündungen bei Lachsen hervorrufen, die Verdaulichkeit von Proteinen herabsetzen oder Mineralstoffe und Spurenelemente binden, sodass sie für den Fisch nicht mehr verfügbar sind. Diese sogenannten anti-nutritiven Effekte treten durch modernes Hochleistungsfutter kaum mehr auf, weshalb der pflanzliche Anteil beispielsweise im Futter für Salmoniden nun auf siebenzig bis achtzig Prozent angestiegen ist. Pflanzenbasierte Futterkomponenten werden dafür allerdings stark prozessiert; fehlende Aminosäuren, Mineralstoffe oder Vitamine müssen zugesetzt oder sekundäre Pflanzenstoffe entfernt werden. Diese Verfahren sind aufwändig und teuer, und mit steigender Aquakulturproduktion steigt deshalb auch langfristig die Nachfrage nach effizienteren Lösungen.

Ein Konzept, welches diesen Problemen entgegenwirken könnte, ist *metabolische Programmierung* und bildet die Grundlage für diese Dissertation. Metabolische Programmierung wird vor allem in der Humanmedizin als eine Ursache für immer weiter ansteigende Zahlen an den an typischen Volkskrankheiten, wie Diabetes und Übergewicht, erkrankten Menschen angesehen. Das Prinzip beruht auf einem sehr früh gesetzten externen Reiz, welcher einen permanenten physiologischen Effekt induziert und damit langfristig physiologische Antworten eines Organismus auf eben jenen Reiz beeinflussen kann. Sowohl in der Human- als auch in der Tierernährung wird das Wissen um den Einfluss solch früher Reize von immer größerer Bedeutung. Es gibt nur wenige Untersuchungen über die Effekte metabolischer Programmierung bei Fischen. Allerdings konnte gezeigt werden, dass der Kohlenhydratstoffwechsel von Forellen beeinflusst werden konnte, wenn bestimmte Kohlenhydrate bereits im Brutfutter oder im Futter der Elterntiere enthalten waren. Dies ist insofern positiv zu bewerten, als dass die Verdauung vieler Kohlenhydrate gerade bei karnivoren Fischen stark herabgesetzt ist. Vor allem langkettige Kohlenhydrate sind jedoch ein natürlicher Bestandteil vieler Landpflanzen. Über die genaue Wirkungsweise metabolischer Programmierung ist noch sehr wenig bekannt und auch darüber, welche physiologischen Komponenten davon beeinflusst werden können. Das Ziel dieser Dissertation war es deshalb, die Möglichkeiten metabolischer Programmierung durch nutritive Reize während der frühen Entwicklungsphase für die Nutzung pflanzlicher Rohstoffe in der Ernährung karnivorer Fische zu untersuchen. Dazu wurde ein Versuch mit Regenbogenforellen und ein Versuch mit Nachzuchten wilder Bachforellen durchgeführt. Für jeden Versuch wurden Futtermittel konzipiert, die sich in ihrem Prozentsatz pflanzlicher Rohstoffe unterschieden. Diese Futtermittel wurden anschließend ab der ersten Aufnahme von exogenem Futter durch die Forellenbrut verabreicht und deren Auswirkungen auf das Wachstum der Tiere, die Aktivität der Verdauungsenzyme Amylase und Pepsin, und auf die Zusammensetzung des Magen-Darm-Mikrobioms untersucht.

Die beiden ersten Kapitel dieser Dissertation beschreiben den Aufbau und die Ergebnisse des Fütterungsversuchs mit wilden Bachforellen. Für diesen Versuch wurden drei Futtermittel mit einem Anteil pflanzlicher Rohstoffe von 0%, 50% und 90% gemeinsam mit dem Aquakultur-Forschungszentrum des Futtermittelherstellers Skretting in Stavanger, Norwegen, entwickelt und produziert. Als Proteinlieferanten wurde eine Mischung aus verschiedenen pflanzlichen Rohstoffen genutzt, um im Futter eine optimale Nährstoffzusammensetzung zu gewährleisten. Diese drei Futtermittel wurden in verschiedenen Körnergrößen produziert, damit sie den Bedürfnissen der wachsenden Forellenbrut entsprechen. In Kapitel 1 stand das Wachstum der Tiere im Vordergrund, sowie die Aktivitätslevel der Verdauungsenzyme Amylase und Pepsin. Amylase ist ein

Kohlenhydrat-spaltendes Enzym, welches hauptsächlich Stärke abbaut und deshalb ein wichtiges Enzym für die Verdauung pflanzlicher Rohstoffe ist. Pepsin ist ein proteinspaltendes Enzym und wird im Magen sezerniert. Dort verdaut es Proteine, die mit der Nahrung aufgenommen werden und spaltet diese in Peptide auf, welche anschließend im Darm von weiteren Proteasen, wie Trypsin, zu Aminosäuren verdaut und resorbiert werden. Da Pepsin das erste Enzym in der Kette der Verdauungsenzyme für die Verdauung pflanzlicher Proteine ist, war es für diese Dissertation von besonderem Interesse. Verdauungsenzyme spalten ihr Substrat an ganz spezifischen Stellen und die Häufigkeit und Ausprägung dieser Schnittstellen innerhalb eines Substrates kann über die Aktivität des Enzyms entscheiden. Aus diesem Grund sollte untersucht werden, ob die verschiedenen Formen pflanzlicher und tierischer Proteine einen Effekt auf die Aktivität von Pepsin haben. Die Ergebnisse des ersten Kapitels zeigen jedoch ganz klar, dass zwar die Futteraufnahme an sich einen Anstieg der Amylase- und Pepsin-Aktivitäten erzeugte, jedoch die Art des Futters und der damit verbundene Anteil pflanzlicher oder tierischer Proteinquellen keinerlei Auswirkungen auf die Aktivität der beiden Verdauungsenzyme hatte. Stattdessen scheinen die Aktivitäten dieser Enzyme intrinsisch gesteuert und abhängig vom jeweiligen Entwicklungsstand der Tiere zu sein. Kerngedanke des Versuches war es, eine metabolische Programmierung hinsichtlich der Proteinquelle zu induzieren. Eine solche Programmierung sollte durch ein kreuzweises Versuchsdesign erzeugt werden, in welchem nach einem Zeitraum von etwa zwei Monaten alle Futtermittel durchgetauscht wurden. So ergaben sich insgesamt neun Fütterungsregime. Da es jedoch zu keinem Zeitpunkt einen Effekt auf die Enzymaktivitäten durch die Art des Futtermittels gab, konnte auch keine metabolische Programmierung festgestellt werden. Ein weiterer Untersuchungsparameter war die Gewichtszunahme der Fische, welche in unterschiedlichen Abständen während des gesamten Versuches gemessen wurde. Hierbei konnte gezeigt werden, dass ein Anteil von 50% pflanzlicher Rohstoffe im Futter sich in den frühen Entwicklungsstadien der wilden Bachforellen wachstumssteigernd im Vergleich zu rein fischmehlbasiertem Futter auswirkte. Nach dem Futtertausch, im juvenilen Stadium, führte allerdings das fischmehlbasierte Futter zu einem stärkeren Anstieg im Wachstum. Jedoch blieb der wachstumssteigernde Effekt aus der früheren Fütterung erhalten und wirkte sich positiv auf die Endgewichte der Tiere aus. Dies war allerdings – wie bereits bei den Verdauungsenzymen – kein metabolischer Programmierungseffekt im klassischen Sinne. Die Fische zeigten, im Gegenteil, eine hohe Plastizität hinsichtlich des Futters.

Wachstum ist jedoch nur das Resultat verschiedenster Stoffwechselwege. Neben Enzymen spielt bei der Verdauung von Nährstoffen auch das intestinale Mikrobiom eine äußerst wichtige Rolle. In Studien konnte bereits gezeigt werden, dass die bakterielle

Darmgemeinschaft nicht nur essentiell für das Immunsystem und die Abwehr pathogener Bakterien ist. Man konnte auch beobachten, dass Darmbakterien in Fischen weitere Verdauungsenzyme sezernieren oder ganze Stoffwechselwege bereitstellen, die dem Fisch die Möglichkeit bieten ansonsten unverdauliche Futterbestandteile zu verwerten. Kapitel 2 dieser Dissertation befasste sich ausführlich mit den Effekten pflanzlicher Rohstoffe auf das Darmmikrobiom der Bachforellen. So konnte beobachtet werden, dass der Futtertyp einen eklatanten Einfluss auf die bakterielle Gemeinschaft besitzt. Verschiedene Analysemethoden zeigten, dass sich die Bakterienzusammensetzung des Magen-Darm-Traktes stark an die jeweilige Futterzusammensetzung angepasst hat. Interessanterweise konnte dabei festgestellt werden, dass sich vor allem solche Bakterien etablieren, die auch bei anderen Fischarten mit einer bestimmten Nahrungsstrategie assoziiert sind. So stieg beispielsweise der Anteil an Milchsäurebakterien stark an, wenn die jungen Forellen mit einem hohen Anteil pflanzlicher Proteinquellen gefüttert wurden. Milchsäurebakterien werden in herbivoren oder omnivoren Fischen, wie dem Karpfen, vor allem mit der Fermentation langkettiger Kohlenhydrate oder Pflanzenfasern in Verbindung gebracht. Diese Ergebnisse zeigen also ganz deutlich, dass die Art des Futtermittels einen großen Einfluss auf das Darmmikrobiom hat. Die bakterielle Zusammensetzung wurde nach der ersten Fütterungsperiode zum Zeitpunkt der Futterumstellung erfasst und am Ende der zweiten Fütterungsperiode. Auch hier wurde wieder überprüft, ob das jeweilige Brutfutter einen permanenten und langfristigen Einfluss auf das sich formende Mikrobiom juveniler Forellen hatte. Doch wie bereits die Verdauungsenzyme und Wachstumsdaten, zeigte die bakterielle Zusammensetzung eine so hohe Plastizität gegenüber dem jeweiligen Futtermittel, sodass nicht von einer metabolischen Programmierung der bakteriellen Gemeinschaft ausgegangen werden kann.

Ähnliche Fragestellungen sollten in einem zweiten Versuch beantwortet werden, welcher in den Kapiteln 3 und 4 dieser Dissertation vorgestellt wird. In diesem Versuch wurden Regenbogenforellen aus einem etablierten nordamerikanischen Elternbestand als Versuchstiere genutzt. Die Futtermittel für diesen Versuch wurden manuell und mit wenig prozessierten Rohstoffen hergestellt. Erneut wurden drei Futtermittel konzipiert, die entweder 0%, 50% oder knapp 100% pflanzliche Proteinquellen beinhalten. Diese drei Futtermittel wurden in verschiedenen Größen hergestellt, die dem jeweiligen Wachstumsstand der Tiere während des 4-monatigen Versuches entsprachen. Auch in diesem Versuch war das Ziel eine metabolische Programmierung durch einen früh gesetzten Reiz in Form des ersten Brutfutters zu etablieren. In Kapitel 3 werden die Ergebnisse aus der Analyse der Wachstumsdaten, sowie der Einfluss der Futtermittel auf die beiden Verdauungsenzyme Amylase und Pepsin diskutiert. Die Ergebnisse zeigten, dass nicht nur die Forellenbrut, sondern auch die juvenilen Regenbogenforellen eine deutlich bessere Wachstumsleistung

zeigten, wenn das Futter mindestens 50% pflanzliche Rohstoffe enthielt. Im Gegensatz dazu steht das Futtermittel, welches fast ausschließlich aus pflanzlichen Rohstoffen bestand. Hier zeigten sich starke Wachstumseinbrüche, die vermutlich auf die bekannten anti-nutritiven Effekte zurückzuführen sind. Interessanterweise zeigt der vorliegende Versuch, dass Fischmehl als alleinige Proteinquelle nicht notwendig ist, obwohl Fischmehl jahrzehntelang als optimale Proteinquelle für Salmoniden angesehen wurde. Wie bereits bei den Bachforellen beobachtet werden konnte, war es jedoch auch in diesem Versuch nicht möglich eine metabolische Programmierung zu induzieren. Die Messung der Aktivitäten von Amylase und Pepsin zeigte erneut keinen Zusammenhang mit dem Anteil pflanzlicher Rohstoffe im Futter.

Interessanterweise konnte ein klarer, positiver Zusammenhang der Amylase-Aktivität mit dem Stärkegehalt des Futtermittels festgestellt werden. Stärke ist ein bei der Herstellung von Fischfutter unentbehrlicher Bestandteil, weil diese aufquillt und so dem Futterpellet seine stabile Form gibt. Anders als bei den extrudierten Futtermitteln, welche im ersten Versuch verwendet wurden, wurden für den zweiten Versuch pelletierte Futtermittel verwendet. Das Extrudieren (ein mit starker Hitze und hohem Druck verbundener Prozess) wirkt sich positiv auf die Verdaulichkeit von Stärke aus, weil die reine Form der Stärke – wie sie normalerweise im Pellet zu finden ist – für Salmoniden schwer verdaulich ist. Der positive Zusammenhang von Amylase-Aktivität und Stärkegehalt des Futters während der ersten Fütterungsperiode wirkte sich auch auf die Amylase-Aktivitäten aus, die nach der Futterumstellung gemessen wurden. Hierbei eine deutliche metabolische Programmierung zu beobachten.

Auch in diesem Versuch sollten mögliche Effekte der Futtermittel auf das Darmmikrobiom untersucht werden. Die Ergebnisse sind detailliert in Kapitel 4 dargestellt und decken sich mit den Ergebnissen der Untersuchungen aus dem Fütterungsversuch mit Bachforellen. Interessanterweise gibt es hier jedoch starke Unterschiede zwischen der Probenahme nach der ersten Fütterungsperiode und der Probenahme nach der zweiten Fütterungsperiode. So steigen die Anzahl gefundener Mikroorganismen und die Diversität der verschiedenen Arten mit zunehmendem Alter deutlich an. Die strikte Auftrennung der bakteriellen Zusammensetzung je nach Futtermittel-Typ ist jedoch auch hier gut zu beobachten.

Zusammenfassend kann festgestellt werden, dass unter den gegebenen experimentellen Parametern keine metabolische Programmierung der Fische möglich war – mit Ausnahme der Amylase-Aktivitäten im zweiten Versuch. Somit konnten keine positiven Effekte auf eine spätere Verwertung pflanzlicher Rohstoffe erzeugt werden. Stattdessen war zu beobachten, dass Regenbogenforellen und wilde Bachforellen während der ersten Monate sehr plastisch

gegenüber nutritiven Reizen reagieren. Dies war insbesondere bei der Zusammensetzung des Darmmikrobioms zu beobachten. Die Tatsache, dass sich pflanzliche Rohstoffe positiv auf das Wachstum beider Fischarten auch im frühen Entwicklungsstadium ausgewirkt hatten sollte weiter untersucht werden. Die Aquakultur würde dabei vor allem von Ergebnissen zum metabolischen Beitrag des Mikrobioms profitieren. In Hinsicht auf die große Anzahl verfügbarer Pro- und Präbiotika und dem starken Effekt des Futters auf das intestinale Mikrobiom, wäre es von Vorteil diese Produkte stärker mit dem jeweiligen Futtermittel abzustimmen. So können eventuelle negative Wechselwirkungen vermieden und positive Interaktionen erzeugt werden.

Appendix A – Methodology for microbiome analysis

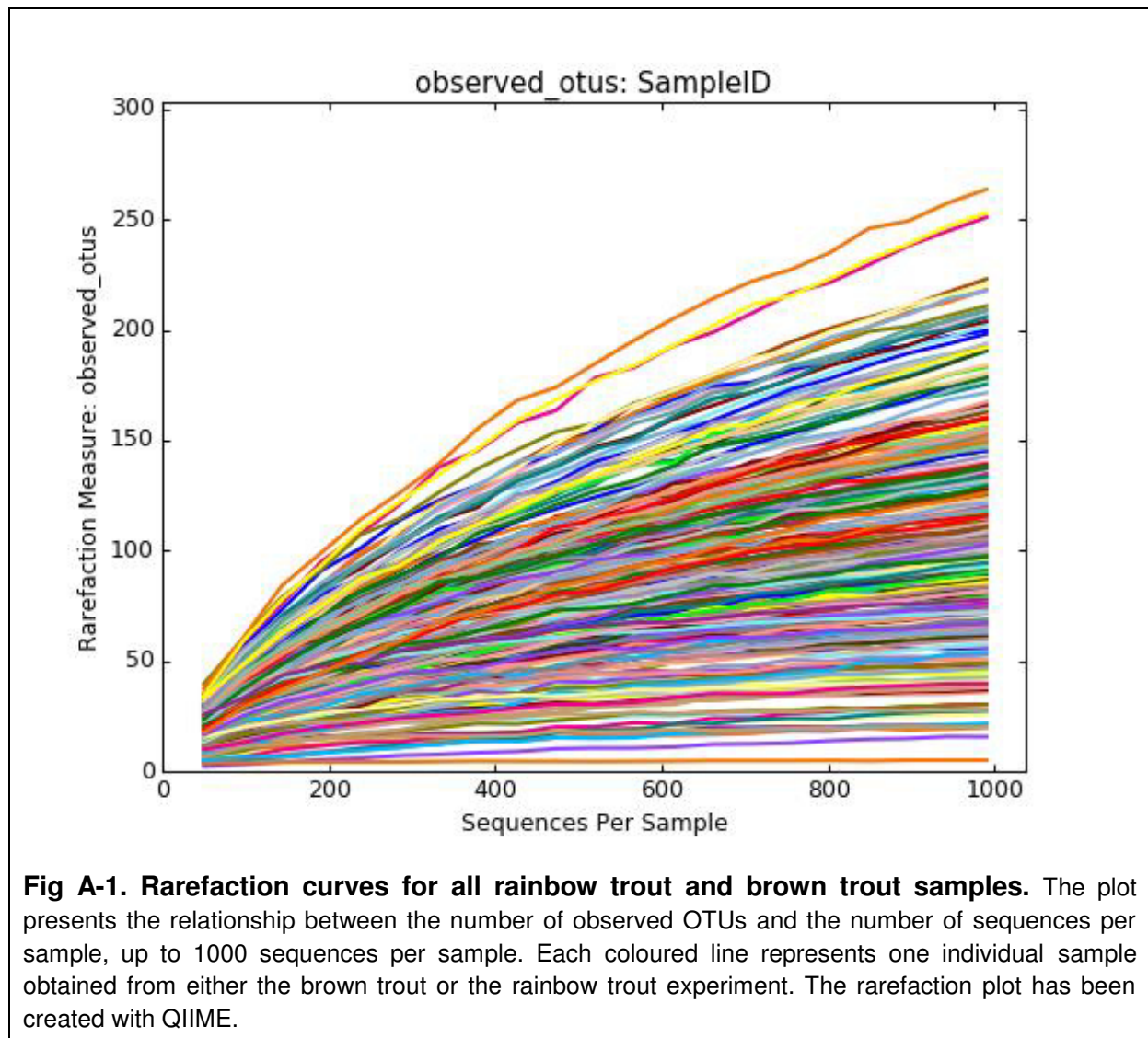
16S rRNA Sequencing

The microbial ribosome consists of two subunits, the 30S and the 50S subunit, and the 16S unit is part of the smaller ribosome subunit. The gene which is encoding for the ribosomal 16S RNA, can be used for phylogenetic studies of prokaryotes, because of the slow evolutionary rates of this gene. The 16S rRNA gene is ubiquitous in microorganisms and is divided in several conserved and variable DNA regions. The variable DNA regions (nine in total) are unique to bacterial taxa, which makes the sequencing of these regions a good approach for bacterial community profiling [1]. However, there is no gold-standard region and in fact, several variable regions or various combinations are used for studying a microbial community [2]. Several primers are available for amplifying the 16S rDNA, but sequencing results depend strongly on the choice of primer and region [3], and not all primers are precise or unbiased [2]. The decision which primers and regions are used varies between studies, research questions and labs. Once the desired region is amplified and sequenced, all sequences obtained for a sample are compared to online databases, such as Greengenes [4], in order to match the sequences to a bacterial taxon. Nevertheless, the success of these matches is based on the number of specifically defined sequences deposited in such databases and on the quality of obtained sequences. The best sequencing results are useless if the desired taxon is not deposited in any database and a microbe can be present in all databases, but it will not be discovered if the error rates of a given sequence are too big.

Rarefaction

Rarefaction is part of the quality control that is implemented in QIIME to ensure best results for further phylogenetic and non-phylogenetic analysis. All sequences that were generated on the MiSeq™ sequencing platform were rarified to 1000 reads per sample. One read is defined as one sequenced DNA strand that has been amplified by PCR with the primers described in Chapter 2 and Chapter 4. The sequencing workflow generates a different number of reads for every sample due to different binding characteristics of sequencing primers or the quality of amplification products. However, the more sequences for one sample are obtained, the greater the chance of finding a new OTU (Operational Taxonomic Unit). Fig A-1 demonstrates the positive relationship between the number of sequences (i.e. reads) per sample and the number of observed OTUs. In order to remove this bias for further downstream analysis, the rarefaction procedure is applied. This procedure randomly selects a distinct number of sequences from the sequence pool of every sample (in this case 1000),

which equalises the number of sequences for all samples and allows the direct comparison of observed OTUs between samples. In general, the rarefaction curve flattens at a certain number of sequences obtained, which would make a good threshold. It is beneficial to set this threshold as high as possible, however there is the risk of losing too many samples. For the current study the threshold was set to 1000 reads per sample, because it is supposed to be sufficient for 16S rRNA sequencing with a good sampling depth [2] and at least three samples per replicate remained in the dataset to ensure an adequate statistical assessment.



Alpha diversity indices

Four alpha diversity measures were calculated for Chapter 2 and Chapter 4 based on the OTU table generated during the QIIME workflow. The number of observed distinct OTUs, the Chao1 richness estimator, the Simpson's evenness measure E and the Shannon diversity index H' were selected for the purpose of estimating the diversity of microbial species within the GI tract samples. The number of observed distinct OTUs is by nature the simplest form of

species *richness* (one essential component of diversity) in a given sample or environment [5]. The non-parametric Chao1 richness estimator corrects the plain number of observed OTUs with a factor to take into account that it is rather unlikely to observe a species more than once in a very rich (i.e. rich in species) environment [6]. Nevertheless, richness is highly dependent on sample size, because it cannot be assumed that all possible OTUs of a specific environment have been detected during sampling [6]. The Shannon diversity index H' , not only takes richness but also *evenness* into account (the second essential component of diversity, which reflects the relative abundance of the observed OTUs) and summarises both into a single measure. H' is defined as

$$H' = - \sum_{i=1}^m p_i \ln p_i$$

where p_i is the proportion of individuals belonging to the i th OTU of a sample with m different OTUs [5]. However, the Shannon index increases with the number of observed OTUs and therefore it can be difficult to compare samples that greatly differ in richness [5]. The Simpson's evenness measure E in contrast, standardises the Shannon diversity index by the richness of the sample; values are usually between 0 and 1, with estimates close to 1 indicating completely evenly distributed species. E is calculated as

$$E = H' / H_{max}$$

where H_{max} is the maximum possible H' , i.e. where the abundance of all OTUs in a sample is equal. Thus, the lower the Simpson's evenness measure, the lower is the diversity in terms of evenness [5]. In this case, only a few OTUs would account for the bulk of sequences obtained.

Nonmetric multidimensional scaling (NMDS)

Differences between bacterial communities in relation to the dietary treatment or the sampling point were investigated by nonmetric multidimensional scaling (NMDS). NMDS is based on the principle that samples are placed as objects in a two-dimensional ordination space according to the (dis)similarity of their microbial community. The dissimilarity of the microbial community between two objects can be calculated in a dissimilarity matrix, based on the Bray-Curtis dissimilarity δ_{jk} [7]. The distances between these objects reflect the (dis)similarity of their microbial communities, i.e. objects are in close proximity if their microbiomes are similar [8]. It was necessary to transform the data beforehand via Hellinger-

transformation, due to the high number of zeros within the dataset, which is quite common for species abundance data, but problematic for subsequent linear analysis as NMDS or Principal Component Analysis [8]. The stress factor (a value between 0 and 1, but preferably close to zero) was additionally calculated to provide a way of determining how well the original data is represented in the ordination space [7].

Principal Coordinate Analysis (PCoA)

The Principal Coordinate Analysis (PCoA) is a very similar procedure to NMDS and also used to determine differences between groups and objects [8]. In contrast to *nonmetric* multidimensional scaling (NMDS), PCoA is *metric* multidimensional scaling (MDS). It is based on linear (Euclidian) distances and thus very similar to the Principal Component Analysis (PCA). The main difference is the fact that PCoA components are complex functions of the original variables, contrasting PCA, where principal components are linear combinations of the original variables [8].

DNA purification procedures

DNA extraction from gastro-intestinal tract samples was performed with the Qiagen DNeasy[®] Blood & Tissue DNA extraction kit¹ according to the manufacturer's protocol. The use of such extraction kits is based on selective binding of DNA to a specific silica-based membrane applied to a small centrifugation column (spin-column). After lysis of cells present in the sample, the DNA binds to this membrane due to specific buffer conditions during centrifugation while cell-components, peptides, RNAs or any other contaminants are washed through the spin-column. Afterwards the bound DNA is eluted by water or a low-salt buffer, due to a change in the selective binding conditions within the spin-column. In order to especially lyse all bacterial cells present in the sample, lysozyme was applied beforehand. To further clean DNA samples to be suitable for PCR, a second DNA clean-up step was performed using the NucleoSpin[®] gDNA clean-up kit² (Machery-Nagel) following the manufacturer's protocol including all recommended steps. The results of the PCR were evaluated on an agarose gel and afterwards, samples were gel extracted using the QIAquick Gel Extraction Kit³ (Qiagen) following the manufacturer's protocol. Both procedures are

¹ <https://www.qiagen.com/de/resources/resourcedetail?id=6b09dfb8-6319-464d-996c-79e8c7045a50&lang=en>

² http://www.mn-net.com/Portals/8/attachments/Redakteure_Bio/Protocols/DNA%20clean-up/UM_GenomicDNACleanup_NS.pdf

³ <https://www.qiagen.com/us/resources/resourcedetail?id=3987caa6-ef28-4abd-927e-d5759d986658&lang=en>

based on the same principles as the DNA extraction kit: selective binding of DNA to a specific centrifugation column in order to clean the DNA from contaminants.

Illumina Sequencing Technology

The sequencing of all PCR products generated was performed on an Illumina MiSeq sequencing platform. Illumina sequencing is based on the so called *sequencing by synthesis* technology. In this procedure an individually labelling adapter is added to the PCR products of every sample by amplification, accompanied by further motifs necessary for the sequencing procedure, such as binding sites for the reaction cell. Afterwards, all samples are combined into a sequencing library and applied to the reaction cell (usually a small glass slide). Here, each DNA fragment present in the library is isothermally amplified (via so called *bridge-amplification*), which produces clusters of cloned copies of the DNA fragments. This procedure is called clustering. At the end of clustering all reverse DNA strands are washed off the glass slide and only the forward strands remain. Sequencing is then performed by adding fluorescently marked nucleotides, which bind to the corresponding nucleotide of the DNA strand. Each nucleotide is added individually after each other and an excitation light is applied after every nucleotide to induce fluorescence of the labelled nucleotide (refer to Appendix B for an introduction into fluorescence). The characteristic fluorescence of each added nucleotide is emitted by the amplification clusters and can be detected. This is the sequencing by synthesis procedure. Afterwards, the DNA fragments of each cluster are amplified again, the forward strands are washed away and the reverse strands are sequenced according to the sequencing by synthesis procedure. The detected order of characteristic fluorescence for every cluster is then adjusted to the labelling adapter and mapped for every sample. More information about the Illumina Sequencing Technology is provided by the Illumina YouTube Channel⁴.

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Appendix B – Fluorescence-based enzyme activity assays

Enzyme kinetics

Enzymes are catalytically active proteins, with very specific target molecules. Digestive enzymes, for example, also need precise cleavage sites within those target molecules. In general, enzymes need to directly interact with the target and this interaction is termed catalytic activity. The target molecule in an enzymatic reaction is called substrate and the velocity of an enzymatic reaction can be described by Michaelis Menten-kinetics. Michaelis Menten-kinetics demonstrates that the velocity of an enzymatic reaction is directly dependent on the substrate concentration. The SI unit to describe enzymatic activity is 1 unit (U), which is the amount of enzyme needed to catalyse 1 μmol of substrate within 1 minute. Enzyme activity assays are built upon those principles to determine the catalytic activity of an enzyme *in vitro*.

Fluorescence-based activity assays

Measuring the catalytic activity of an enzyme requires accurately defined reaction conditions, which supports the usage of commercial reaction kits. The kits used in this dissertation to measure amylase and pepsin activity were purchased from Molecular Probes™, a brand of Thermo Fisher Scientific. Both kits (EnzChek® Ultra Amylase Assay Kit; EnzChek® Protease Assay Kit *green fluorescence*) are fluorescence-based – a fluorimetric measurement technique. This technique is based on the change of a given fluorescence intensity due to the activity of an enzyme, which can be measured in a fluorescence plate reader. The substrate used in each kit is labelled with a fluorophore in high amounts, which enables intramolecular self-quenching of its fluorescence. When the substrate is cleaved by the enzyme, quenching is relieved and the fluorescent fragment can be measured. Quenching is the reduction in fluorescence intensity of a fluorescent molecule. In this case, the close proximity of fluorophores prevents fluorescence of the substrate before being cleaved by the enzyme. Thermo Fisher Scientific uses BODIPY fluorophore conjugates for its enzymatic activity assays (detailed description of all BODIPY fluorophores can be found on the Thermo Fisher Scientific webpage⁵). The substrate used in the EnzChek® Ultra Amylase Assay Kit is a specific starch derivate (DQ™ starch), which is hydrolysed by amylase into a mix of maltose, maltotriose and dextrans (EnzChek® Ultra Amylase Assay Kit, product information). The substrate used in the EnzChek® Protease Assay Kit *green fluorescence* is the

⁵ <https://www.thermofisher.com/de/de/home/references/molecular-probes-the-handbook/fluorophores-and-their-amine-reactive-derivatives/bodipy-dye-series.html>

BODIPY FL casein (EnzChek® Protease Assay Kit *green fluorescence*, product information). The cleavage of the substrate by the enzyme induces an increase in fluorescence, which is proportional to the enzymatic activity. The fluorescence was measured with the TECAN infinite M200 spectrophotometric plate reader. In order to measure fluorescence, the fluorophore needs to be excited by light of a given wavelength (optimal excitation wavelength for BODIPY is 505 nm). Excitation is the absorption of energy in the form of light by the outmost electrons of an atom, which elevates these electrons to a higher energetic state. Immediately afterwards, the electrons fall back to the energetic ground state and simultaneously emit light that can be detected by the plate reader (usual emission wavelength of BODIPY is 512 nm). By using an enzyme standard, such as α -amylase from *Bacillus* spp. or pepsin from porcine gastric mucosa, the increase in fluorescence of a sample can be monitored over time and (at a specific time point) related to the fluorescence intensity of defined enzymatic activities (Fig B-1).

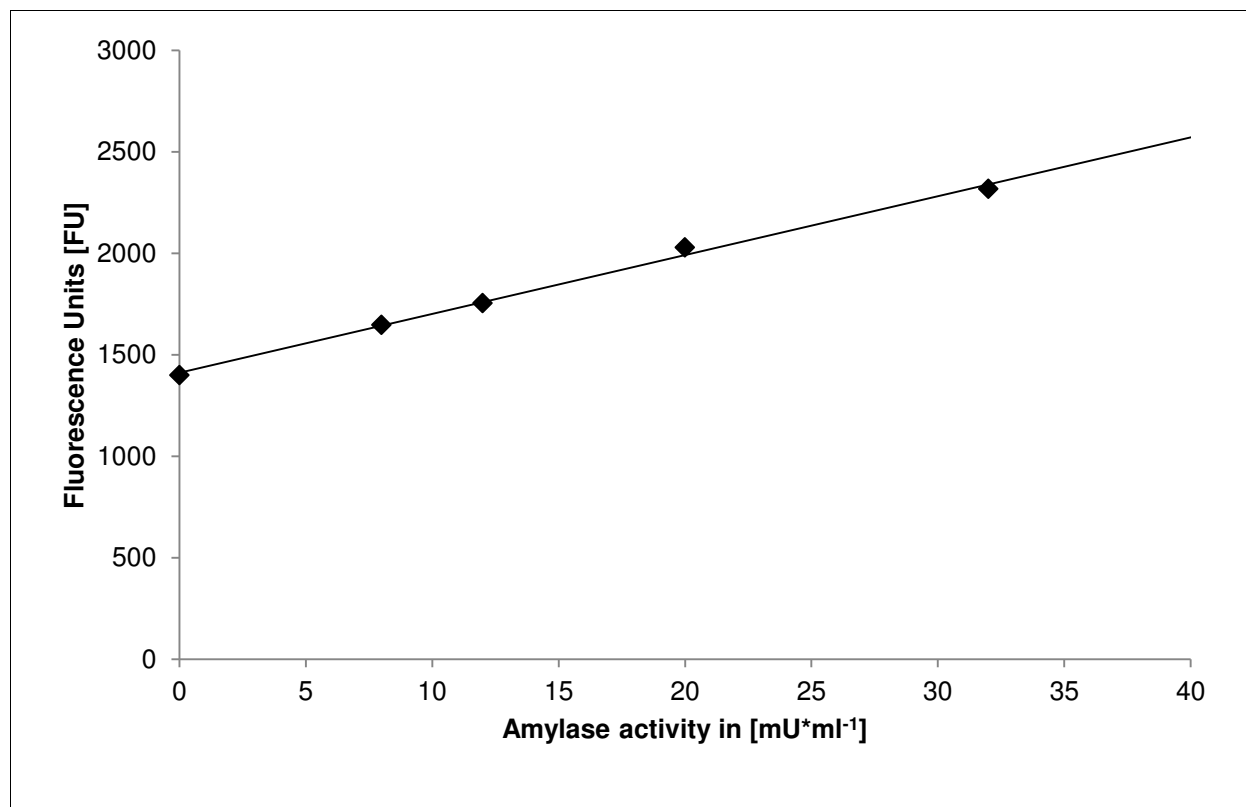


Fig B-1. Standard curve of the enzymatic activity of *Bacillus* spp. α -amylase. Presented are the fluorescence intensities of five α -amylase solutions in relation to their activity (in $\text{mU}\cdot\text{ml}^{-1}$), measured after 30 min of incubation with the EnzChek® Ultra Amylase Assay Kit. α -amylase activity is directly proportional to the fluorescence intensity of the substrate (DQ™ starch), with a linear relationship ($R^2=0.996$). Reactions were performed with $200\mu\text{g}\cdot\text{ml}^{-1}$ DQ starch and the indicated concentration of α -amylase in 20 mM $\text{H}_2\text{PO}_4\text{Na}$, 6.7 mM NaCl and 0.5 mM $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$ (pH 6.9).

Appendix C – Feed production and analysis

Feed production for the rainbow trout experiment

The experimental diets for the first feeding period during the rainbow trout experiment were produced at GMA. All ingredients were finely ground to be suitable for the smallest feed size. Afterwards ingredients were mixed according to the feed formulation presented in Chapter 3 and 4 and moistened with water until the matter coagulated with the gelatine. The matter was pressed to small roles with a meat grinder and dried at room temperature for at least 72 hours. The dried feed roles were ground with a professional mill and sieved to the desired particle size of <0.4 mm, 0.6 mm and 1.2 mm. Resulting feed particles had the characteristics of swimming feed with low sinking time. In order to produce feed particles with a diameter of 2.0 mm (used during the second feeding period) all ingredients were pelletized with a pellet machine L 14 – 175 (AMANDUS KAHL GmbH & Co. KG, Reinbek, Germany). The ingredients were mixed, but not finely ground and moistened, before added to the pellet machine.

Chemical nutrient analysis

The chemical nutrient analysis of all experimental diets was performed according to the European Commission Regulation (EC) No 152/2009 (European Union 2009). Dry matter content of feed samples was determined by drying samples directly at 103°C for 4 hours (ED 53, Binder GmbH, Germany). Afterwards, samples were cooled down in the exsiccator for 45 min and weighed. Crude ash content of feed and body homogenates was determined in a combustion oven at 550°C for 4 hours (P300, Nabertherm, Germany). The method after Kjeldahl (InKjel 1225 M, WD 30, Behr, Germany) was used to determine the crude protein content of diets. The nitrogen (N) present in organic material is quantitatively converted to ammonium sulphate by adding concentrated sulphuric acid (catalytic converter needed). Ammonia is released by adding concentrated sodium hydroxide solution at boiling temperature. The released ammonia is distilled off and transferred to an acid. The amount of ammonia can then be determined via titration and is further used to calculate the protein content of a sample. Crude lipid content of experimental diets was determined by using methods after Soxhlet (Soxtherm, C. Gerhardt GmbH & Co. KG, Germany). The fat within a sample is extracted by petrol ether, which is subsequently distilled off and the remaining fat can be weighed. Gross energy was determined in a bomb calorimeter (C200; IKA, Germany).

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Curriculum Vitae

Stéphanie Céline Michl

Geboren am 06.01.1988 in Fontainebleau, Frankreich

Staatsangehörigkeit: deutsch, österreichisch



- Jan. 2013 – Jan. 2017 Promotion in der Aquakultur**
- Thesis: Evaluation of plastic responses to nutritional programming by various feed sources in brown and rainbow trout fry
- Gesellschaft für Marine Aquakultur mbH Büsum
Christian-Albrechts-Universität zu Kiel
GEOMAR (Helmholtz Research School for Ocean Systems Science and Technology)
- Jan. 2013 – März 2013 Trainee Research & Development**
- Skretting Aquaculture Research Centre
Stavanger, Norwegen
- Okt. 2010 – Dez. 2012 Master of Science der Biologie**
- Thesis: Digestive Physiology of Freshwater Mussels
- Technische Universität München
Wissenschaftszentrum Weihenstephan
- Aug. 2010 – Feb. 2011 Forschungsprojekt**
- Lautproduktion des Atlantischen Dorsches
- Universität Island
Department für Marine Biologie, Reykjavik, Island
- Aug. 2009 – Dez. 2012 Studentische Mitarbeiterin in der Pressestelle der TUM**
- Technische Universität München
Corporate Communications Center Weihenstephan
- Okt. 2007 – Sept. 2010 Bachelor of Science der Biologie**
- Thesis: Indikationspotenzial von Auwaldvögeln für das Monitoring von Auenrenaturierungen
- Technische Universität München
Wissenschaftszentrum Weihenstephan
- Sept. 1998 – Mai 2007 Allgemeine Hochschulzugangsberechtigung (Abitur)**
- Anne-Frank-Gymnasium Erding